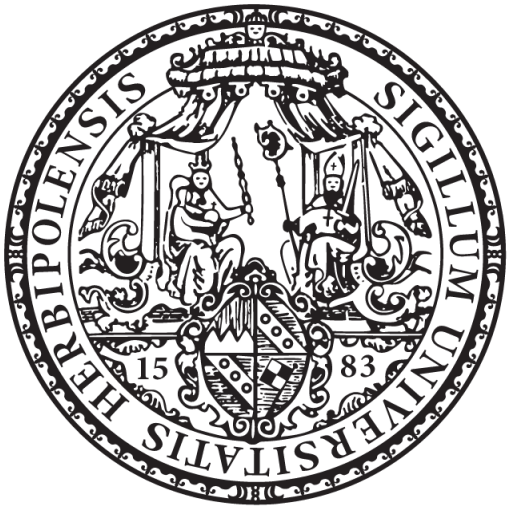
Targeting of M_2 and M_4 Muscarinic Receptor Subtypes with New Dualsteric Ligands



Dissertation

zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Julius-Maximilians-Universität Würzburg

vorgelegt von

Florian Geyer

aus Landshut

Würzburg 2022

Eingereicht bei der Fakultät für Chemie und Pharmazie am

_____

Gutachter der schriftlichen Arbeit:

1. Gutachter: _____

2. Gutachter: _____

Prüfer des öffentlichen Promotionskolloquiums:

1. Prüfer: _____

2. Prüfer: _____

3. Prüfer: _____

Datum des öffentlichen Promotionskolloquiums

_____

Doktorurkunde ausgehändigt am

_____

Die vorliegende Arbeit wurde am Institut für Pharmazie und Lebensmittelchemie der Bayerischen Julius-Maximilians-Universität Würzburg auf Anregung und unter der Anleitung von

Frau Prof. Dr. Ulrike Holzgrabe

angefertigt.

Ihr gilt mein besonderer Dank für die Aufnahme in den Arbeitskreis, das Anvertrauen des spannenden Themas und die Unterstützung im Verlauf der Promotion. Bedanken möchte ich mich zudem für das in mich gesetzte Vertrauen und den gewährten wissenschaftlichen Freiraum.

Weiterhin möchte ich mich bei meinen Kooperationspartnern bedanken:

- Prof. Dr. Carsten Hoffmann, Dr. Michael Kauk und Carolin Große, Universitätsklinikum Jena, für die Durchführung der FRET- und BRET-Messungen sowie die Hilfe bei der Auswertung und Interpretation der Daten.
- Prof. Dr. Marcel Bermudez, Universität Münster, für die wertvollen Diskussionen bei der Identifizierung der Verknüpfungspunkte.

Darüber hinaus danke ich Prof. Dr. Subhabrata Sen für die Mithilfe bei der Syntheseplanung zu Beginn der Doktorarbeit und Prof. Dr. Michael Decker für die Unterstützung des Xanomelin-Teilprojekts. Mein herzlicher Dank gilt auch Liana Pogorelaja für das Nachsynthetisieren meiner Vorstufen, das Aufstocken der Laborvorräte und die vielen netten Gespräche. Ebenso danke ich Gülşah Bayraktar für ihr großes Engagement bei der Herstellung der Xanomelin-Linker und Dr. Curd Schollmeyer, Dr. Jens Schmitz, Dr. Ludwig Höllein, Lieselotte Möhler und Martina Wecklein-Weidinger für die Hilfe bei technischen und organisatorischen Fragen.

Ganz besonders möchte ich mich bei allen Labor- und Arbeitskollegen sowie den Assistenten des 4. Semesters für die hervorragende Zusammenarbeit, die tolle Arbeitsatmosphäre, die vielen gemeinsamen Abende und die unvergesslichen Ski- und Wanderausflüge bedanken: Adrian, Alex, Alena, Anja, Antonio, Cristian, Christian, Christine E., Christine H., Christiane, Daniela, Emilie, Jogi, Jonas W., Jonas U., Joseph, Joshua, Klaus, Laura, Lukas, Markus, Michi, Mohamed, Nelson, Niclas, Nicolas, Nina, Patrick, Rasmus, Regina, Ruben, Sebastian, Sylvia, Theresa, Thomas.

Abschließend möchte ich meiner Familie, Chrissi und meinen Freunden für die großartige Unterstützung während der letzten Jahre danken.

“Satisfaction of one’s curiosity is one of the greatest sources of happiness in life.”

Linus Pauling (1901-1994)

Meinen Eltern, meiner Schwester
und meiner Verlobten

Table of contents

1 Introduction 1
1.1 Classification and structural characterization of muscarinic receptors 1
1.2 Muscarinic receptor subtypes 2
1.3 Activation and deactivation of muscarinic receptors 3
1.4 Allosteric modulation of muscarinic receptors 6
1.5 Dualsteric targeting of muscarinic receptors 10
1.6 Structural insights into allosteric modulation of LY2119620 and LY2033298 at M_2 and M_4 receptors 12
2 Aims and objectives 16
3 Synthesis of LY-hybrid ligands 18
3.1 Evaluation of adequate linking points 18
3.2 Preparation of amide functionalized LY2033298-hybrids 20
3.3 Preparation of amide functionalized LY2119620-hybrids 28
3.4 Preparation of LY2033298-hybrids by using the methyl side chain as functionalization unit 35
3.5 Attempted preparation of LY2119620-hybrids by using the methyl side chain as functionalization unit 46
4 Pharmacological investigations of LY-hybrid ligands 50
4.1 Theoretical background of the assays 50
4.2 Investigation of amide functionalized LY2033298- and LY2119620-hybrid ligands 54
4.3 Investigation of LY20-MQn-iper hybrid ligands 64
4.4 Comparison of most active hybrid ligands 67
4.5 Preliminary results of LY20-MAn series and β-arrestin measurements 70
4.6 Overall discussion of pharmacological results 71
5 Summary 72
6 Zusammenfassung 75
7 Experimental section 78
7.1 General experimental procedures and equipment 78
7.2 Synthesis of LY20-An hybrids 81
7.3 Synthesis of LY21-An hybrids 136
7.4 Synthesis of LY20-MQn hybrids 163
7.5 Synthesis of LY20-MAn hybrids 178
7.6 Attempted preparation of LY21-MAn hybrids 194
7.7 Solubility 196
8 Appendix 197

9 Literature 205

1 Introduction

In human physiology, muscarinic acetylcholine receptors (mAChR) play a crucial role as part of the parasympathetic nervous system. Muscarinic receptors regulate functions ranging from cardiac rate or smooth muscle contraction in the peripheral nervous system to cognition or motor control in the central nervous system (CNS). Drugs acting as agonists at mAChRs are classified as direct parasympathomimetic drugs and are used for the treatment of glaucoma or to reduce postoperative urinary retention. In contrary, mAChR antagonists belong to the parasympatholytic agents. These are applied, for example, as antispasmodics for smooth muscle relaxation in tubular organs of the gastrointestinal tract, for the treatment of chronic obstructive pulmonary disease, or to reduce secretion of gastric acid.[1] Besides, the utilization of muscarinic ligands has been discussed for cancer therapy, diabetes, schizophrenia, or pain management.[2, 3, 4] To avoid side effects due to this broad field of mAChR-related pharmacological actions, highly selective ligands for the different mAChR subtypes are needed. In the last decade, the determination of several mAChRs crystal structures allowed to gain a better insight on the molecular level of the receptors.[5, 6, 7, 8] Furthermore, the generation and phenotypic analysis of different muscarinic receptor knockout mice has improved the biological understanding of mAChRs.[9, 10]

1.1 Classification and structural characterization of muscarinic receptors

Muscarinic receptors belong to the superfamily of G-protein-coupled receptors (GPCRs). Mainly located at the cell surface, GPCRs translate extracellular signals delivered by, for example, light energy, neurotransmitters, or peptides into intracellular signaling cascades. Even though the GPCR superfamily is very diverse in structure and function, all GPCRs consist of an unbranched protein chain with an extracellular *N*-terminal domain, an intracellular *C*-terminal domain, and seven transmembrane-spanning α-helical domains (TM1–TM7). The seven domains are linked by three extracellular loops (ECL) and three intracellular loops (ICL). On the cytosolic side of the membrane, a guanine nucleotide binding protein (G-protein), comprised of an α-, β-, and γ-subunit, is bound to the receptor.[11] Among the five main families of GPCRs, the rhodopsin-like receptors (class A) constitute the largest class, including mAChRs.[12] The binding pocket of the respective endogenous ligand in class A GPCRs is typically located in a cavity that is formed by the seven TMs. Depending on the ligand, the different receptors bear specific amino acid residues within the cavity. In mAChRs, the binding

site of the natural agonist acetylcholine (ACh) can be found in a depth of 15 Å near the extracellular domain.[13, 14]

Together with nicotinic acetylcholine receptors (nAChRs), mAChRs form the cholinergic system. The activation of this network by ACh leads to stimulation of the parasympathetic nervous system. The latter is a division of the autonomic nervous system and controls "rest and digest" activities, such as a reduced heart rate, contraction of the urinary bladder wall, or secretion in salivary glands. Within this system, nAChRs transfer signals from preganglionic to postganglionic neurons, whereas mAChRs are located at the target organs.[1]

1.2 Muscarinic receptor subtypes

The intronless genes *CHRM1* to *CHRM5* encode for the five distinct subtypes of muscarinic receptors. Despite having a similar ACh-binding affinity, the subtypes differ in appearance as well as in physiological function and location.[15, 16, 17] At the molecular level, the five subtypes show a significant divergence in the amino acid sequence of the third internal loop. This deviation in the G-protein binding area contributes to a preferred signaling through $G\alpha_{i/o}$-proteins for the M_2/M_4-subtypes, whereas the $M_1/M_3/M_5$-group predominantly couples G-proteins of the $G\alpha_{q/11}$-type.[18, 19, 20, 21] By favoring the recruitment of $G\alpha_{i/o}$-proteins, adenylate cyclase (AC) activity is reduced upon receptor activation in the M_2/M_4-group, thus lowering the intra-cellular cyclic adenosine monophosphate (cAMP) level. Furthermore, down regulation of AC enhances blockage of voltage-gated calcium channels. In the $M_1/M_3/M_5$-group, $G\alpha_{q/11}$-proteins mediate phospholipase C (PLC) activation. Subsequent formation of inositol 1,4,5-trisphosphate (IP_3) by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) increases the intracellular calcium concentration. Diacylglycerol (DAG) is also formed by PIP_2-cleavage and serves as activator of protein kinase C (PKC) (Figure 1).[22]

However, the evidence of $G\alpha_{q/11}$- and $G\alpha_s$-protein recruitment by M_2-receptors demonstrates that despite showing coupling preferences, the different subtypes do not solely bind their preferred G-proteins.[23] The five subtypes of mAChRs are distributed in distinct areas of the central and peripheral nervous system as well as in non-neuronal tissues. M_1 receptors are abundantly expressed in the forebrain, especially in regions of the hippocampus, striatum, and prefrontal cortex.[24, 25, 26] Both M_2 and M_3 mAChRs are distributed in different brain regions, such as the basal forebrain, thalamus, and hippocampus. In peripheral tissues, M_2 receptors are found in the heart and, along with M_3 receptors, in smooth muscle tissues.[16, 17, 24, 25, 26, 27, 28] M_4 and M_5 receptors have predominantly been tracked down in the CNS. The M_4 subtype has

mainly been detected co-localized with dopamine receptors in the striatum, whereas the M_5 mAChRs are expressed in the substantia nigra.[24, 29]

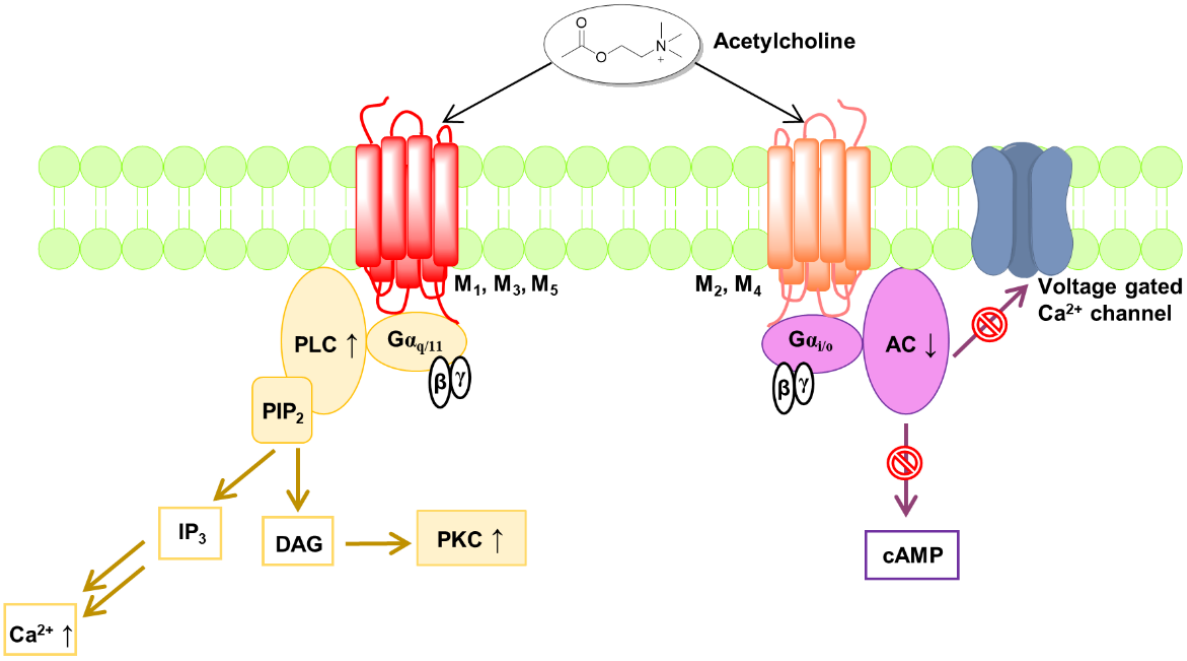


Figure 1: Signal transduction pathways of the $M_1/M_3/M_5$- and M_2/M_4-group.

Despite these differences, the binding pocket of the endogenous ligand acetylcholine, the so-called orthosteric binding site, shows a high level of homologous sequence regions among mAChR subtypes.[9, 19] This explains why drugs targeting the orthosteric binding site of mAChRs usually show insufficient subtype selectivity and thus often cause intolerable side effects.

1.3 Activation and deactivation of muscarinic receptors

Even though no crystal structure of an mAChR in combination with its endogenous transmitter acetylcholine has been obtained so far, the involvement of several amino acids in receptor stimulation at the orthosteric binding site can be assumed due to point mutation studies or structural characterization of agonist-bound muscarinic receptors. An ionic interaction between a negatively charged aspartate (D103) in TM3 and the quaternary amine of ACh is supposed to be crucial for the binding process.[30] The choline group is presumably enclosed in an aromatic cage which is formed by a hydrogen bond network between three tyrosine residues (Y104, Y403, and Y426), often referred to as tyrosine lid.[31] Moreover, the carbonyl function of ACh

is likely to interact with an asparagine residue (N404) of TM6 (Figure 2).[6] Due to these interactions, extensive conformational changes are induced within the receptor upon ligand binding, whereby the formation of a hydrogen bond between the intracellular domains TM5 and TM7 and an outward tilting of the cytoplasmic end of TM6 are most notably.[32, 33, 34]

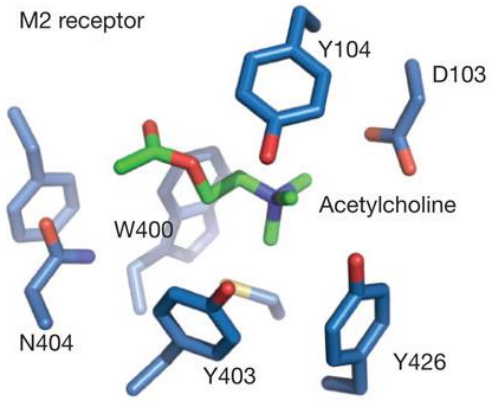


Figure 2: Model of acetylcholine docked into the orthosteric binding site of the M_2 receptor. Reprinted with permission of Springer Nature; Copyright © 2012.[6]

Such rearrangements of α-transmembrane helices promote interactions of the receptor with heterotrimeric G-proteins, thereby inducing an exchange of GDP (guanosine diphosphate) against GTP (guanosine triphosphate). This causes dissociation of the G-protein into an α subunit and a βγ dimeric subunit, followed by activation of the already mentioned effector systems. The leisurely hydrolyzation of GTP by GTPase activity of the Gα-subunit and the thus initiated reassociation of the heterotrimeric G-protein terminate the activation cycle (Figure 3).[35, 36] The switch from an inactive to an active receptor state also enables G-protein coupled receptor kinases (GRKs) to phosphorylate serine or threonine residues of the intracellular loops and the C-terminal ending.[37, 38] Subsequently, β-arrestin proteins bind to the phosphorylated receptor. Since so-called clathrin proteins can cause internalization of β-arrestin bound mAChRs, this represents an endogenous mechanism for down regulation of mAChR activity.[39] Thereby, the amplification of GRK activity by the released Gβγ dimer prompts a negative feedback loop.[40] Unlike class B GPCRs, class A GPCRs release β-arrestin proteins after receptor internalization and return to the plasma membrane after dephosphorylation.[41] M_1, M_3 and M_4 receptors have shown to internalize clathrin-mediated. In contrast, the internalization pathway of M_2 receptors is independent of β-arrestin.[42, 43]

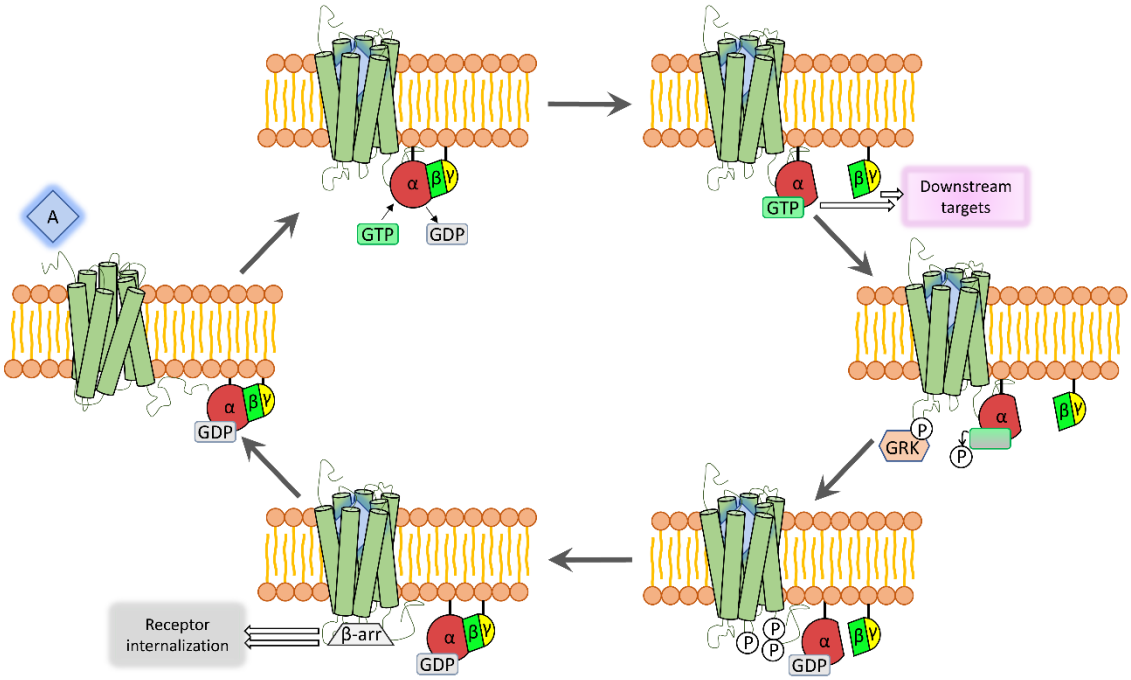


Figure 3: Activation cycle of muscarinic receptors. The cycle starts on the left side with agonist (A) binding. This leads to the exchange of GDP by GTP at the Gα-subunit and a subsequent dissociation of the heterotrimeric G protein, thereby causing different downstream effects. GRKs phosphorylate intracellular domains of the receptor, thus enabling the binding of β-arrestin (β-arr) and subsequent receptor internalization. The hydrolysis of GTP at the Gα subunit allows anew formation of the heterotrimeric G protein and returns the receptor to the original state.

Due to the hydrolytic instability of the ester group, the endogenous ligand acetylcholine bears almost no therapeutic relevance. Besides acetylcholine, several orthosteric agonists have been identified so far (Figure 4). The alkaloid muscarine binds nonselectively to all mAChR subtypes and is basically known for its toxic effects upon mushroom consumption.[44] Pilocarpine is used in eye drops for the treatment of glaucoma and preferentially stimulates M_2 and M_3 subtypes.[45, 46]

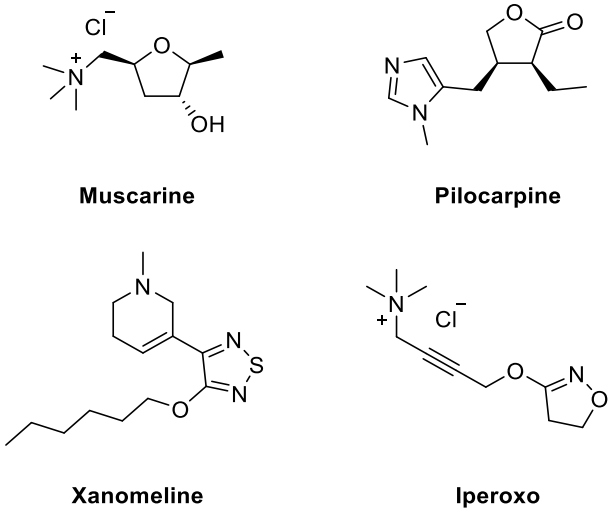


Figure 4: Structures of selected orthosteric agonists at muscarinic receptors.

The so-called superagonist iperoxo actually causes a higher activation of M_2 receptors than acetylcholine.[47] However, the lack of subtype selectivity prevents its application as a drug. The M_1/M_4-preferring agonist xanomeline is under discussion for the treatment of schizophrenia and neurodegeneration.[48, 49, 50, 51] In clinical trials, xanomeline improved symptoms in schizophrenic patients. Unfortunately, a range of peripheral side effects still hampers an application in therapeutics. In a recent study, xanomeline was combined with the tropin-type mAChR antagonist trospium. The latter exclusively acts in the body periphery. The results of this phase IIb clinical trial showed that unwanted xanomeline-related secondary effects were reduced.[52, 53]

1.4 Allosteric modulation of muscarinic receptors

To enhance subtype selectivity at mAChRs, newly developed drug candidates often address binding sites distinct to the highly conserved orthosteric site. The stimulation of mAChRs by so-called allosteric ligands in combination with an orthosteric agonist leads to the formation of a ternary complex. Depending on the nature of the allosteric ligand, the binding affinity of the orthosteric agonist and the receptor activation can thus be influenced (Figure 5).[54, 55, 56] Direct activation of muscarinic receptors by allosteric agonists has also been reported.[57]

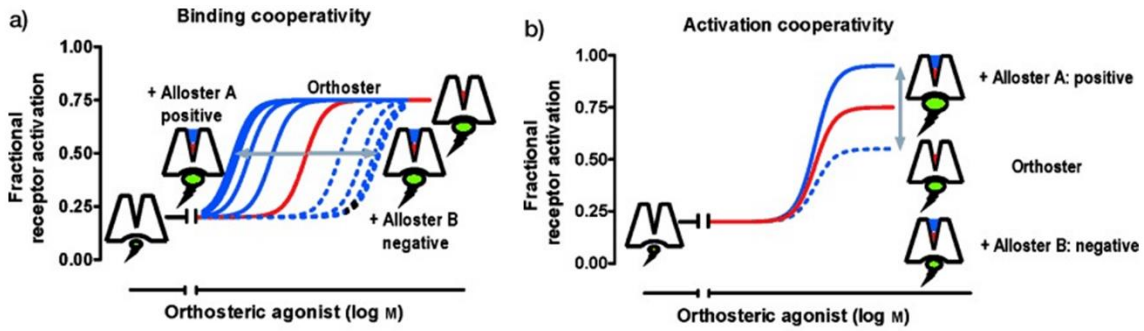


Figure 5: Influence of positive (blue curves) and negative (blue dashed curves) allosteric ligands on a) binding affinity of orthosteric ligands and b) receptor activation. The red curve represents receptor activation in absence of any allosteric modulator. Reprinted with permission of John Wiley and Sons; Copyright © 2012.[55]

The ternary complex model according to Ehlert describes the cooperative interactions between orthosteric and allosteric ligands (Figure 6).[54] Both ligands can bind separately to the free receptor (Figure 6, I) with their respective dissociation constants K_a (Figure 6, II) and K_b (Figure 6, IV). When a ternary complex is formed (Figure 6, III), the reciprocal effect of orthosteric and allosteric unit is referred to as cooperativity factor α.

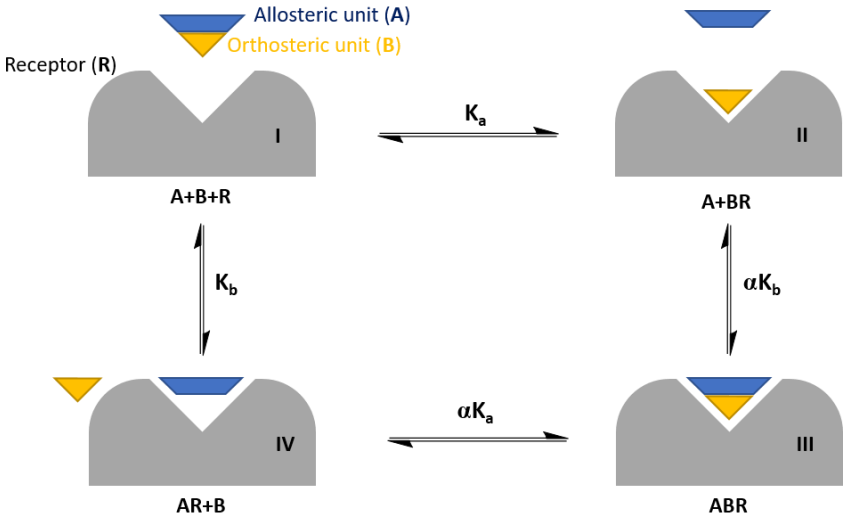


Figure 6: Illustration of binding cooperativity between orthosteric and allosteric ligands based on the ternary complex model according to Ehlert.[54]

For $\alpha < 1$, the dissociation constant is decreased which leads to an enhanced binding affinity and is related to positive cooperativity. Furthermore, a negative cooperative effect is induced for $\alpha > 1$, causing a reduced binding affinity of the orthosteric ligand. When no binding cooperativity is induced ($\alpha = 1$), the ligands are classified as neutral allosteric ligands.[58, 59] However, this model solely describes binding cooperativity and neglects activation cooperativity.

A comparison of the amino acid sequence of the five muscarinic subtypes reveals significant variability within the extracellular vestibule. Additionally, crystal structures of M_1–M_5 receptors show differences of the tertiary structure in this region.[8, 60, 61] Since such deviations are crucial for the implementation of subtype selective mAChR activation, several allosteric ligands targeting this part of the receptor have been investigated (Figure 7). In 1968, the alkane bis-ammonium compound W84 was the first substance that showed interactions between an allosteric and an orthosteric binding site at mAChRs. While trying to improve the therapy of organophosphate intoxications, Lüllmann and coworkers observed a cooperative effect when the muscarinic antagonist atropine was combined with W84.[62, 63] Later, the alkaloid brucine was shown to selectively enhance the binding affinity of acetylcholine at M_1 receptors, thus also proofing the feasibility of subtype selective mAChR activation.[64, 65] Subsequently, investigations of the binding mode of M_1 selective ligand AC-42 by mutation studies confirmed the existence of an allosteric binding site. The results demonstrated that AC-42 definitely addresses a receptor region aside from the orthosteric site.[66] In the 2000s, a series of thienopyridine derivatives was identified by Eli Lilly scientists as powerful positive allosteric

modulators from a small molecule screening at M_4 receptors and a following hit expansion.[67] Within this series, the compound LY2033298 was extensively examined due to its high M_4 selectivity.[68, 69, 70] Furthermore, the M_2/M_4 selective compound LY2119620 had a huge impact on mAChRs research as it allowed the crystallization and structural characterization of an agonist-bound M_2 receptor.[7, 71] However, allosterism at mAChRs is not limited to interactions between two small molecules at the extracellular part and the transmembrane core. G-protein binding at the intracellular receptor surface also generates binding cooperativity with the orthosteric ligand.[72] A positive allosteric effect of Na^+ ions towards ACh recruitment has further been detected at the M_2 receptor.[73]

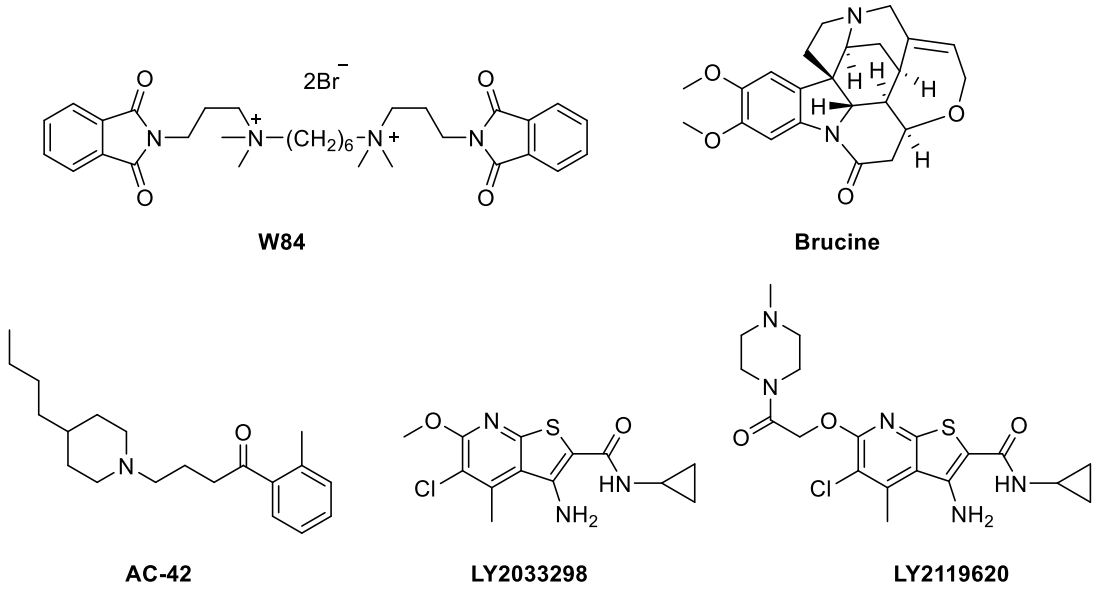


Figure 7: Selection of allosteric modulators.

Two different mechanisms contribute to cooperativity between allosteric modulators in the extracellular vestibule and orthosteric ligands. On the one hand, the conformations of both binding sites are associated. Therefore, ligand stimulation of one binding site induces a structural shift at the other. On the other hand, direct charge-charge interactions between the two ligands also affect binding cooperativity, such that the combination of two positively charged molecules reduces the mutual effect through electrostatic repulsion.[74]

Besides the improvement of subtype selectivity, allosteric ligands have shown several beneficial characteristics. While orthosteric agonists stimulate a receptor for as long as they are available, the effect of allosteric modulators is mostly saturable. Due to the cooperative mechanism, an upper limit for receptor response is reached when the allosteric site is entirely occupied.[55] Moreover, the scope and the direction of an allosteric effect released by the same modulator at the same receptor subtype varies as a function of the utilized orthosteric ligand.[75]

This correlation is called probe dependence and has been extensively studied for the allosteric modulator LY2033298. When applied in combination with acetylcholine at rodent M_4 receptors, LY2033298 strongly potentiated the action of ACh, whereas alongside with the orthosteric ligand xanomeline only a weak positive allosteric effect was detected.[76] Interestingly, an investigation of cooperativity between LY2033298 and xanomeline at human M_2 receptors revealed that augmenting concentrations of the modulator led to an amplification of agonist affinity on the one hand, but to a reduction of receptor response on the other. These findings indicate a stabilization of an inactive receptor state by the formed ternary complex. Thus, the allosteric modulator LY2033298 changes the agonistic nature of xanomeline at the M_2 receptor.[77] In consequence, an allosteric modulator can only be classified in context to a specific orthosteric ligand. A closely related property of some allosteric ligands is the release of an affinity shift towards specific signaling pathways by preserving a receptor conformation that initiates the respective signal cascade (Figure 8a). This effect is referred to as biased modulation [78]

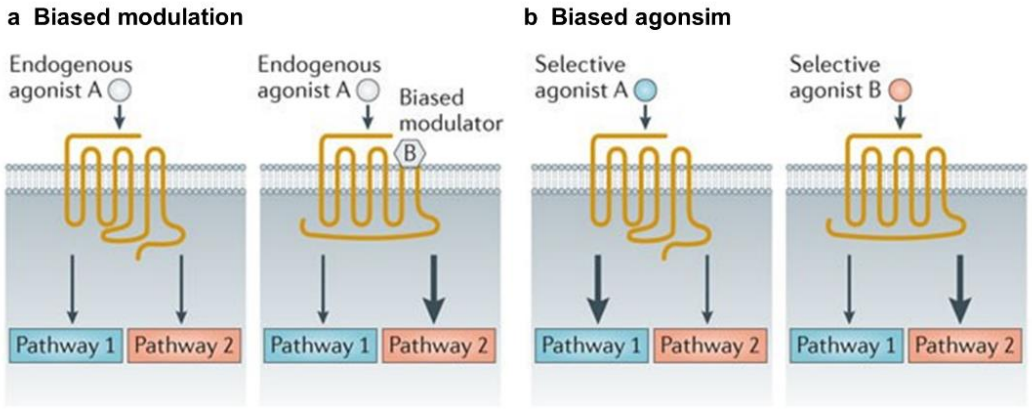


Figure 8: Pathway selective receptor activation through (a) biased modulation or (b) biased agonism. Reprinted with permission of Springer Nature; Copyright © 2012.[78]

Biased modulation has been described exemplarily for the M_1 selective positive allosteric modulator VU0029767. Marlo and coworkers reported an enhancement of phospholipase C activity but not phospholipase D activity when VU0029767 was applied in combination with ACh.[79] Biased modulation at mAChRs may allow a more selective addressing of therapeutically relevant signaling cascades, whereas other pathways that are related to unwanted on-target side effects stay unaffected. Noteworthy, orthosteric ligands can also exhibit a pathway bias (Figure 8b). For example, the muscarinic agonist pilocarpine does not activate $G\alpha_{q/11}$-proteins at the M_3 receptor but mediates the recruitment of β-arrestin.[80]

1.5 Dualsteric targeting of muscarinic receptors

A further approach in the design of highly selective ligands for muscarinic receptors is the combination of an orthosteric unit and an allosteric modulator within one molecule (Figure 9a). The resulting hybrids are called dualsteric or bitopic ligands.[55, 81, 82] This idea derives from the so-called "message-address concept" established by Schwyzer in 1977.[83] In this context, the orthosteric moiety delivers the desired message by stimulation of the receptor and the release of appropriate downstream effects. The allosteric part of the molecule interacts with less conserved receptor sites and thus contains the recipient address. The two parts of the molecule need to be connected by a linker of adequate chain length at the appropriate connection points. In this way, the dualsteric strategy aims to unite high affinity with high subtype-selectivity. A pathway-selective receptor activation through biased agonism is also a desirable property of bitopic ligands.

Disingrini and coworkers followed this approach by combining the orthosteric agonist iperoxo with a phthalimide or a naphthalimide moiety as M_2-selective allosteric modulator (Figure 9c).[84] The resulting hybrid compounds showed the expected subtype selectivity towards M_2 receptors as well as an affinity and efficacy comparable to the endogenous transmitter acetylcholine. Moreover, preferential activation of the G_i signaling pathway was observed. However, the negative binding cooperativity of the investigated bis(ammonio)alkane-type modulators towards orthosteric agonists did not result in an additive affinity gain.[85] Following binding studies of these compounds revealed that the same ligand can stabilize the M_2 receptor in two distinct orientations.[86] The preservation of a conformation with a reduced efficacy for downstream signaling leads to partial agonism. The proportion between the occupied active and inactive receptor states thereby dictates the activity of a partial agonist (Figure 9b).[87, 88] Hence, rational design of partial agonists may allow to control the extent of muscarinic receptor activation, paving the way for drugs with an improved side effect profile. Moreover, the receptor response upon partial agonist binding is context-dependent. Distinct pharmacological phenotypes of one and the same receptor subtype arise in different cell populations. The released downstream effect is therefore not only depending on agonist activity and concentration, but also on parameters such as the intracellular cAMP level and the number of receptors within the membrane.[89] Recently, a Naph-iper hybrid ligand was shown to increase cytotoxicity and apoptosis in glioblastoma cancer stem cells. M_2 receptor activation by the hybrid ligand generated DNA damage within the cancer cells and reduced their survival rate. Since the Naph-iper ligand also prevented drug-resistance, this hybrid may allow the application of lower doses of chemotherapeutic agents.[90, 91]

A promising application of bitopic ligands was investigated by Keller and coworkers. Through combination of dibenzodiazepinone derivatives with a fluorescence-labeled unit, M_2 preferring antagonists were designed which bind the receptor in a dualsteric fashion. These ligands can serve as tools to map M_2 receptors in cells and may be helpful to determine binding affinities of allosteric or orthosteric ligands.[92] Furthermore, the M_2 selectivity of dibenzodiazepinone type hybrids was modulated by addition of different peptide conjugates.[93, 94]

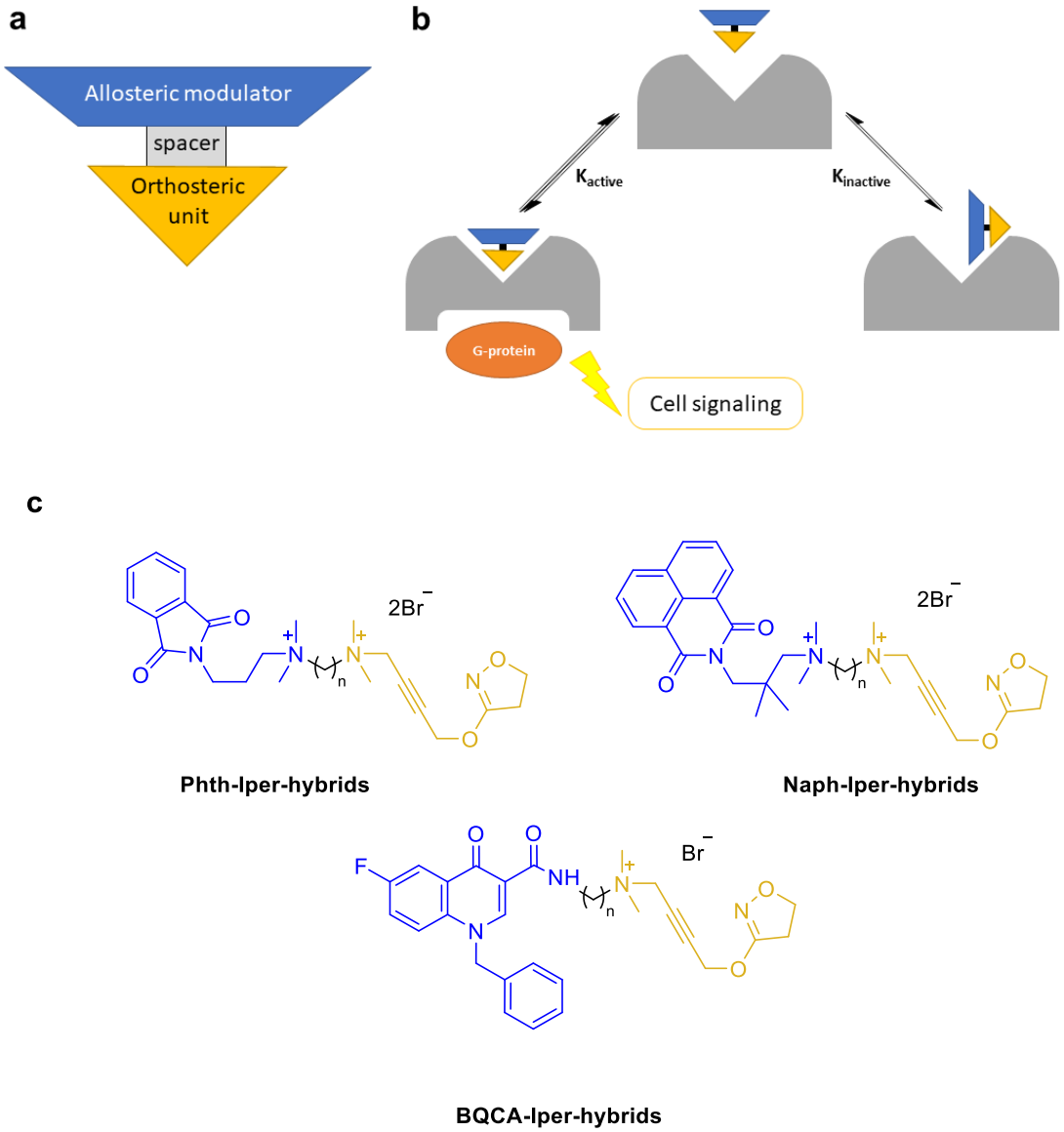


Figure 9: a) Schematic depiction of bitopic ligands. b) Dynamic ligand binding of dualsteric compounds in an active and an inactive binding mode. K_{active} and $K_{inactive}$ represent the respective equilibrium dissociation constants. Cell signaling can only be triggered in the active conformation. c) Structures of bitopic ligands based on iperoxo as orthosteric unit (yellow) and phthalimide (Phth-Iper-hybrids), naphthalimide (Naph-Iper-hybrids), or BQCA (BQCA-Iper-hybrids) as allosteric modulators (blue).

Another attempt to apply the dualsteric concept to muscarinic receptors was undertaken with a highly M_1 selective benzyl quinolone carboxylic acid (BQCA) derivative as positive allosteric

modulator (Figure 9c). Hybrids composed of BQCA and iperoxo were found to act as partial agonists at human M_1 receptors, activating both G-protein and β-arrestin signaling pathways. Remarkably, when the orthosteric moiety was minimized to an ammonium ion (TMA), only G-protein stimulation was observed. These findings strongly suggest that a positively charged ammonium ion as orthosteric unit is sufficient for G-protein activation, whereas β-arrestin binding requires further interactions within the orthosteric site.[95, 96] Instead of applying an alkane chain as linker, BQCA and iperoxo were further combined by a photoswitchable azobenzene unit. The activity of the thus formed hybrids can be controlled by light.[97]

1.6 Structural insights into allosteric modulation of LY2119620 and LY2033298 at M_2 and M_4 receptors

In 2013, nobel prize laureate Brian Kobilka and coworkers reported the X-ray crystal structure of the M_2 receptor bound to the agonist iperoxo and, additionally, as ternary complex with the allosteric modulator LY2119620 (Figure 10a+b). The high degree of similarity between the two structures indicated that binding of an orthosteric agonist pre-forms the binding site of the modulator to a great extent.[7] By comparing the conformation of the ternary complex with a former published crystal structure of an inactive M_2 receptor, important insights into structural changes that occur upon receptor stimulation were obtained. The agonist-induced movements of transmembrane domains lead to the formation of a “slot-like” allosteric vestibule which can subsequently interact with the planar molecule LY2119620 (Figure 10d+e).[6, 7, 61] Moreover, a closure of the tyrosine lid was observed, thus separating the orthosteric from the allosteric site.[7] LY2119620 sits above the closed lid and stabilizes the active conformation by several interactions with the receptor (Figure 10c).

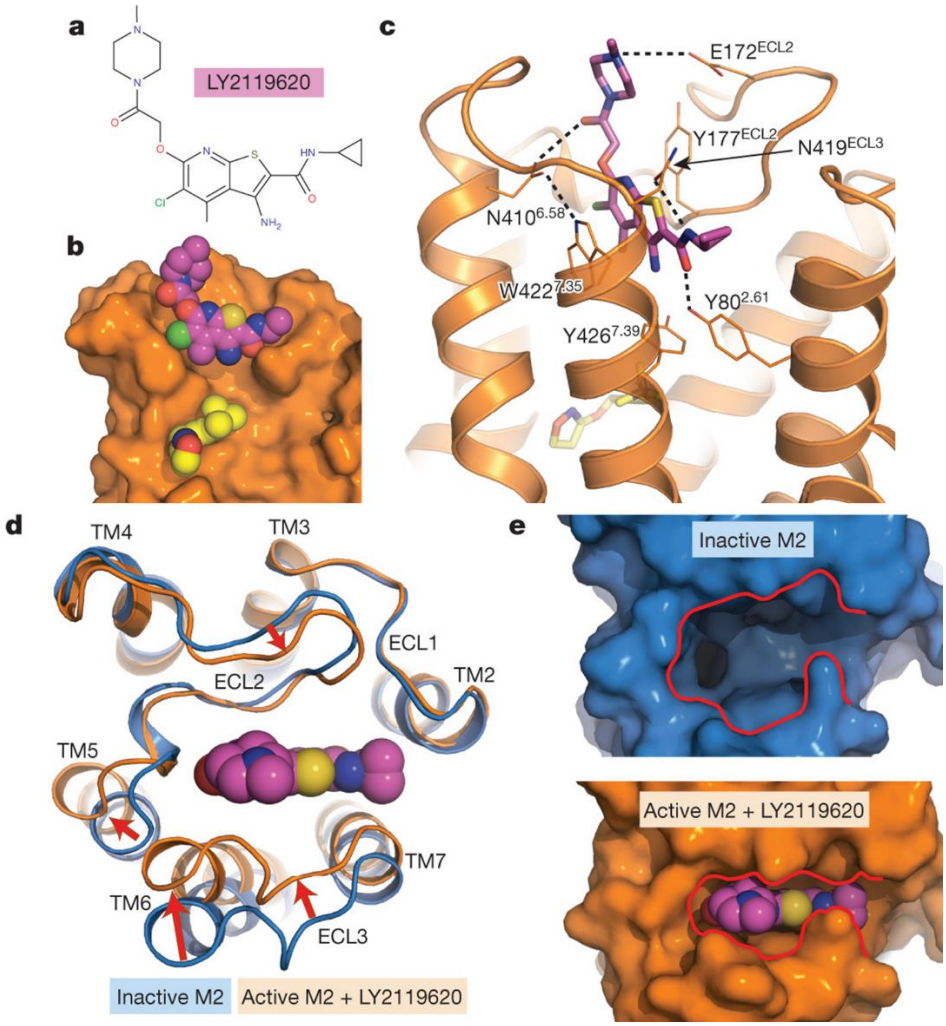


Figure 10: a) Structure of LY2119620. b) Ternary complex of LY2119620 (purple) and iperoxo (yellow) at the M_2 receptor. c) Interactions of LY2119620 in the allosteric vestibule. d) Comparison of the extracellular site between an inactive and an active M_2 receptor. Red arrows indicate movements of the transmembrane domains upon ligand binding. e) Contraction of the allosteric binding site upon receptor activation indicated by red lines. Reprinted with permission of Springer Nature; Copyright © 2013.[7]

The contribution of each allosteric contact was evaluated by molecular dynamics simulations (Figure 11).[98] The amino acids Asn410$^{6.58}$, Tyr80$^{2.61}$, and Asn419$^{7.32}$ form direct polar contacts with the allosteric modulator, whereas the piperazine ring of LY2119620 is presumably involved in an interaction with the residues Glu172 and Tyr177 of ECL2 and the backbone of ECL2 and ECL3. Furthermore, the thienopyridine ring system lays in a three-way π-π-stacking complex between Trp422$^{7.35}$ and Tyr177. Remarkably, Trp422$^{7.35}$ possesses different orientations in the presence or absence of LY2119620. Since Trp422$^{7.35}$ also influences the position of amino acids within the tyrosine lid, these findings suggest a direct impact of LY2119620 on the orthosteric binding site.[7, 98]

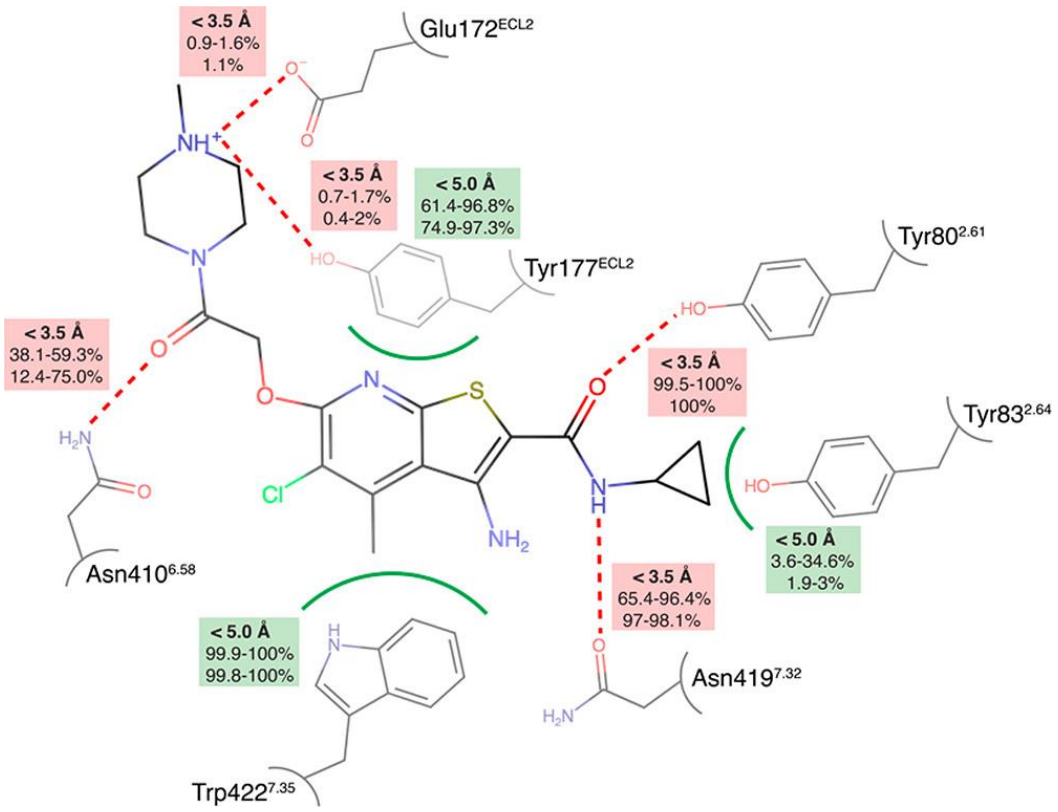


Figure 11: Polar (red) and hydrophobic (green) interactions between LY2119620 and the iperoxo-bound M_2 receptor based on molecular dynamics simulations. The percentages indicate how often the respective interaction occurs in a simulation of a LY211960-iperoxo receptor complex (first line) or a simulation with an additionally bound "G-protein mimetic" nanobody (second line). Reprinted with permission of American Chemical Society; Copyright © 2018.[98]

Several amino acids surrounding LY2119620 in the allosteric binding pocket of the human M_2 receptor are nonconserved within the five subtypes.[61] Firstly, Asn419$^{7.32}$ is solely present at the M_2 receptor. It is noteworthy that at the M_4 receptor, this residue is exchanged by aspartic acid which likely forms a similar polar interaction with LY2119620. Secondly, Tyr80$^{2.61}$ is exchanged by phenylalanine at the M_3 subtype. Hence, the M_3 receptor is not able to build up a polar interaction at this position. Moreover, the M_2 receptor exclusively contains both residues Glu172 and Tyr177 and is consequently the only subtype that can interact effectively with the piperazine ring. In combination with two adjacent acidic residues, Glu172 forms the so-called EDGE sequence. This motif is uniquely present at the M_2 receptor and has earlier been shown to interact with allosteric modulators.[99, 100]

The dissociation of iperoxo from the M_2 receptor was simulated *in silicio* by metadynamics simulations, leading to the identification of two distinct dissociation pathways. The modulator LY2119620 was shown to inhibit ligand dissociation for both escape routes and thus extends the unbinding process.[101]

Due to the lack of active state M_4 crystal structures, the binding of allosteric modulator LY2033298 at the M_4 receptor was investigated by mutagenesis studies.[60, 102] Thereby, alanine mutation of $Trp422^{7.35}$ led to a complete loss in affinity of the modulator. Furthermore, mutations of the tyrosine-lid forming residues also caused a significant decrease in cooperativity. Taken together, M_4 mutation studies allowed the identification of an allosteric network that mediates interactions between the allosteric and the orthosteric site. This network involves residues of TMs 2, 3, 6 and 7 as well as some residues of the second extracellular loop. Based on the previously discussed active M_2 crystal structure, a homology model of the M_4 receptor bound to acetylcholine and LY2033298 was developed (Figure 12).[60, 61]

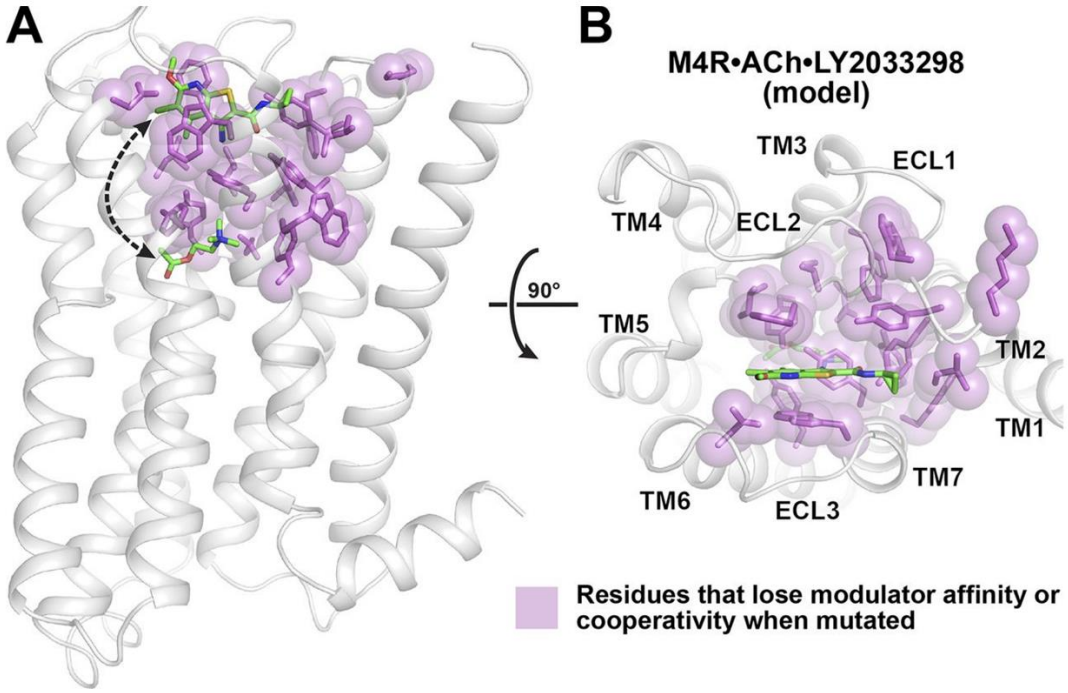


Figure 12: Homology model of the active M_4 receptor bound to acetylcholine and LY2033298. An allosteric network mediates interactions between the allosteric and the orthosteric site. Residues that are involved in this network are shown in violet. The receptor is displayed from side view (A) and as seen from the extracellular site (B). Reprinted with permission of Rockefeller University Press; Copyright © 2018.[61]

A comparison of this model with the inactive state crystal structure of the M_4 receptor indicated that the residues of the allosteric network within TMs 2, 3, and 7 remain largely unmoved. However, residues that are situated closer to the extracellular vestibule show a noticeable rearrangement. The residues of TMs 2, 3, and 7 may thus serve as a hinge which mediates movements within the extracellular loops upon receptor activation. In this context, the allosteric modulator acts as a stabilizer of the hinge and helps to switch the receptor from a dynamic state to a more fixed position.[60, 61]

2 Aims and objectives

Muscarinic receptors represent a promising target for the treatment of multiple diseases. A lack of subtype selectivity is the main reason why many drugs targeting muscarinic receptors suffer from a poor side effect profile. In this context, dualsteric ligands that address two different binding sites simultaneously can improve selectivity and affinity. The binding properties and allosteric interactions of the M_2/M_4 selective modulators LY2033298 and LY2119620 at muscarinic receptors have been studied extensively in the past decade. However, no direct linkage of these thienopyridine moieties with an orthosteric muscarinic agonist has been reported so far. A first approach undertaken by J. Klöckner did not lead to the formation of bitopic ligands of this type.[103] Nevertheless, the investigation of such hybrid ligands could reveal whether the rational design of dualsteric ligands from building blocks with a well-known binding mode is possible.

The aim of this thesis was to re-examine the synthetic feasibility of dualsteric compounds for muscarinic receptors that involve LY2033298 or LY2119620. Hence, appropriate linkage points at the thienopyridine moieties were identified based on former published literature. A synthetic pathway for the introduction of a linker was then created and different orthosteric units were attached. For this purpose, the agonists iperoxo, xanomeline, and TMA were chosen. Iperoxo and xanomeline should lead to an enhanced M_2 or M_4 selectivity, respectively, whereas TMA was selected to cause biased signaling (Figure 13). Moreover, two different linkage points within xanomeline were examined. Furthermore, the chain length of the alkyl linkers was varied to optimize binding properties of the dualsteric ligands.

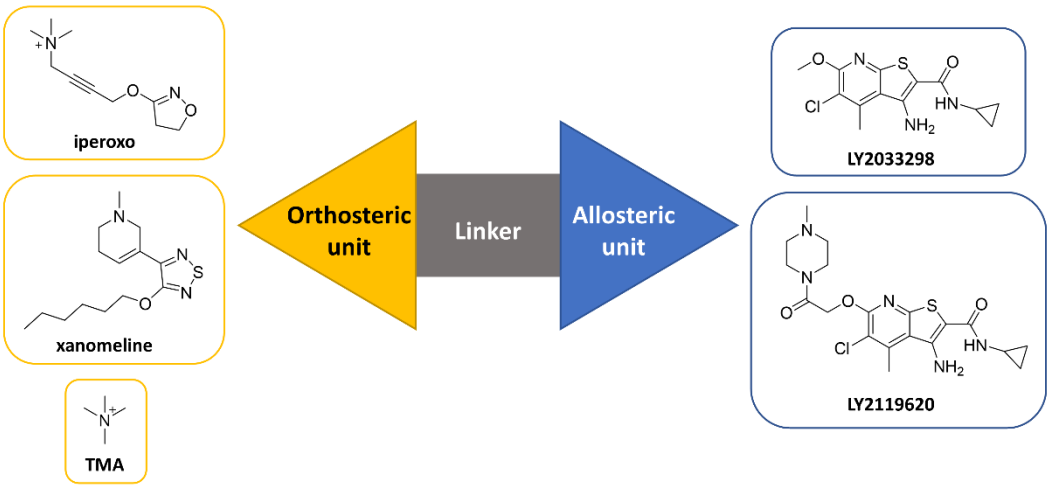


Figure 13: Overview of the investigated orthosteric and allosteric building blocks.

To find out whether the synthesized ligands release conformational changes or G protein recruitment at the M_2 or M_4 receptor, the obtained dualsteric compounds should be studied by FRET (Förster resonance energy transfer)- and mini-G protein BRET (bioluminescence resonance energy transfer) experiments. The respective assays were performed in cooperation with Prof. Dr. Carsten Hoffmann by Dr. Michael Kauk and Carolin Grosse.

3 Synthesis of LY-hybrid ligands

3.1 Evaluation of adequate linking points

Prior to the synthetic part of this thesis, the allosteric modulators LY2033298 and LY2119620 were evaluated in terms of adequate attachment positions to an alkyl linker. In the design of hybrid molecules, the choice of suitable linking points is crucial to avoid a significant decrease in binding affinity. Therefore, the linker should be introduced in positions that allow minimization of the relative distance between allosteric and orthosteric building blocks within the receptor. The active state crystal structure of the M_2 receptor co-bound to iperoxo and LY2119620 showed that the latter is inserted in a slot-like channel above the orthosteric binding site (Figure 10).[7] Since the modulator LY2033298 forms similar interactions in the allosteric binding pocket, an alkyl chain could either be induced in 5-position of the thienopyridine ring or at the methyl-, amino-, or amido-group (Figure 14). Otherwise, the linker would have to wind round the allosteric unit and could thereby disturb for example π-π-interactions between the thienopyridine ring and the receptor residues Tyr177 and Trp422$^{7.35}$.

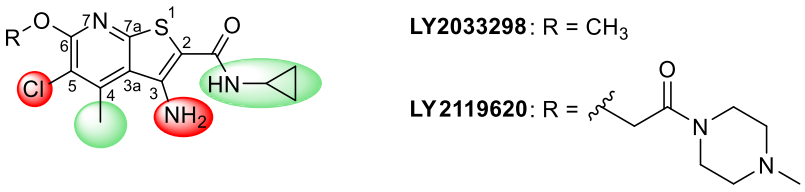


Figure 14: Possible attachment points for linker introduction at the thienopyridine scaffold of LY2033298 and LY2119620. The green linking points were investigated within this thesis.

In 2015, Szabo and coworkers performed a structure-activity relationship (SAR) study of LY2033298 at the M_4 receptor.[104] Therein, the impact of an acylation of the amino group, halogen exchange of the chloro-substituent in 5-position, and cyclopropyl replacement by different alkyl groups were investigated amongst others. Additionally, the following evaluation also includes the methyl group in 4-position.

1) Acylation of the amino group led to a complete loss of PAM activity, hence excluding this type of attachment for the synthesis of dualsteric ligands. However, an alkylation of the aromatic amino group has not been described. Due to delocalization of the nitrogen's lone electron pair within the thienopyridine ring system, the nucleophilicity of the vinylogous amide is strongly reduced. Hence, a nucleophilic substitution reaction between the amine and a haloalkane is presumably difficult to realize.

2) Halogen exchange of the chloro-group at 5-position by bromine or iodine barely affected binding affinity of LY2033298 at the M_4 receptor, even though a decrease in efficacy and PAM activity was observed. A replacement of the chloro-substituted carbon atom by a nitrogen atom gave similar results.[104] Consequently, modifications that decrease electronegativity at this position should be circumvented. This issue complicates the attachment of a linker at 5-position. Besides, regarding the active state model of the ternary LY2033298-acetylcholine-M_4 receptor complex, a linker at 5-position would likely interfere TM4 and TM6.[60]
3) Replacement of the cyclopropyl group by an alkyl chain led to a reduction of the allosteric properties with increasing linker chain length. Thereby, the extension up to a hexyl chain clearly diminished PAM properties.[104] Since uncontrolled movements of the linker probably occur to a smaller extent when the alkyl chain is integrated on both endings into a dualsteric ligand, this effect may diminish for hybrid molecules. Seen from a synthetic viewpoint, functionalization at the amide position represents the easiest way to produce dualsteric ligands derived from LY2033298 or LY2119620. Nonetheless, it is hard to predict whether full agonism can be achieved by such compounds.
4) The M_4 active state model as well as the M_2 active state crystal structure both indicate that the methyl group in 4-position of the thienopyridine ring directly points towards the orthosteric site.[7, 60] However, no structure-activity studies involving this promising attachment point have been performed so far. Firstly, that might be due to a challenging synthetic accessibility of this substituent. Secondly, modifications at the methyl group require the insertion of a further functional group, thus complicating an estimation between the synthetic input and the desired pharmacological output.

Considering these findings, the amido group and the methyl substituent were chosen as adequate linkage points of LY2033298 and LY2119620 for the synthesis of dualsteric ligands targeting muscarinic receptors. With regard to the orthosteric units, functionalization of iperoxo is known to be convenient at the quaternary amine[7, 105, 106], whereas xanomeline can be modified at the tertiary amine or at the terminal ending of the hexyl group (Figure 15).[107, 108]

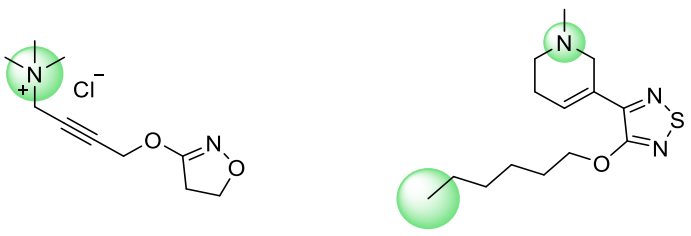


Figure 15: Attachment points of iperoxo (left) and xanomeline (right).

3.2 Preparation of amide functionalized LY2033298-hybrids

In a first approach, the amide position of LY2033298 was functionalized to introduce an orthosteric linker moiety (Figure 16). These ligands are further referred to as LY20-An series. Retrosynthetic analysis revealed that compounds of the LY20-An series are accessible through amide coupling of thienopyridine carboxylic acid **1** with different amine linkers. Comparable modifications at LY2033298 have been reported before in the literature.[104, 109] Because of the high degree of flexibility of the alkyl linker, dualsteric ligands may generally show a complex behavior in chemical reactions. Thus, the connection of orthosteric and allosteric units should preferably occur in the last step of the total synthesis under relatively mild reaction conditions. Therefore, nucleophilic substitution reactions or amide coupling reactions that take place at room temperature or, at least, at an only slightly increased temperature are suitable.

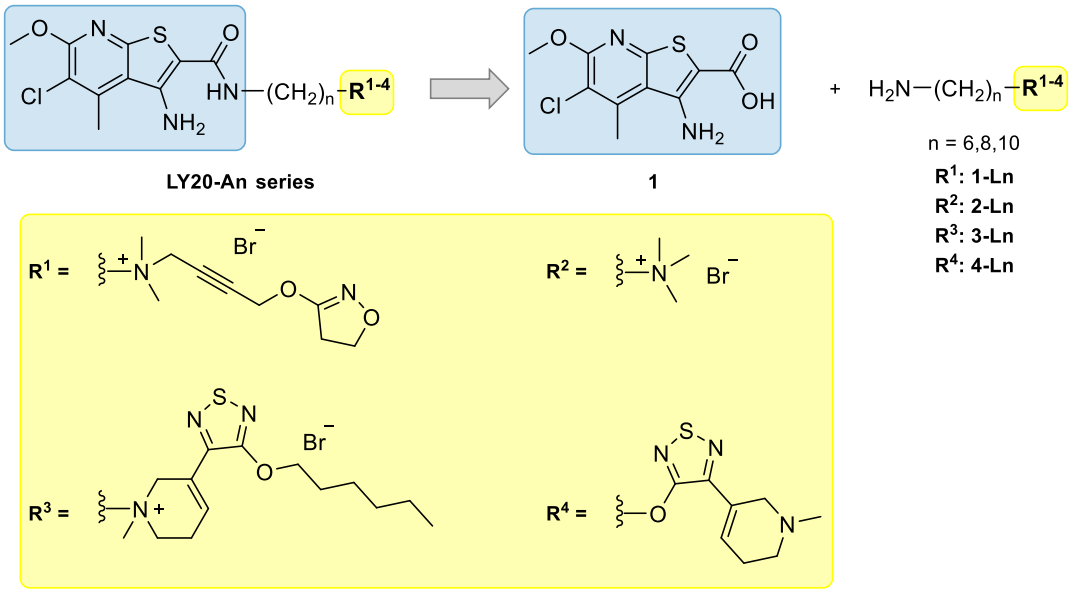


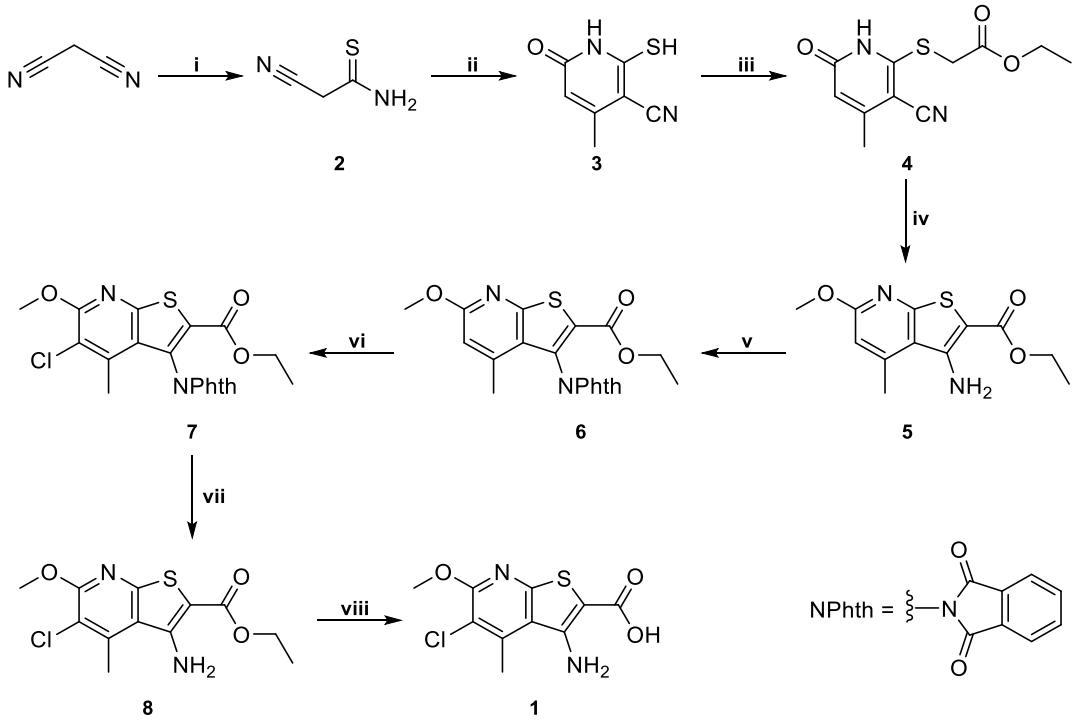
Figure 16: Retrosynthesis of the LY20-An series.

The herein applied orthosteric units iperoxo, xanomeline, and TMA were previously attached to the opposite ending of the respective linker. Additionally, both xanomeline binding sites were explored within the LY20-An series. Furthermore, the chain length of the alkyl linker was varied between six, eight, and ten methylene units. Notably, target compounds containing residues R^1, R^2, or R^3 all bear a positively charged nitrogen atom at the orthosteric site, whereas bitopic ligands involving R^4 are uncharged. A positively charged target molecule can be beneficial in terms of water solubility on the one hand but is unlikely to pass the blood brain barrier in living organisms on the other.

3.2.1 Synthesis of thienopyridine carboxylic acid 1

The synthesis of key thienopyridine scaffold **1** was achieved in eight steps, starting from the simple building block malononitrile (Scheme 1). Following a reaction protocol from Dotsenko and coworkers, hydrogen sulfide gas, generated in a separate flask by dropwise addition of 6 M hydrochloric acid to sodium sulfide, was induced into an ethanolic solution of malononitrile and triethylamine (TEA).[110] Absorption of H_2S by malononitrile led to the formation of cyanothioacetamide (**2**). Subsequently, a synthesis pathway from Szabo *et al*. was followed.[109] In a Guareschi-Thorpe like condensation reaction, compound **2** was reacted with methyl acetoacetate under basic conditions. Reaction work-up by addition of hydrochloric acid afforded pyridone derivative **3**. Next, the thiol group of **3** was deprotonated by addition of TEA and the formed thiolate was reacted with ethyl-2-chloroacetate in a nucleophilic displacement reaction to give ethyl ester **4**.

Scheme 1: Synthesis of thienopyridine carboxylic acid scaffold **1**.



Reagents and conditions: (i) H_2S, TEA, CH_3CH_2OH, 15-20 °C; (ii) methyl acetoacetate, morpholine, CH_3CH_2OH, reflux; (iii) ethyl-2-chloroacetate, TEA, DMF, 0 °C → rt; (iv) a) CH_3I, K_2CO_3, DMF, rt; b) 1 M KOH, rt; (v) phthalic anhydride, acetic acid, reflux; (vi) *N*-chlorosuccinimide, concentrated HCl, CH_3CH_2OH, reflux; (vii) hydrazine monohydrate, CH_3CH_2OH, reflux; (viii) 2 M NaOH, CH_3CH_2OH, reflux.

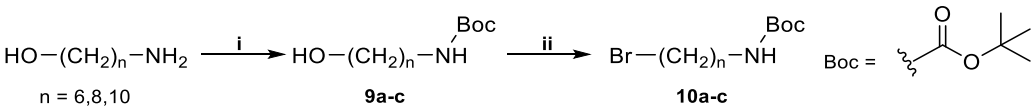
To convert the obtained pyridone into a pyridine methyl ether, compound **4** was methylated by iodomethane. Subsequent addition of 1 M potassium hydroxide solution converted the monocyclic structure into the bicyclic thienopyridine ring **5**. The amino group of **5** was

protected with a phthalimide group to circumvent *N*-halogenation in the following step. Therefore, compound **5** was reacted with phthalic anhydride in a solution of acetic acid. The obtained *N*-protected product **6** was chlorinated in 5-position by *N*-chlorosuccinimide in an electrophilic aromatic substitution reaction to give compound **7**. Following *N*-deprotection of **7** was performed with hydrazine monohydrate and afforded intermediate compound **8**. Finally, sodium hydroxide mediated hydrolysis of the ester group and successive acidic reaction work-up yielded the desired thienopyridine carboxylic acid scaffold **1**.

3.2.2 Synthesis of orthosteric amine linkers

For the preparation of orthosteric amine linkers **1-Ln**, **2-Ln**, and **3-Ln**, three unbranched terminal bromoalkanes with a *tert*-butyloxycarbonyl protected amine group (Boc) at the opposite ending of the chain were synthesized as intermediate compounds. At first, a Boc protection of the respective alkanolamine was performed in the presence of di-*tert*-butyl dicarbonate and TEA to give linkers **9a-c**. Intermediates **9a-c** were also required for the synthesis of tertiary xanomeline linkers **4-Ln**.[104] Thereafter, the alcohol was converted into a good leaving group by addition of methanesulfonyl chloride under basic reaction conditions. To ensure a suitable counterion in later steps of the total synthesis, the mesylate was directly substituted by a bromine group through conversion with lithium bromide (Scheme 2). For the following nucleophilic substitution reactions of the thus obtained linkers **10a-c**, the bromine residue represents a sufficient good leaving group.

Scheme 2: Synthesis of alkyl linker moieties **10a-c**.

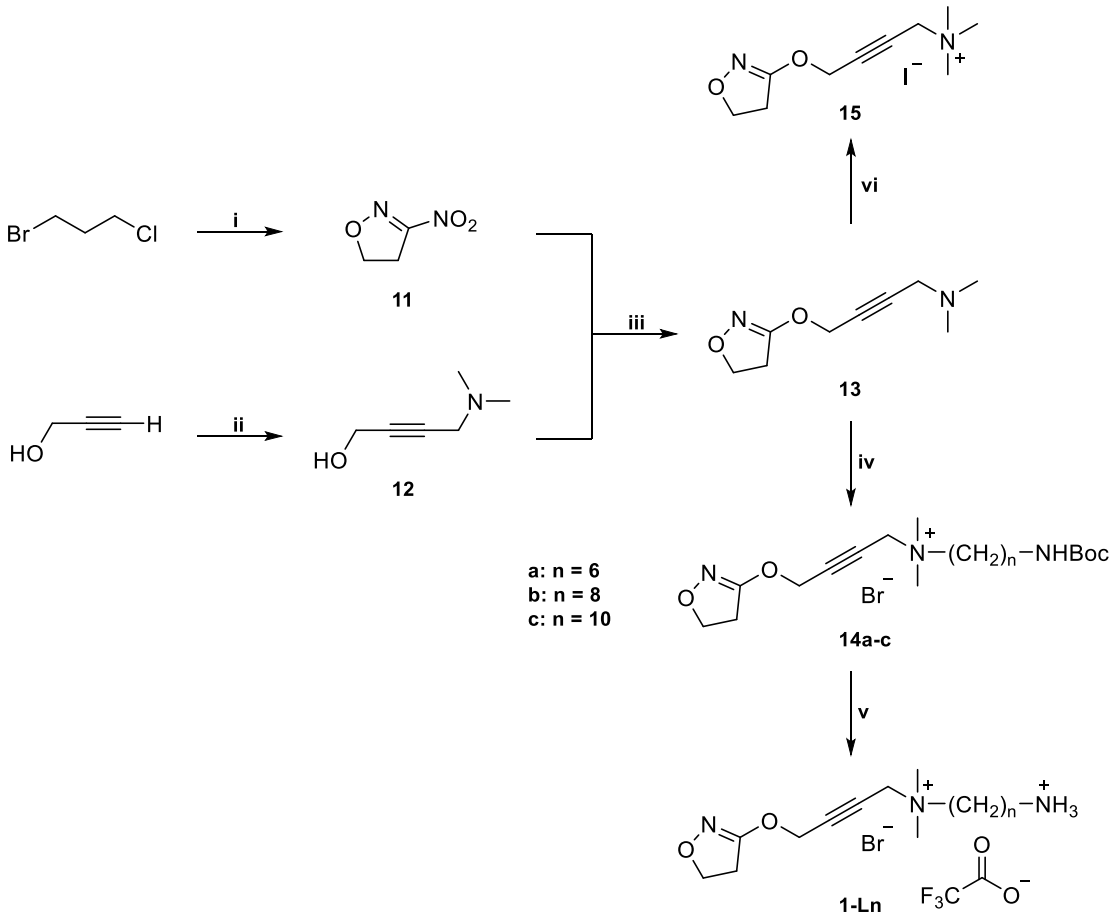


Reagents and conditions: (i) Di-*tert*-butyl dicarbonate, TEA, DCM, rt; (ii) a) methanesulfonyl chloride, TEA, DCM, 0 °C → rt; b) LiBr, THF, reflux.

*3.2.2.1 Synthesis of iperoxo linkers **1-Ln***

According to a literature procedure from Klöckner *et al*., iperoxo base was obtained in a three-step synthesis.[111] Then, iperoxo base was connected to linkers **10a-c**, followed by Boc-deprotection (Scheme 3).

Scheme 3: Synthesis of iperoxo linkers **1-Ln** and iperoxo (**15**).



Reagents and conditions: (i) $NaNO_2$, isopentyl nitrite, DMSO, rt; (ii) dimethylammonium chloride, aqueous formaldehyde solution, $CuSO_4 \cdot 5H_2O$, pH = 8, H_2O, 80 °C; (iii) NaH, THF, reflux; (iv) **10a-c**, CH_3CN, 78 °C (microwave); (v) TFA, DCM, -20 °C → rt; (vi) CH_3I, $CHCl_3$, rt.

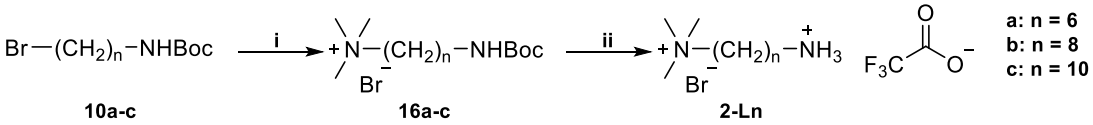
The synthesis started from 1-bromo-3-chloropropane which was reacted with sodium nitrite to replace the bromide substituent by a nitro group. Thereupon, the addition of isopentyl nitrite led to the formation of intermediate compound 3-nitro-Δ^2-isoxazoline (**11**). The mechanism of this isoxazoline formation presumably begins with deprotonation of the acidic proton in α-position of the nitro group and a subsequent insertion of the NO-group. The reaction is completed by a further deprotonation in α-position of the nitro group, followed by intramolecular ring closure. Concurrently, a Mannich reaction with 2-propyn-1-ol, dimethylammonium hydrochloride, and an aqueous formaldehyde solution delivered 4-dimethylaminobut-2-yn-1-ol (**12**). Compounds **11** and **12** were combined by deprotonation

of the alcohol group of **12** with sodium hydride and an ensuing nucleophilic displacement of the nitro group of **11** to afford iperoxo base (**13**). The latter served as nucleophile in the following substitution reaction with linkers **10a-c**. To avoid excessively long reaction times and the formation of small amounts of undesirable side products in this step, the substitution was performed under microwave irradiation. For the same reason, iperoxo base (**13**) was used in a small excess. Purification of the formed quaternary amines **14a-c** was shown to deliver highest yields by column chromatography on deactivated basic aluminum oxide. Finally, iperoxo linkers **1-Ln** were obtained as trifluoroacetate salts after trifluoro acetic acid (TFA) mediated Boc deprotection. Besides to this reaction sequence, iperoxo (**15**) was synthesized to serve as reference substance for later pharmacological investigations. Hence, iperoxo base (**13**) was methylated with iodomethane according to Klöckner *et al*. to afford **15** quantitatively.[111]

*3.2.2.2 Synthesis of trimethyl amine linkers **2-Ln***

TMA linkers **2-Ln** were obtained in a two-step synthesis (Scheme 4). At first, linkers **10a-c** were heated in an ethanolic solution of trimethylamine in a sealed tube. The occurring nucleophilic displacement reaction afforded the Boc-protected TMA linkers **16a-c**. Subsequently, the protecting group was removed by stirring **16a-c** in TFA to give the desired linkers **2-Ln**.

Scheme 4: Synthesis of TMA linkers **2-Ln**.



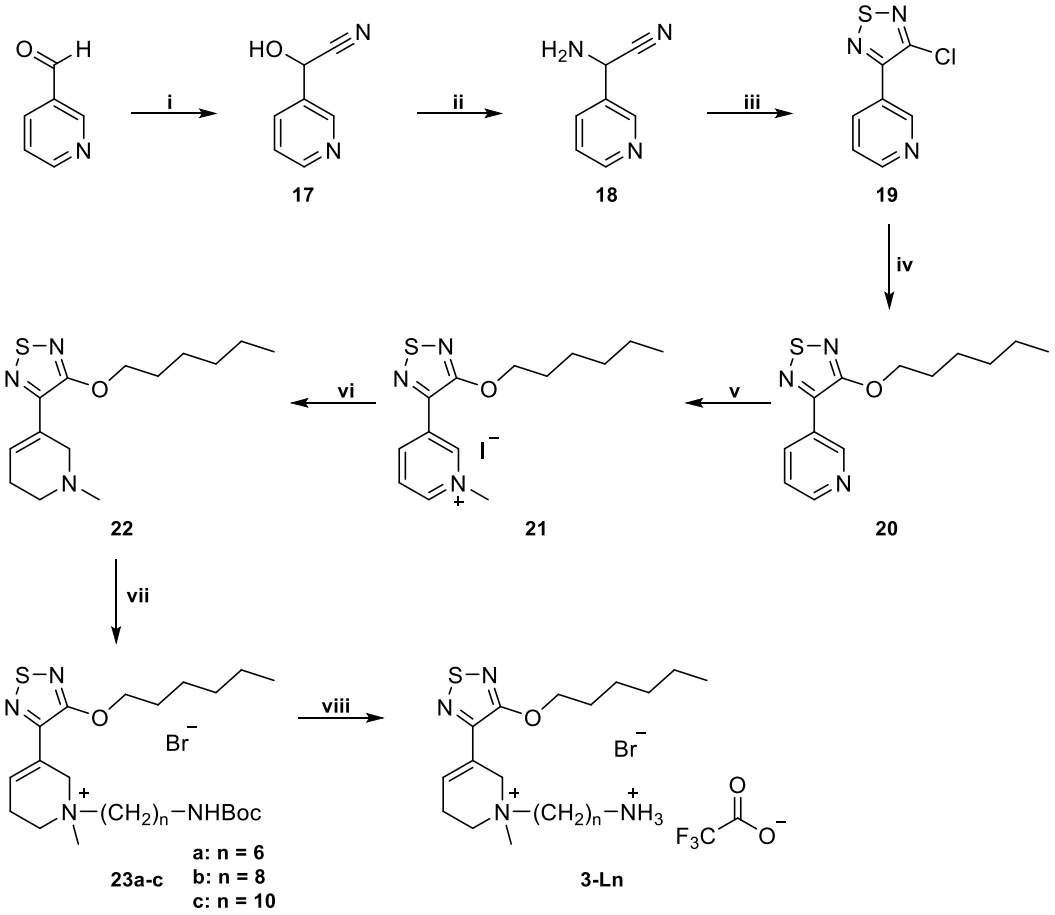
Reagents and conditions: (i) trimethylamine, CH_3CH_2OH, sealed tube, 100 °C; (ii) TFA, DCM, -20 °C → rt.

*3.2.2.3 Synthesis of quaternary xanomeline linkers **3-Ln***

The preparation of quaternary xanomeline linkers **3-Ln** was achieved in eight steps (Scheme 5). The first six steps thereof were conducted according to a literature procedure.[112] The total synthesis started from pyridine-3-carboxaldehyde which was converted to cyanohydrin **17** upon treatment with potassium cyanide and acetic acid. Crude product **17** was then reacted with ammonium chloride in the presence of an aqueous ammonia solution. Thereby, the alcohol group was exchanged by an amino group to give the aminonitrile **18** which was again used without further purification. Next, the formation of the thiadiazole ring was achieved through a

cyclization reaction of intermediate **18** with sulfur monochloride. Unlike intermediate compounds **17** and **18**, thiadiazole **19** did not decompose during purification by column chromatography and, furthermore, can be stored at room temperature.

Scheme 5: Synthesis of quaternary xanomeline linkers **3-Ln**.



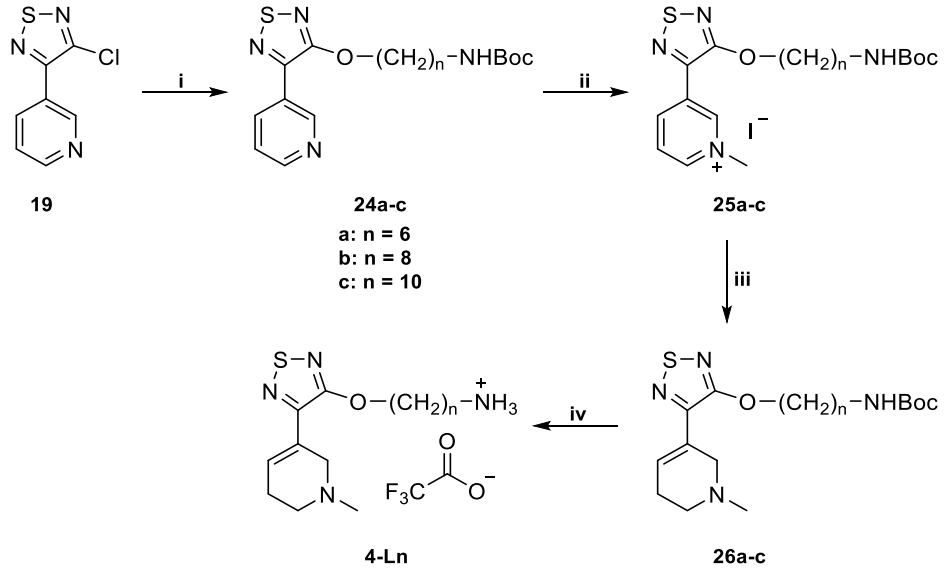
Reagents and conditions: (i) KCN, acetic acid, ethyl acetate, 5 °C → rt; (ii) NH_3/NH_4Cl, H_2O, rt; (iii) S_2Cl_2, DMF, 0 °C; (iv) 1-hexanol, NaH, THF, reflux; (v) CH_3I, acetone, rt; (vi) $NaBH_4$, CH_3CH_2OH, 0 °C → rt; (vii) **10a-c**, CH_3CN, 78 °C (microwave); (viii) TFA, DCM, -20 °C → rt

The chloro substituent of compound **19** was then replaced in a nucleophilic substitution reaction by hexanolate. The latter was formed previously by deprotonation of 1-hexanol using sodium hydride as strong, non-nucleophilic base. The thereby formed intermediate **20** was methylated at the nitrogen atom of the pyridine ring by treatment with methyl iodide to afford the quaternary ammonium salt **21**. A subsequent reduction of the quaternary pyridinium salt to a tetrahydropyridine ring with sodium borohydride resulted in the formation of xanomeline (**22**). In the following step, linkers **10a-c** were introduced by a nucleophilic substitution reaction under assistance of microwave irradiation to give the Boc protected amines **23a-c**. Finally, TFA-mediated removal of the Boc group delivered quaternary xanomeline amine linkers **3-Ln** as trifluoroacetate salts.

*3.2.2.4 Synthesis of tertiary xanomeline linkers **4-Ln***

Tertiary xanomeline linkers **4-Ln** were prepared in cooperation with Gülşah Bayraktar according to She *et al*.[113] and Kane *et al*.[114] The respective Boc-protected alkanolamine **9a-c** was deprotonated by sodium hydride and then reacted with intermediate **19** to substitute the chlorine atom in a nucleophilic displacement reaction. The obtained compounds **24a-c** were methylated at the pyridine nitrogen atom by addition of methyl iodine to give pyridinium salts **25a-c**. A subsequent reduction with sodium borohydride gave tetrahydropyridine derivatives **26a-c** which were then converted to the tertiary xanomeline linkers **4-Ln** in a TFA mediated Boc deprotection reaction (Scheme 6).

Scheme 6: Synthesis of tertiary xanomeline linkers **4-Ln**.

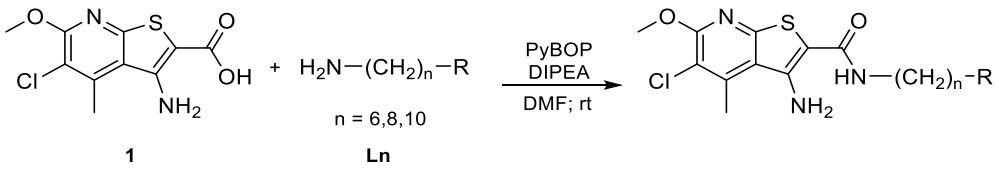


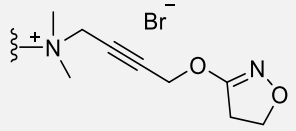
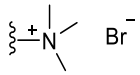
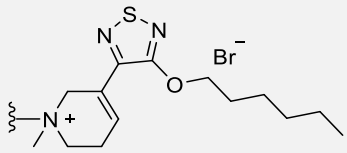
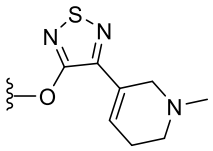
Reagents and conditions: (i) **9a-c**, NaH, THF, reflux; (ii) CH_3I, acetone, rt; (iii) $NaBH_4$, CH_3CH_2OH, 0 °C → rt; (iv) TFA, DCM, -20 °C → rt.

3.2.3 Synthesis of LY20-An hybrid compounds

The final step in the preparation of LY20-An hybrids was a PyBOP (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate) mediated amide coupling reaction between thienopyridine carboxylic acid **1** and the respective orthosteric amine linkers **Ln**. A comparable procedure has already been described by Szabo *et al*. for the synthesis of hybrid ligands at dopamine receptors.[104] At first, one equivalent of DIPEA was used to deprotonate **1**. The formed carboxylate was activated by PyBOP and reacted with the respective **Ln** linkers at room temperature. Since the amines **1-Ln**, **2-Ln**, and **3-Ln** were formerly isolated as the corresponding TFA salts, these linkers were also treated with one equivalent of DIPEA prior to addition to the reaction mixture. PyBOP serves as peptide coupling reagent within the reaction. Unlike the related coupling reagent BOP, the use of PyBOP does not lead to the formation of hexamethylphosphoramide as carcinogenic waste product and is thus preferential.[115] Purification of the crude products was achieved by column chromatography on basic alumina by using different ratios of chloroform and methanol. Since especially **LY20-An-iper** and **LY20-An-TMA** hybrids only show a very weak absorbance of ultraviolet light at a wavelength of 254 nm, Dragendorff's reagent was used as color reagent to detect product spots on thin-layer chromatography (TLC) plates. The formed insoluble ammonium tetraiodobismuthate complex salts can be detected as orange precipitate. That way, twelve hybrid ligands were produced and purified (Table 1). Additionally, thermodynamic solubility of **LY20-A6-iper** in PBS buffer (phosphate-buffered saline) (pH 7.4) was investigated. Following a continuous shake flask protocol[116], a moderate solubility of 0.36 mg/ml in PBS buffer was determined for **LY20-A6-iper**.

Table 1: Synthesis of amide functionalized LY2033298-hybrid compounds.



R	n	Substance code
	6	LY20-A6-iper
	8	LY20-A8-iper
	10	LY20-A10-iper
	6	LY20-A6-TMA
	8	LY20-A8-TMA
	10	LY20-A10-TMA
	6	LY20-A6-XanA
	8	LY20-A8-XanA
	10	LY20-A10-XanA
	6	LY20-A6-XanB
	8	LY20-A8-XanB
	10	LY20-A10-XanB

3.3 Preparation of amide functionalized LY2119620-hybrids

The synthesis of bitopic ligands through functionalization of the amido group of LY2119620 was evaluated as well. So far, no hybrid ligands based on the allosteric modulator LY2119620 have been reported. Iperoxo and TMA were selected as adequate orthosteric building blocks, whereas xanomeline was not inserted within the LY21-An series. Xanomeline shows a preferred binding for M_1 and M_4 subtypes and is thus less attractive for the combination with LY2119620 which is known to interact effectively with an EDGE sequence of the M_2 receptor through the piperazine ring.[7, 50, 51, 98] The general approach for the preparation of LY21-An hybrids is derived from the synthesis of LY2119620.[67] Therein, a thiomethyl group in 6-position of the thienopyridine ring was converted into a leaving group through mono-oxidation. Subsequently, the obtained sulfoxide can be substituted in a nucleophilic aromatic displacement reaction. This strategy allows the integration of the piperazine ring into the

molecule (Figure 17). To follow the described reaction pathway, thienopyridine carboxylic acid **27** was synthesized at first.

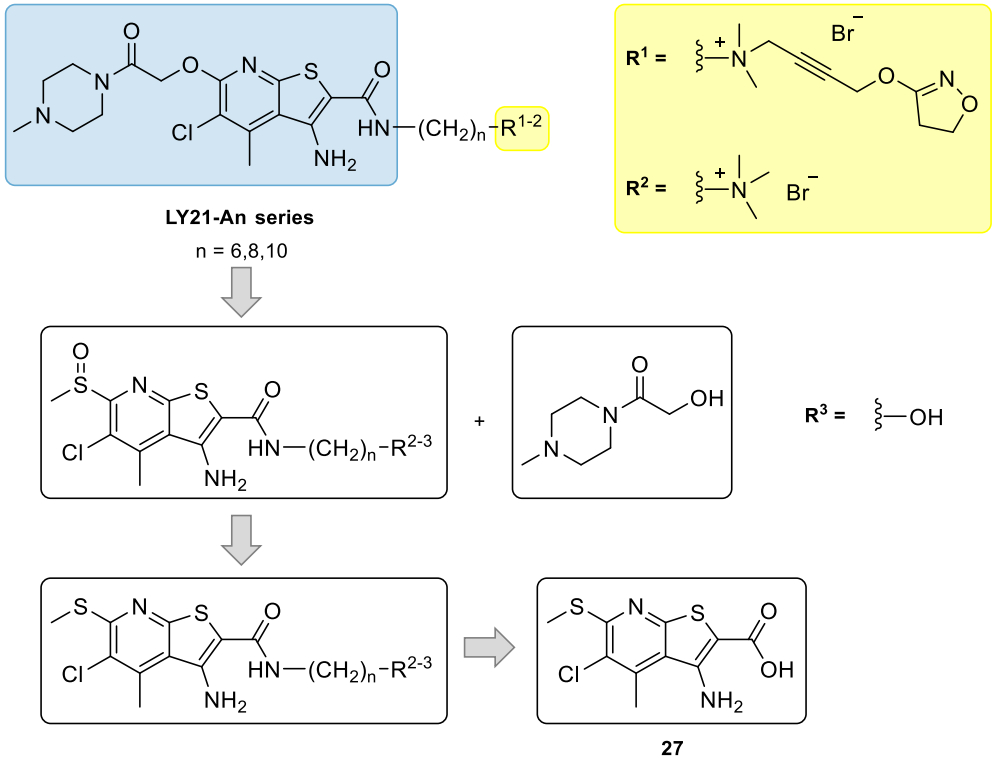
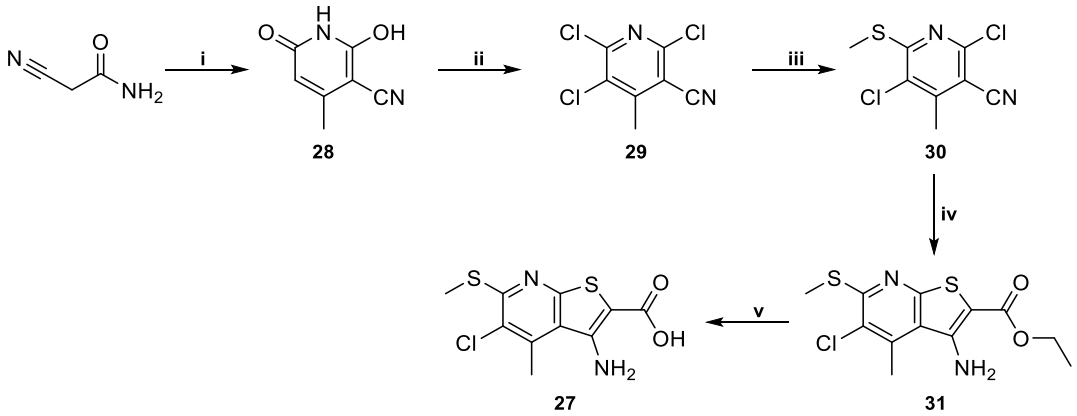


Figure 17: Retrosynthesis of LY21-An series.

3.3.1 Synthesis of thienopyridine carboxylic acid 27

The synthesis route of thienopyridine carboxylic acid scaffold **27** is depicted in Scheme 7. Firstly, cyanoacetamide was reacted with ethyl chloroacetate according to Renck *et al*. in a Guareschi-Thorpe condensation in the presence of potassium hydroxide to give pyridone derivative **28**.[117] Next, three chlorine substituents were induced in one synthesis step by following a literature procedure by Lounasmaa *et al*.[118] Therefore, pyridone **28** was grained with three equivalents of phosphorous pentachloride, followed by heating at 160 °C. The reaction mechanism includes an electrophilic aromatic substitution in meta position of **28** and two nucleophilic aromatic substitutions in ortho position. This reaction suffers from poor yields, ranging between 10 and 20 %. However, neither a variation of PCl_5 equivalents between two and five, nor a reduction of the reaction temperature to 150 °C resulted in better yields. Extension of the reaction time showed no positive effect, whereas a reduction of the reaction time even led to less product formation.

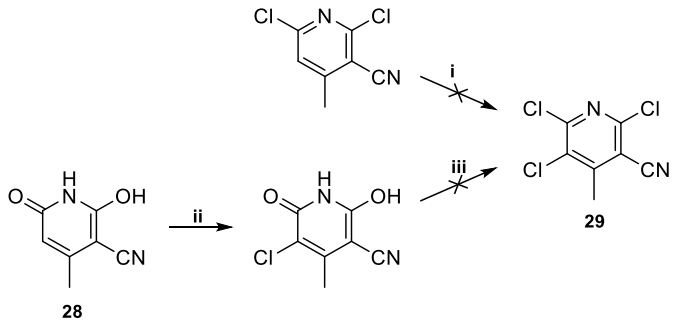
Scheme 7: Synthesis of thienopyridine carboxylic acid scaffold **27**.



Reagents and conditions: (i) ethyl acetoacetate, KOH, CH_3OH, reflux; (ii) PCl_5, 160 °C; (iii) sodium methanethiolate, CH_3OH, 0 °C → rt; (iv) ethyl 2-mercaptoacetate, Na_2CO_3, CH_3CH_2OH, reflux; (v) 2 M NaOH, CH_3CH_2OH, reflux.

For that reason, alternative synthesis routes leading to the formation of compound **29** were investigated. A chlorination of commercially available 2,6-dichloro-4-methyl-3-pyridinecarbonitrile by PCl_5 was tested but did not result in product formation (Scheme 8). Consequently, the electronic withdrawing chlorine substituents in ortho position of the pyridine ring seem to disadvantage an electrophilic aromatic substitution reaction in meta position. Accordingly, when pyridone **28** is converted to compound **29**, the electrophilic aromatic substitution must take place first. Thus, intermediate **28** was reacted with sulfuryl chloride to give a pyridine derivative which only bears one chlorine substituent in meta position. The latter was then heated in phosphoryl chloride according to a literature procedure by Dyadyuchenko *et al.*[119] Once again, no product formation was observed. Even variation of the reaction temperature did not yield pyridine derivative **29**. Eventually, no synthetic route delivering compound **29** in a higher yield with less effort was found (Scheme 8). The conversion of pyridone **28** with PCl_5 is accompanied by the formation of several side products, thus making the following purification challenging. Therefore, a new approach involving purification by column chromatography under the use of a mixture of toluene and cyclohexane as eluent, followed by crystallization of the obtained yellow oil in ice-cooled methanol proved to be more efficient than the reported literature procedure by Lounasmaa *et al.*[118]

Scheme 8: Investigated alternative synthetic pathways for compound **29**.



Reagents and conditions: (i) PCl_5, 160 °C; (ii) sulfuryl chloride, CH_3CN, 0 °C → rt; (iii) $POCl_3$, 120-180 °C, sealed tube.

According to a literature procedure by Rubio *et al.*, compound **29** was thiomethylated in ortho position.[67] The more reactive chlorine substituent in 6-position was thereby addressed selectively by maintaining the reaction temperature at 0 °C. When the reaction was performed at an elevated temperature, side product formation through a nucleophilic aromatic substitution in 2-position was observed. Notably, the applied nucleophile sodium methanethiolate must be stored under exclusion of moisture to avoid formation of methanethiol. The highest yield of **30** was obtained when exactly one equivalent of sodium methanethiolate was added. Therefore, it was advantageous to use sodium methanethiolate of high purity.

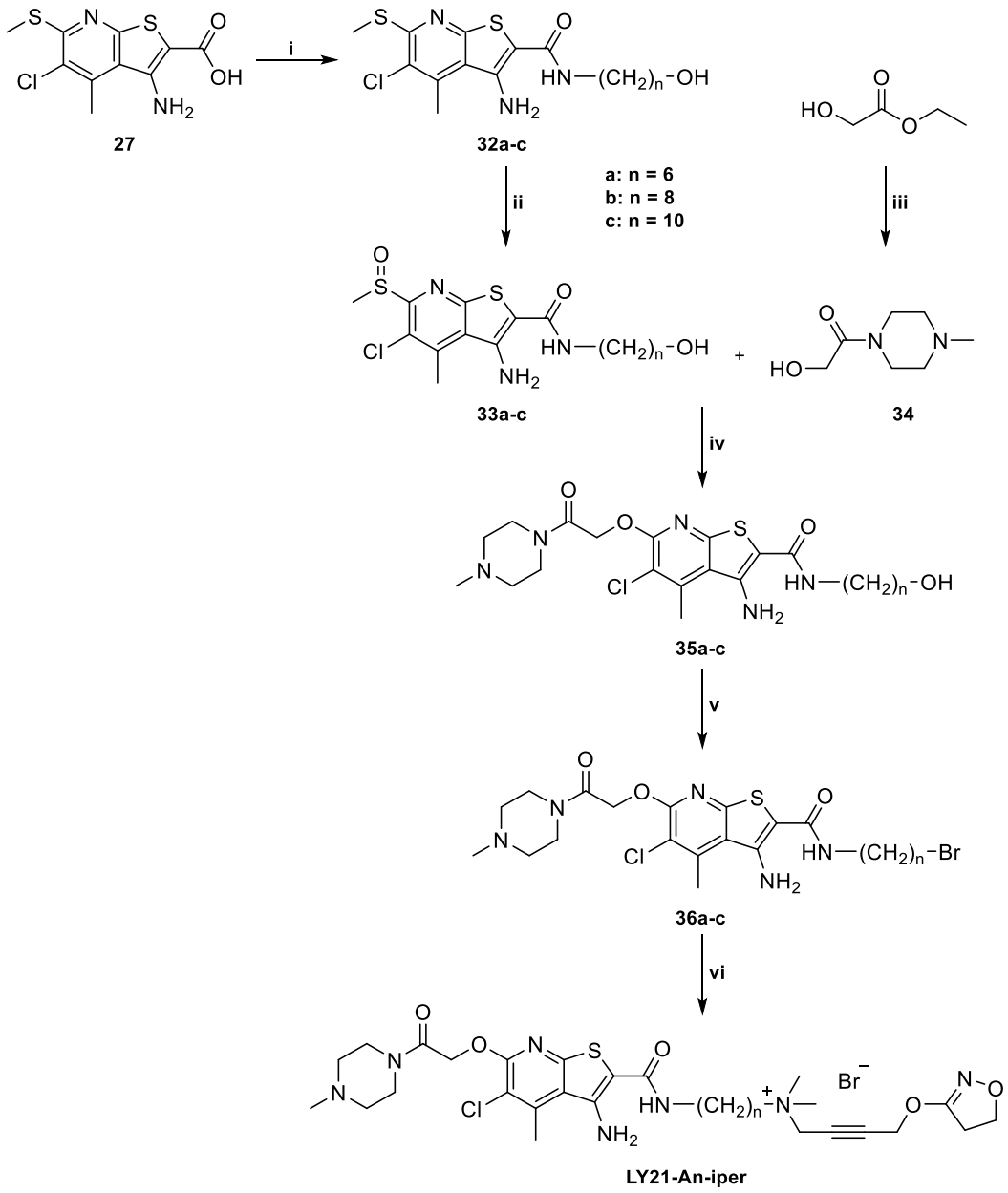
Next, compound **30** was reacted with ethyl-2-mercaptoacetate under the presence of sodium carbonate. This leads to deprotonation of the thiol group, followed by a nucleophilic aromatic substitution of the chlorine substituent in 2-position of **30** by the formed thiolate. Thereafter, thienopyridine ring formation occurs through twofold base catalyzed deprotonation in α-position of the carbonyl group. The nitrile group in 3-position is thereby converted into a primary aromatic amine. Finally, the ester of the thus obtained thienopyridine derivative **31** was cleaved by addition of sodium hydroxide solution to give compound **27**. The latter was further used for the synthesis of **LY21-An-iper** and **LY21-An-TMA** hybrid ligands.

3.3.2 Synthesis of LY21-An-iper hybrid compounds

The accomplished pathway for the synthesis of **LY21-An-iper** hybrid compounds from thienopyridine derivative **27** is shown in Scheme 9. First of all, compound **27** was linked with alkanolamines of different chain length by following the reaction protocol for PyBOP mediated amide coupling reactions that has formerly been described in the synthesis of LY20-An hybrid ligands. The subsequent oxidation of sulfides **32a-c** to sulfoxides **33a-c** was performed according to a modified procedure from Rubio *et al.*, therein used for the synthesis of

LY2119620.[67] The addition of one equivalent of hydrogen peroxide in acetic acid at 35 °C led to mono-oxidation of the sulfide group. Notably, an only slightly increased temperature of more than 35 °C directly caused the formation of the respective sulfone instead of sulfoxides **33a-c**.

Scheme 9: Synthesis of **LY21-An-iper** hybrid compounds.



Reagents and conditions: (i) $H_2N(CH_2)_nOH$, PyBOP, DIPEA, DMF, rt; (ii) H_2O_2, acetic acid, 35 °C; (iii) dioxane, 120 °C, sealed tube; (iv) LiHMDS, THF, reflux; (v) a) MsCl, DIPEA, DCM, 0 °C, b) LiBr, THF, reflux; (vi) **13**, CH_3CN, 35 °C.

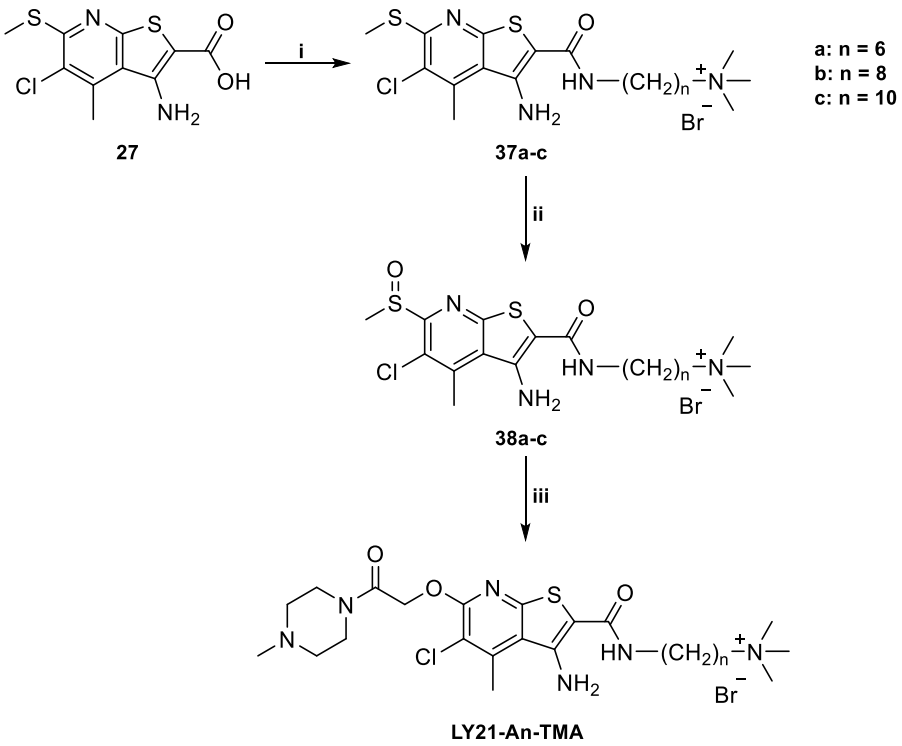
Even at the proposed temperature of 35 °C and in the presence of only one equivalent of hydrogen peroxide, sulfone formation was observed in relevant amounts. Moreover, longer reaction times promoted an esterification of the alcohol group of compounds **33a-c** with acetic acid. Consequently, the reaction had to be stopped before full conversion of the starting material

was observed. Because of the presence of the mentioned side products, purification of sulfoxides **33a-c** by column chromatography was necessary. In conclusion, compounds **33a-c** were obtained in good yields between 57 to 64 % when 1.15 equivalents of hydrogen peroxide were used and, at the same time, the reaction time did not exceed 48 h. In the next step, thienopyridine derivatives **33a-c** were linked to the piperazine moiety **34** in a nucleophilic aromatic displacement reaction. The strong non-nucleophilic base LiHMDS deprotonates the alcohol group of **34**. The thus formed alkoxide replaces the sulfoxide substituent of **33a-c** and thereby gives compounds **35a-c**. Intermediate **34** was previously synthesized by formation of an amine bond between ethyl-2-hydroxyacetate and *N*-methyl piperazine according to Castro *et al*.[120] This proceeding allows the addition of the piperazine moiety to the thienopyridine ring within two steps. A different approach, reported by Rubio *et al*. in the synthesis of LY2119620, requires three steps for this reaction sequence.[67] Before the orthosteric ligand iperoxo could be introduced into the molecule, the alcohol group of **35a-c** had to be converted into a good leaving group. Therefore, the alcohol was deprotonated by DIPEA, followed by the addition of mesyl chloride. It is noteworthy that the replacement of DIPEA by TEA resulted in failure of the reaction. As the obtained mesylate was unstable under any storage conditions, the mesyl group was directly substituted by bromide by means of addition of lithium bromide. Additionally, bromide represents a more suitable counterion for the desired hybrid ligands. Still, intermediates **36a-c** must be used within days because the purity of these compounds decreased remarkably over time. This might be due to intermolecular reactions between the amine function of the piperazine ring and the bromide substituent of the alkyl chain. The final step of the synthesis involved a nucleophilic displacement reaction of **36a-c** and iperoxo base **13**. To achieve full conversion of compounds **36a-c**, five equivalents of **13** were added. The temperature during the reaction may not exceed 35 °C, otherwise several undefinable side products were formed. The adequate reaction time was determined to range between eight and ten days. Since purification by column chromatography on silica led to a complete loss of the final product, basic alumina was chosen as stationary phase. In summary, this proceeding allowed the isolation of the three hybrid molecules **LY21-A6-iper**, **LY21-A8-iper**, and **LY21-A10-iper**.

3.3.3 Synthesis of LY21-An-TMA hybrid compounds

Hybrid compounds **LY21-An-TMA** were synthesized from thienopyridine derivative **27** in three steps (Scheme 10). Firstly, an amide coupling reaction of **27** with the respective orthosteric amine linker **2-Ln** allowed the isolation of compounds **37a-c**. The following mono-oxidation of sulfides **37a-c** to sulfoxides **38a-c** was performed by using *m*-chloroperoxybenzoic acid (mCPBA) as oxidizing agent. Thereby, the temperature was kept at -78 °C to prevent the formation of sulfones. The afore described conditions for the oxidation of sulfides **33a-c** were not applied in this step because the utilization of hydrogen peroxide and acetic acid resulted in only low yields between 10 and 20 %. Subsequently, the sulfoxides **38a-c** were added to a solution of the piperazine moiety **34** and LiHMDS. The nucleophilic substitution of the sulfoxide group by the created alkoxide allowed the isolation of the three target molecules **LY21-A6-TMA**, **LY21-A8-TMA**, and **LY21-A10-TMA**.

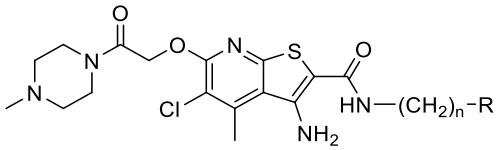
Scheme 10: Synthesis of LY21-An-TMA hybrid compounds.

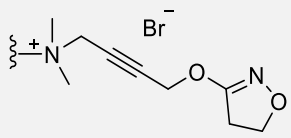
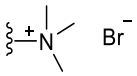


Reagents and conditions: (i) **2-Ln**, PyBOP, DIPEA, DMF, rt; (ii) mCPBA, $NaHCO_3$, DCM, -78 °C; (iii) **34**, LiHMDS, THF, reflux.

In conclusion, Table 2 sums up the six synthesized hybrid ligands within the **LY21-An series**.

Table 2: Overview of synthesized amide functionalized LY2119620-hybrid compounds.



R	n	Substance code
	6	LY21-A6-iper
	8	LY21-A8-iper
	10	LY21-A10-iper
	6	LY21-A6-TMA
	8	LY21-A8-TMA
	10	LY21-A10-TMA

3.4 Preparation of LY2033298-hybrids by using the methyl side chain as functionalization unit

Since functionalization of LY2033298 at the methyl group in 4-position of the thienopyridine ring has not been reported so far, an entirely new synthesis route for the preparation of LY20-MAn hybrids and LY20-MQn hybrids was developed (Figure 18). Therefore, the questions arise of how and at which stage of the synthesis a substituent can be introduced into the molecule. Regarding possible chemical reactions, a Wohl-Ziegler bromination was chosen to be most promising. Here, the bromination only proofed to be successful when performed before the closure of the thienopyridine ring since the latter turned out to be unstable under the required reaction conditions. LY20-MAn hybrids and LY20-MQn hybrids only differ from each other in the type of connection to the orthosteric linker moiety. For LY20-MAn compounds, an amide coupling was performed in the last step of the reaction pathway, whereas the combination of allosteric and orthosteric building blocks during the synthesis of LY20-MQn compounds was achieved by nucleophilic displacement reactions. This led to the formation of a quaternary amine and thus introduced an additional positive charge into the hybrid molecule. The synthesis routes for both series include intermediate compound **39**.

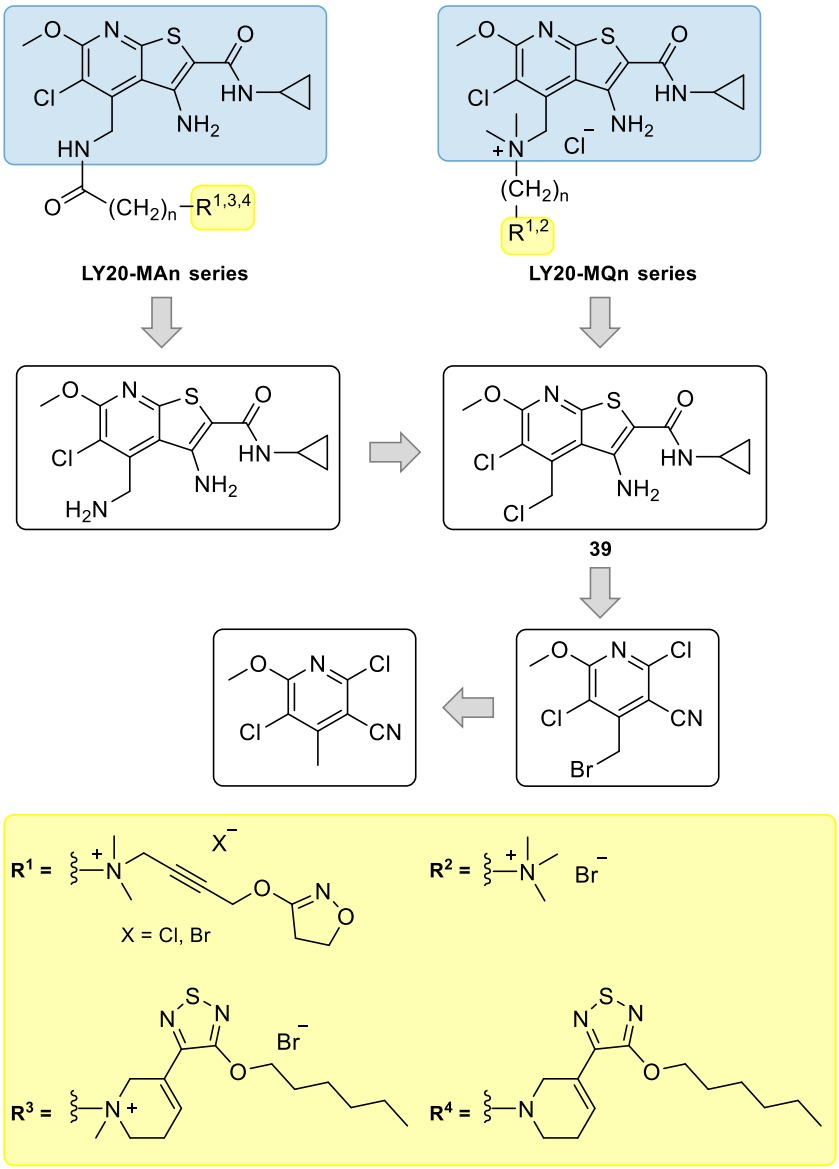


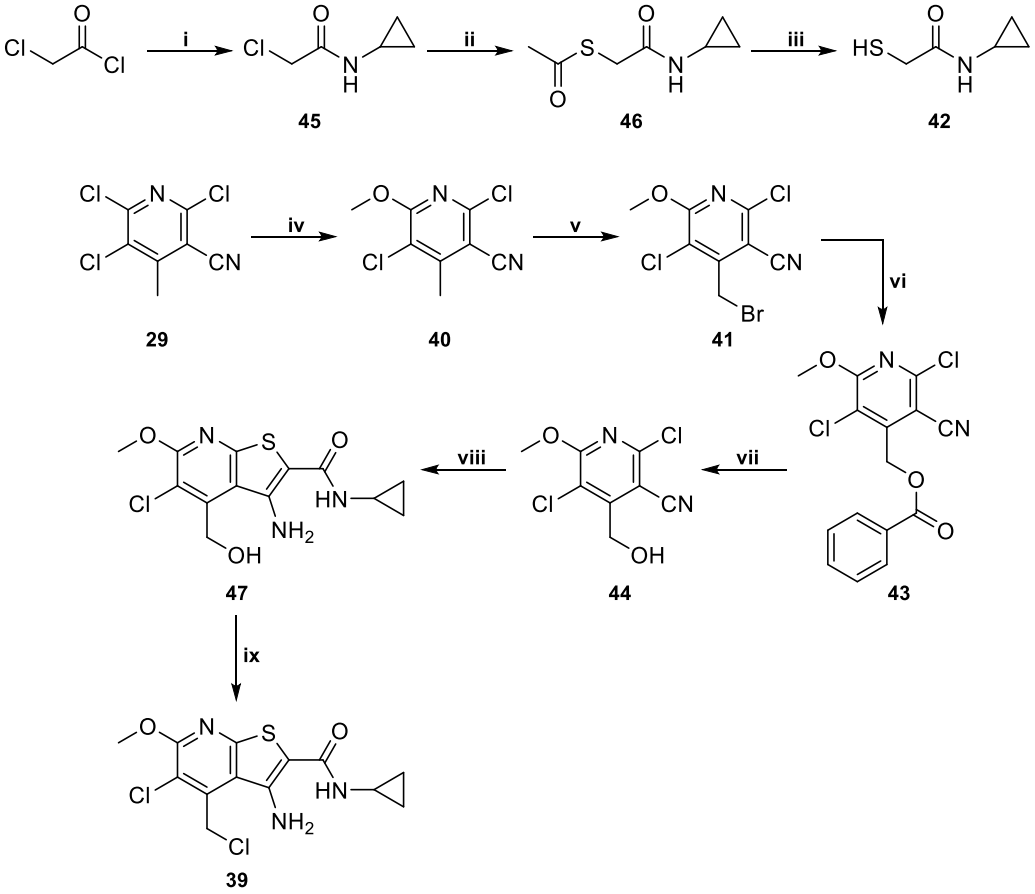
Figure 18: Retrosynthesis of LY20-MQn and LY20-MAn series.

3.4.1 Synthesis of LY2033298 derivative 39

The synthetic pathway for the synthesis of key intermediate **39** is shown in Scheme 11. Therefore, pyridine derivative **29** was firstly converted into compound **40** in a nucleophilic aromatic displacement reaction by using sodium methoxide as nucleophile according to Rubio *et al.*[67] The following Wohl-Ziegler bromination of compound **40** is a crucial step within the synthesis of LY2033298 derivative **39** because dibromination or tribromination can occur as undesired side reaction. Despite its poor safety profile, carbon tetrachloride was applied as solvent therein. A replacement by benzene, dichloromethane, or acetonitrile did not lead to any product formation. *N*-bromo succinimide (NBS) was used as bromination agent and benzoyl peroxide was added as radical initiator. The reaction was performed at a temperature of 110 °C in a sealed pressure tube because no conversion of the starting material was observed at lower

reaction temperatures. After three days of heating, starting material could still be detected by TLC. However, neither longer reaction times, nor further addition of NBS resulted in a full conversion of **40**. Consequently, the following separation of product **41** and starting material **40** by column chromatography proved to be difficult due to very similar retention factors. Compound **41** cannot be applied for a thienopyridine ring closure reaction because the therefore required thiol **42** would displace the bromine substituent of the methyl group instead of the chlorine substituent in 2-position of the pyridine ring after deprotonation. For this reason, the bromine substituent was converted into a primary alcohol in two steps. At first, a S_N2 attack of the nucleophile sodium benzoate led to removal of the bromine group and gave benzoic acid ester **43**.

Scheme 11: Synthesis of LY2033298 derivative **39**.



Reagents and conditions: (i) cyclopropylamine, DCM, 0 °C; (ii) thioacetic acid, TEA, DCM, 0 °C; (iii) NH_4OH, CH_3OH, rt; (iv) sodium methoxide, CH_3OH, 0 °C → rt; (v) NBS, DBO, CCl_4, 110 °C, sealed pressure tube; (vi) sodium benzoate, DMF, rt; (vii) sodium methoxide, CH_3OH, 0 °C → rt; (viii) **42**, Na_2CO_3, CH_3CH_2OH, reflux; (ix) tosyl chloride, TEA, 4-dimethylaminopyridine, DCM, 0 °C → reflux.

The following ester cleavage by means of a sodium methoxide solution allowed the isolation of the primary alcohol **44**. Importantly, exactly one equivalent of sodium methoxide must be

applied during the course of the reaction because a surplus substitutes the chlorine group in 2-position in a nucleophilic aromatic displacement reaction. Simultaneously, chloroacetyl chloride was reacted with cyclopropylamine to give amide **45**. The latter was then converted to compound **46** by addition of thioacetic acid. Both steps were performed according to a procedure by Rubio *et al*.[67] The following cleavage of thioester **46** in the presence of ammonia to form thiol **42** has been described by Rubio *et al.* in the same patent. However, it is not mentioned therein that the applied solvent needs to be degassed prior to use. When methanol was not degassed before the addition of the starting materials, thiol **42** was directly oxidized to give the corresponding disulfide quantitatively. The thus obtained disulfide may then mistakenly be considered as the desired thiol **42**, since the ^{1}H NMR spectra gives adequate signals that also show matching integrals. Solely the expected signal of the thiol group does not appear after disulfide formation, while the spectrum of **42** shows a triplet signal of the thiol proton due to ^{3}J-coupling with the adjacent CH_2-group (Figure 19).

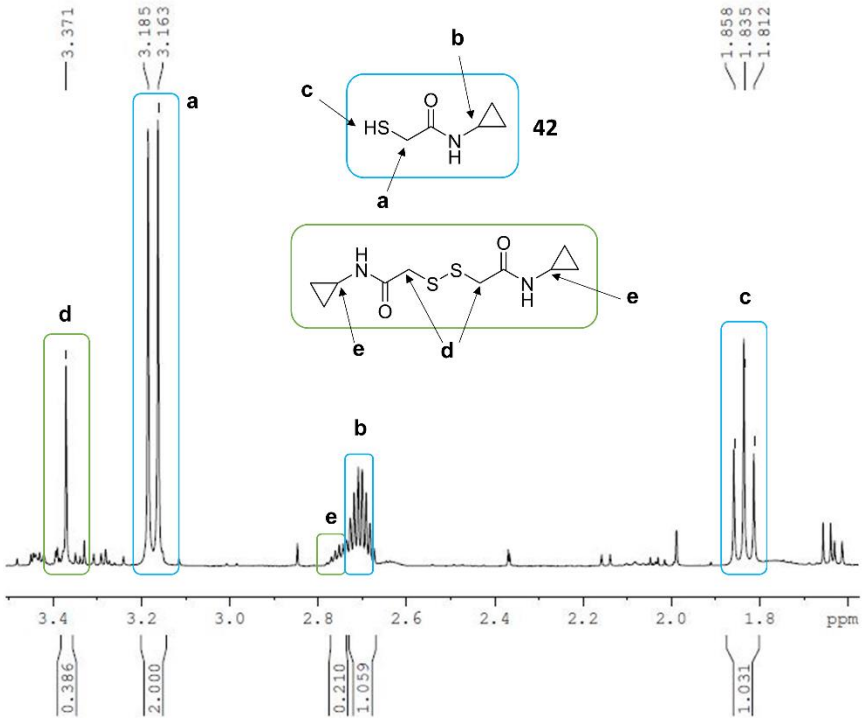


Figure 19: Extract of ^{1}H NMR spectrum of a mixture of thiol **42** and the corresponding disulfide. The signals **a** and **d**, respectively **b** and **e** differ only slightly which complicates the analysis.

Even though degassed solvent was used, disulfide formation could still not be avoided completely. This was probably due to air contact during the reaction work-up. Furthermore, the isolated thiol **42** is not stable under any storage conditions and must thus be converted directly in the next reaction step. Then, thienopyridine ring closure was accomplished by adding freshly

prepared thiol **42** to a solution of **44** and sodium carbonate. Thienopyridine derivative **47** was isolated by simple precipitation in aqueous solution and subsequent filtration.

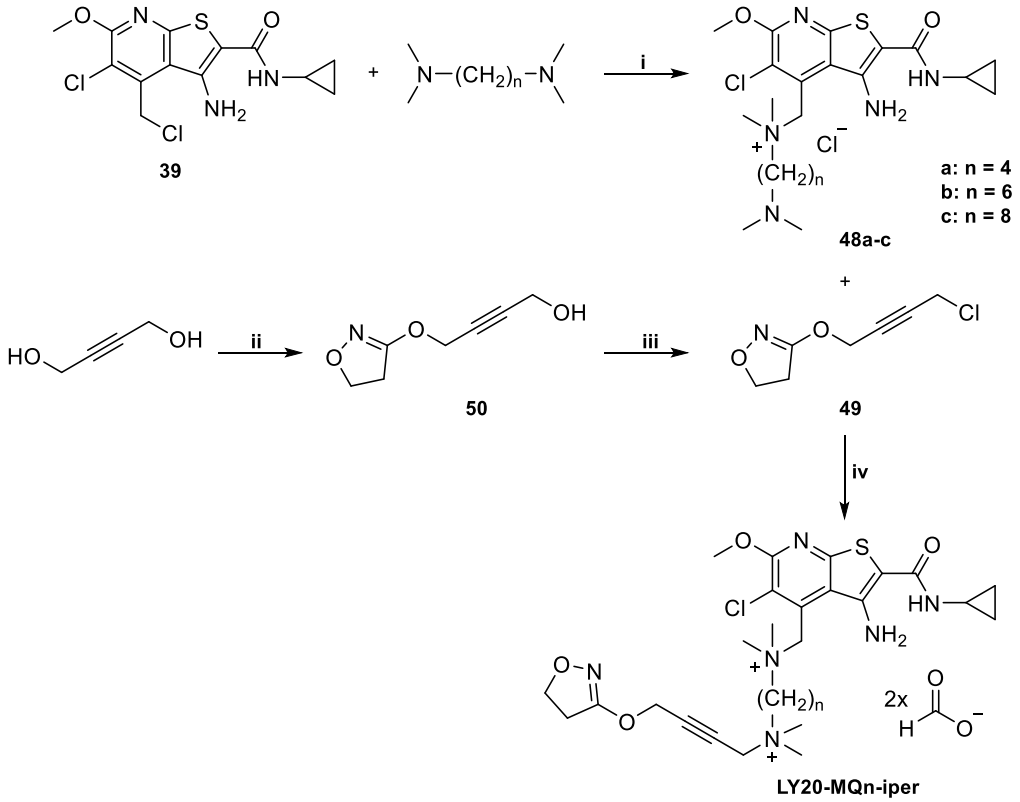
Finally, the primary alcohol of **47** was converted back into a leaving group through addition of tosyl chloride. In the presence of a strong base, the lone pair of the alcohol oxygen displaces the chlorine substituent of tosyl chloride at a temperature of 0 °C. Therefore, 4-dimethylaminopyridine was used as nucleophilic catalyst to accelerate the reaction. However, any attempt to purify the thereby formed tosylate failed due to instability of the product. The tosylate was substituted immediately in the presence of any nucleophile, such as water or the alcohol groups of silica gel. Because a high purity of this intermediate was required for the subsequent steps in the synthesis of LY20-MAn- and LY20-MQn hybrid compounds, this issue proved to be critical for the success of the aimed reaction pathway. Interestingly, reaction control by liquid chromatography-mass spectrometry (LC/MS) indicated the formation of chlorine substituted derivative **39** in small amounts at room temperature. This compound may result from an S_N1 reaction mechanism, triggered by slowly dissociation of the tosylate from the carbon atom. Resonance stabilization of the thus formed carbocation by the thienopyridine ring system presumably enables this reaction mechanism. The amino and methoxy substituents of the thienopyridine ring may thereby serve as strong resonance donors. Afterwards, the carbocation reacts with chloride ions that have previously been released from tosyl chloride.[121] The application of dichloromethane as solvent may also accelerate this reaction pathway because the chloride ions are more exposed in a polar aprotic environment. Heating of the reaction mixture under reflux for three days led to an almost quantitative formation of compound **39**. Unlike the corresponding tosylate, compound **39** was less reactive and could thus be isolated in high purity and good yields. Furthermore, the chlorine substituent proved to be a sufficiently good leaving group during the following reactions.

3.4.2 Synthesis of LY20-MQn hybrid compounds

Within the LY20-MQn hybrid series, iperoxo and TMA were investigated as orthosteric units. For the synthesis of **LY20-MQn-iper** hybrids, key intermediate **39** was reacted with the respective bis(dimethylamino)alkyl linker to give quaternary amines **48a-c** in a nucleophilic displacement reaction (Scheme 12). The crystal structure of the M_2 receptor co-crystallized with LY2119620 and iperoxo had shown that the relative distance between the methyl group of LY2119620 and the quaternary amine of iperoxo is shorter than the respective relative distance of the cyclopropyl group of LY2119620 (Figure 10a).[7] Hence, the required chain length of LY20-MQn hybrids was also assumed to be shorter compared to LY20-An hybrids.

Consequently, the length of the applied bis(dimethylamino)alkyl linkers was ranged between four, six, and eight methylene units. In the final step of the **LY20-MQn-iper** synthesis, compounds **48a-c** were reacted with iperoxo precursor **49**, respectively. The latter was therefore produced according to Dallanoce *et al*. and De Amici *et al.* in two steps.[122, 123] Firstly, one alcohol group of 1,4-butynediol was deprotonated by sodium hydride. The thus formed alkoxide was converted with intermediate **11** to replace the nitro group in a nucleophilic substitution reaction and gave compound **50** which was then treated with thionyl chloride to replace the alcohol group by a chlorine substituent. This yielded the required orthosteric building block **49**. Finally, a nucleophilic attack of tertiary amines **48a-c** at the chlorine substituent of **49** yielded **LY20-MQn-iper** hybrids. Once more, the reaction temperature was optimized to avoid side product formation on the one hand and excessively long reaction times on the other. Best results were obtained at 50 °C, whereas at room temperature no product formation was detected. Reaction control by LC/MS after 3 to 6 hours indicated a product conversion ranging between 65-90 %. However, even longer reaction times did not lead to a full conversion of **48a-c**.

Scheme 12: Synthesis of **LY20-MQn-iper** series.

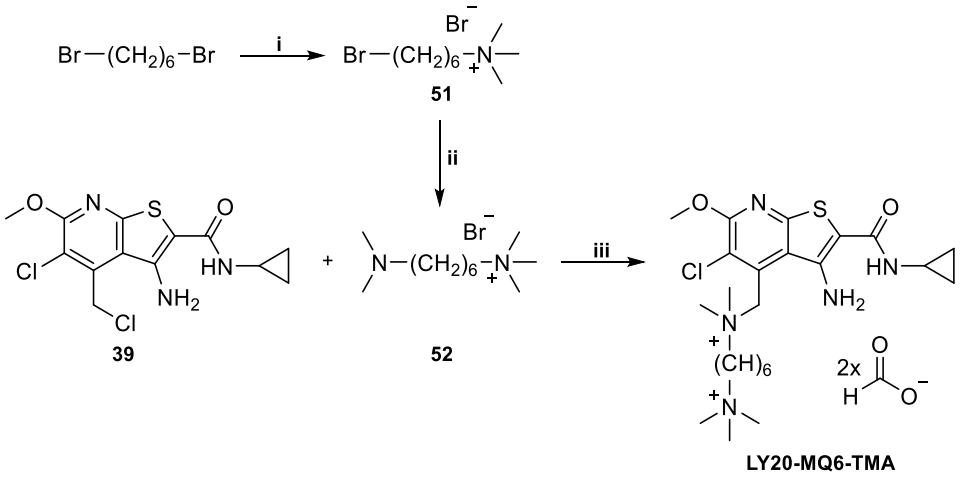


Reagents and conditions: (i) CH_3CN, rt (**48a**), 50 °C (**48b-c**); (ii) **11**, NaH, THF, reflux; (iii) thionyl chloride, pyridine, benzene, DCM, 0 °C → rt; (iv) CH_3CN, 50 °C.

Instead, several spots of undefinable impurities gradually appeared in the LC/MS UV-chromatogram (280 nm), thus indicating a slow decomposition of the hybrid compounds. Due to the two charged quaternary nitrogen atoms, purification of the three hybrids was performed by flash chromatography on a reversed phase (RP) C18 column. Thereby, acid hydrolysis of the isoxazoline ring occurred in the presence of small amounts of formic acid within the solvent mixture and thus resulted in relatively low yields of **LY20-MQn-iper** hybrids ranging between 10-20 %. The high degree of similarity between the desired hybrid ligands and the hydrolyzed side products further complicated the purification. When a solvent mixture without formic acid was used, large amounts of the target compound remained on the C18 column and could only be eluted by flushing the column with a mixture of methanol and 0.1 % of formic acid. Despite the low yields, **LY20-MQn-iper** compounds were isolated in purities of at least 95 %.

Furthermore, the synthesis of **LY20-MQ6-TMA** was achieved in this work (Scheme 13). To obtain an adequate orthosteric linker moiety, 1,6-dibromohexane was reacted with trimethylamine hydrochloride in presence of KOH in a nucleophilic displacement reaction according to Bock *et al*.[124] Separation of compound **51** from the also formed bisquaternary side product was realized by Soxhlet extraction. Then, the bromine group of **51** was substituted by dimethylamine to give the orthosteric TMA linker **52**. The linker was combined with thienopyridine derivative **39** in a nucleophilic displacement reaction and yielded final compound **LY20-MQ6-TMA**.

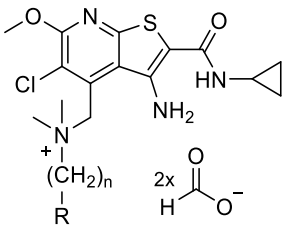
Scheme 13: Synthesis of **LY20-MQ6-TMA**.

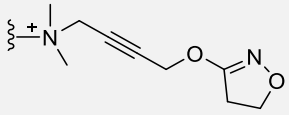


Reagents and conditions: (i) trimethylamine hydrochloride, KOH, CH_3CH_2OH, rt; (ii) dimethyl amine, CH_3CH_2OH, 70 °C; (iii) CH_3CN, 50 °C.

In conclusion, the four synthesized compounds within the LY20-MQn series are summarized in Table 3.

Table 3: Overview of synthesized bis-quaternary LY2033298-hybrid compounds with the methyl side chain as functionalization unit.

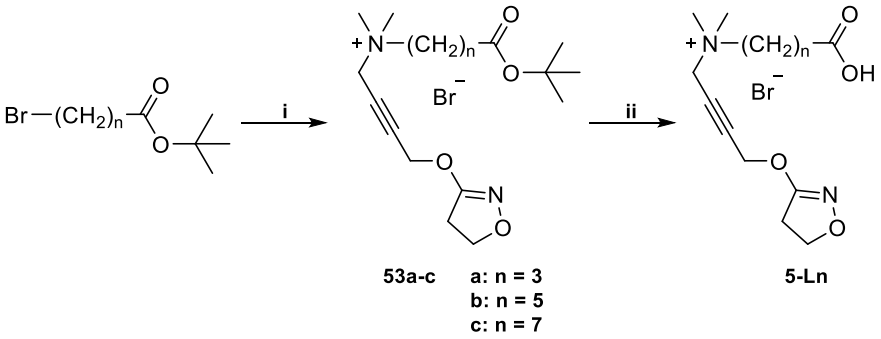


R	n	Substance code
	4	LY20-MQ4-iper
	6	LY20-MQ6-iper
	8	LY20-MQ8-iper
	6	LY20-MQ6-TMA

3.4.3 Synthesis of LY20-MAn hybrid compounds

The functionalization of the methyl group of LY2033298 with an orthosteric linker was further performed through amide coupling reactions. Iperoxo and xanomeline were selected as orthosteric moieties for this purpose. Therefore, a new type of iperoxo linkers (**5-Ln**) with a carboxylic acid function at the end of the alkyl chain was synthesized in two steps (Scheme 14).

Scheme 14: Synthesis of carboxylic acid iperoxo linkers **5-Ln**.

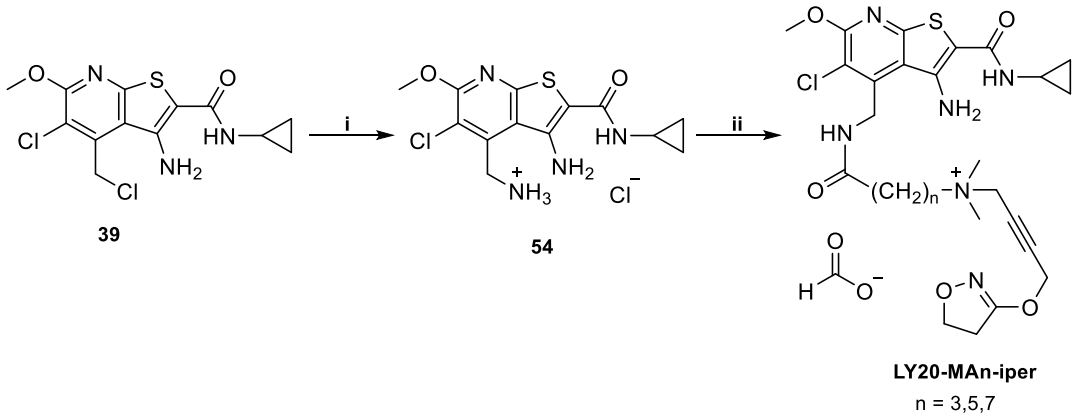


Reagents and conditions: (i) **13**, CH_3CN, 78 °C (microwave); (ii) TFA, DCM, -20 °C → rt.

Firstly, different linkers with a *tert*-butyl ester group at one end of the alkyl chain and a bromine substituent at the opposite end were reacted with iperoxo base **13**. To accelerate the occurring nucleophilic displacement reaction, the mixture was heated using microwave irradiation. After isolation of compounds **53a-c**, the *tert*-butyl group was removed by means of addition of

trifluoroacetic acid, thus resulting in the formation of the required carboxylic acid iperoxo linkers **5-Ln**. Regarding the crystal structure of the M_2 receptor (Figure 10) and the resulting assumption which has already been illuminated in chapter 3.4.2, the chain length of **5-Ln** was varied between three, five, and seven methylene units. Before the implementation of the final amide coupling, compound **39** was reacted with ammonia to convert the chlorine substituent at the methyl group into a primary amine. Intermediate **54** was isolated as the corresponding hydrochloride salt. A PyBOP mediated amide coupling of compound **54** with the respective orthosteric linker **5-Ln** yielded **LY20-MAn-iper** hybrids (Scheme 15). Notably, compound **54** was deprotonated by DIPEA prior to the addition to the reaction mixture. Purification of **LY20-MAn-iper** hybrids was achieved by RP flash chromatography. Unlike **LY20-MQn-iper** hybrids, hydrolysis of the isoxazoline ring in the presence of formic acid was not observed for **LY20-MAn-iper** hybrids. Hence, the additional quaternary amine within the **LY20-MQn-iper** molecules presumably promotes hydrolysis of the isoxazoline ring.

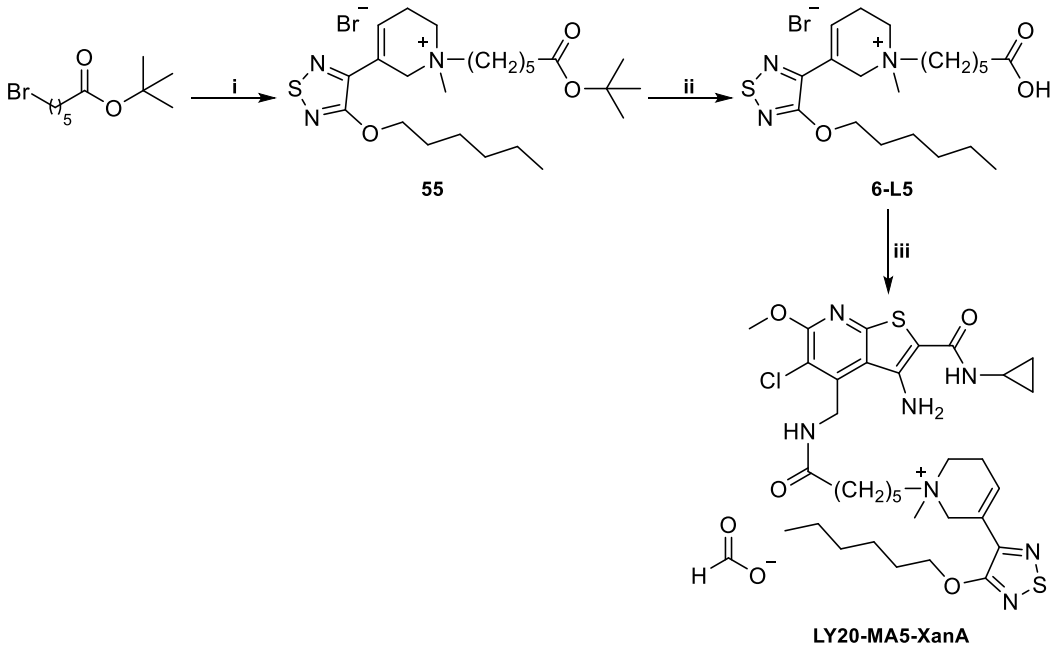
Scheme 15: Synthesis of **LY20-MAn-iper** hybrids.



Reagents and conditions: (i) NH_3, MeOH, reflux, sealed tube; (ii) **5-Ln**, PyBOP, DIPEA, DMF, rt.

In a similar approach, xanomeline **22** was reacted with *tert*-butyl-6-bromohexanoate. The *tert*-butyl group of the obtained quaternary amine **55** was removed and the resulting orthosteric xanomeline linker **6-L5** was reacted with compound **54** via amide coupling to give **LY20-MA5-XanA** (Scheme 16).

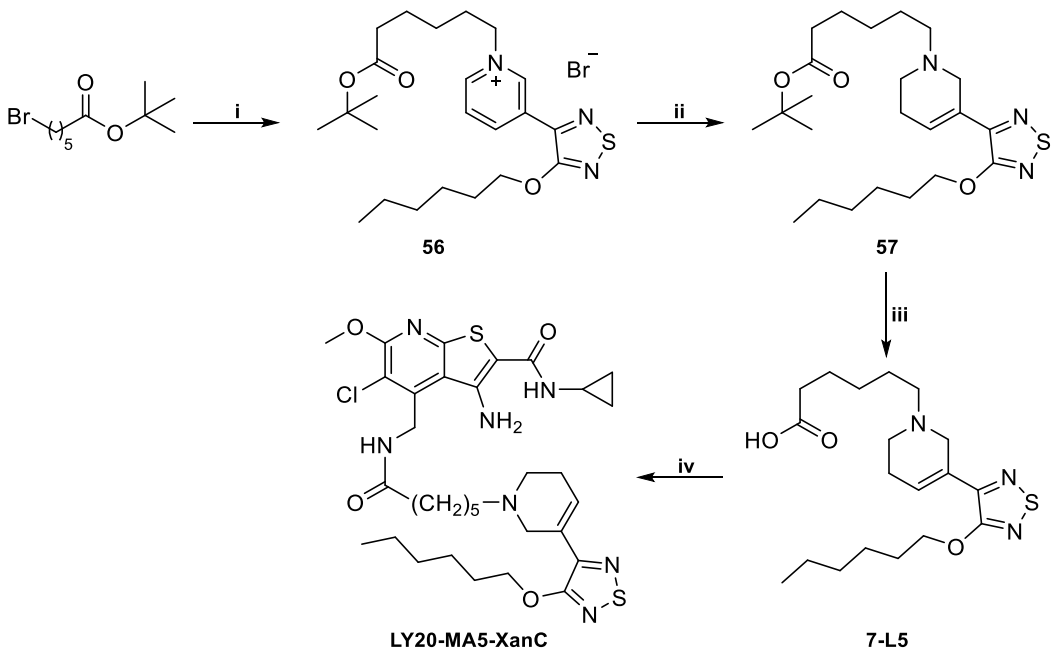
Scheme 16: Synthesis of **LY20-MA5-XanA**.



Reagents and conditions: (i) **22**, CH_3CN, 78 °C (microwave), (ii) TFA, DCM, -20 °C → rt; (iii) **54**, PyBOP, DIPEA, DMF, rt.

Additionally, a further hybrid that differs from **LY20-MA5-XanA** only in the lack of the methyl group at the quaternary amine was synthesized. Since uncharged molecules are more likely to pass the blood-brain barrier, the resulting dualsteric ligand **LY20-MA5-XanC** might be beneficial compared to **LY20-MA5-XanA**. Inspired by a synthesis route towards tacrine-xanomeline hybrids by Maspero *et al.*[108], *tert*-butyl-6-bromohexanoate was reacted with pyridine derivative **20** in a nucleophilic displacement reaction. The resulting pyridinium salt **56** was reduced by sodium borohydride to give tertiary amine **57**. After TFA mediated removal of the *tert*-butyl group, the isolated orthosteric linker **7-L5** was attached to thienopyridine precursor **54** by amide coupling to give **LY20-MA5-XanC** (Scheme 17).

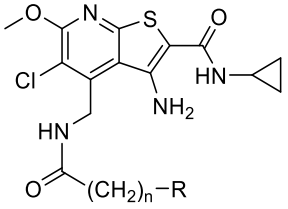
Scheme 17: Synthesis of **LY20-MA5-XanC**.

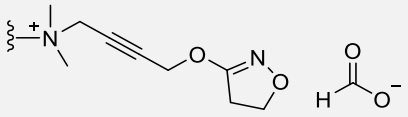
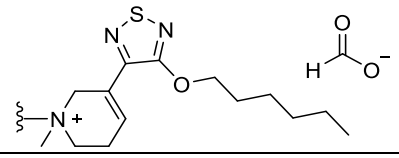
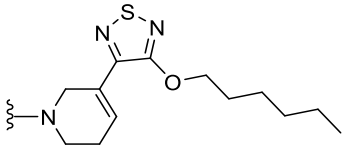


Reagents and conditions: (i) **20**, DMF, 110 °C, sealed tube; (ii) $NaBH_4$, CH_3OH, rt; (iii) TFA, DCM, -20 °C → rt; (iv) **54**, PyBOP, DIPEA, DMF, rt.

Conclusively, the five synthesized compounds within the LY20-MAn series are summarized in Table 4.

Table 4: Overview of synthesized LY20-MAn hybrid compounds.



R	n	Substance code
	3	LY20-MA3-iper
	5	LY20-MA5-iper
	7	LY20-MA7-iper
	5	LY20-MA5-XanA
	5	LY20-MA5-XanC

3.5 Attempted preparation of LY2119620-hybrids by using the methyl side chain as functionalization unit

The strategy for the preparation of LY2119620-hybrids with the methyl side chain as functionalization unit was based on the findings that were made during the synthesis of LY20-MAn-hybrids and LY21-An-hybrids. To evaluate the general feasibility of such ligands, TMA was selected as orthosteric unit because of its higher stability and easier synthetic accessibility compared to iperoxo and xanomeline. Furthermore, hybrids composed of TMA and BQCA have been shown to release G-protein recruitment in a former study.[96] The piperazine moiety should be introduced at the end of the synthetic pathway to prevent cross reactions through nucleophilic attacks of the tertiary amine in earlier steps. Due to their poor solubility in most organic solvents, two-fold charged intermediate compounds should be circumvented within this total synthesis. Consequently, the orthosteric TMA linker needs to be introduced by an amide coupling reaction to avoid formation of a second quaternary amine. Therefore, an approach was planned which is comparable to the preparation of LY20-MAn-hybrids. However, a remarkable difference during this synthetic route represents the insertion of a thiomethyl group at 6-position of the thienopyridine ring instead of a methoxy group (Figure 20).

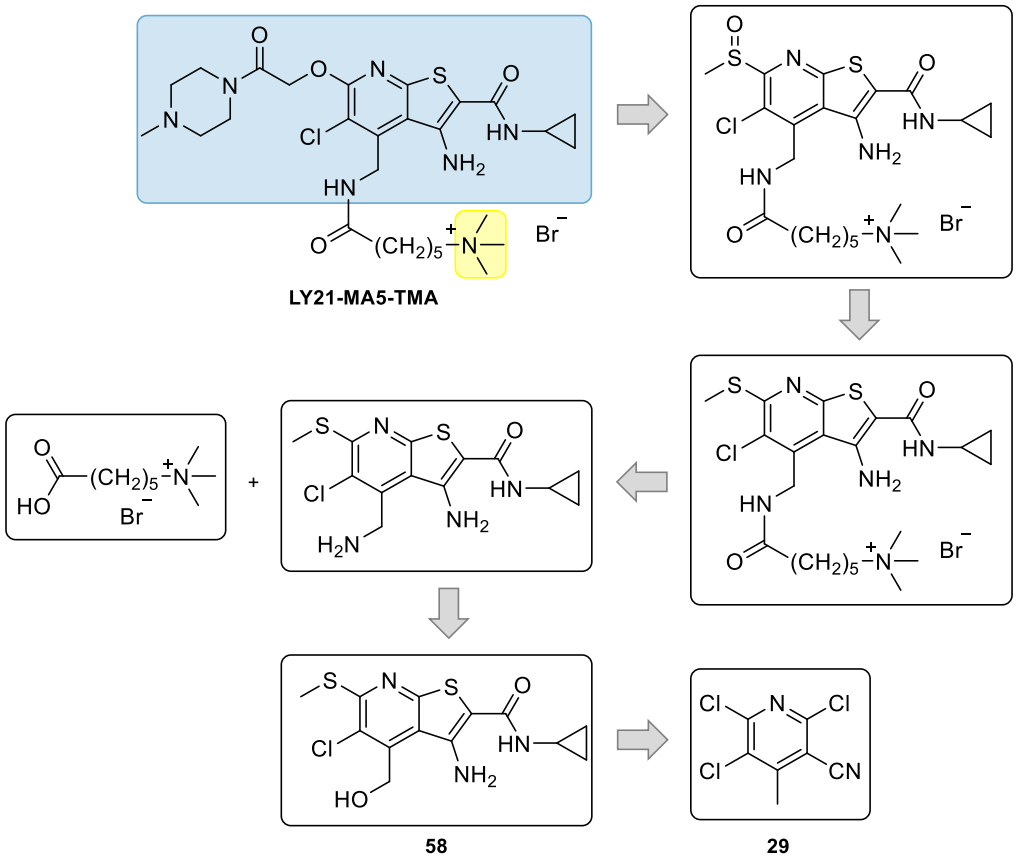


Figure 20: Retrosynthetic approach for synthesis of **LY21-MA5-TMA**.

As seen in the synthesis of LY21-An-hybrids, the thiomethyl group should be converted into a leaving group via mono-oxidation to a sulfoxide. Accordingly, a pathway towards the synthesis of thienopyridine intermediate **58** from compound **29** was evaluated at first.

In a first approach, compound **29** was converted again to sulfide **30**.[67] The latter was reacted with NBS and DBO, following the radical bromination reaction protocol described for the synthesis of compound **41**. Surprisingly, bromination occurred exclusively at the methyl group of the sulfide in ortho position of the pyridine ring (Figure 21). This was confirmed by measurement of a heteronuclear multiple bond correlation (HMBC)-experiment. The signal of the methyl group at 2.60 ppm showed coupling to the carbon atoms in 3-, 4-, and 5-position of the pyridine ring, whereas the CH_2-group at 5.04 ppm coupled exclusively to the carbon atom in ortho-position of the pyridine ring which is attached to the sulfur atom.

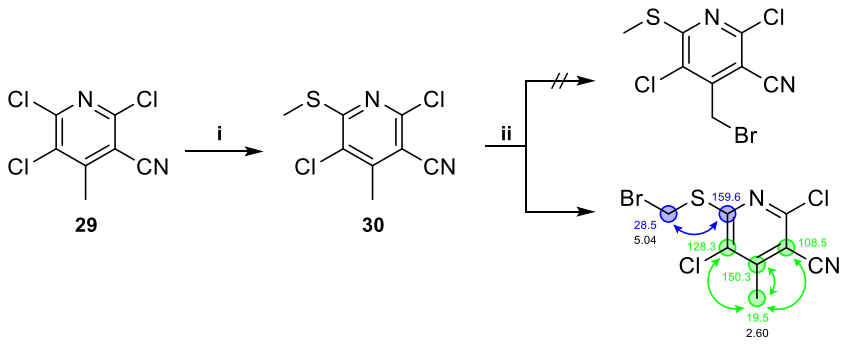
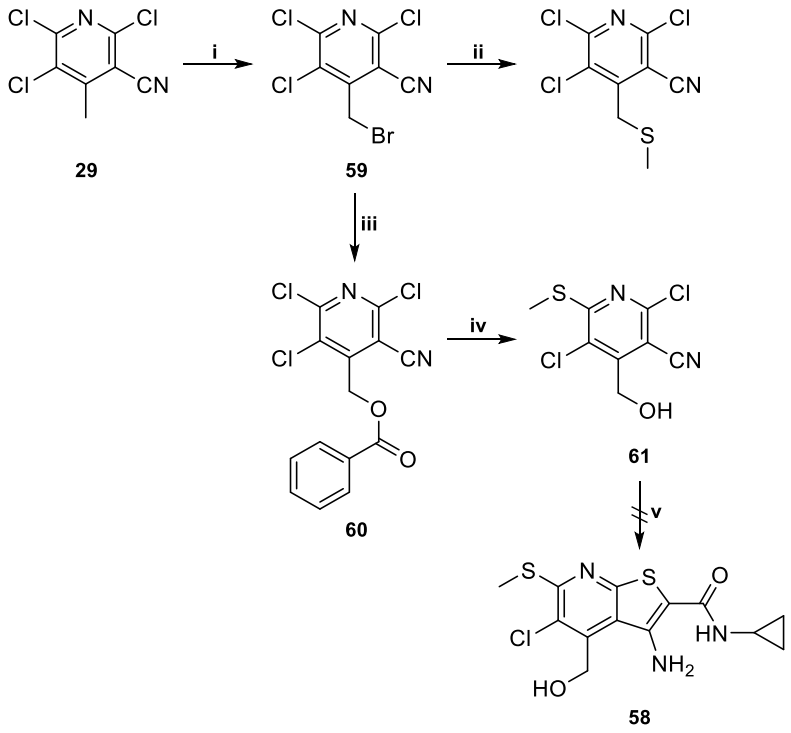


Figure 21: Attempted bromination of compound **30**. Double arrows indicate long range ^{1}H-^{13}C coupling in the HMBC spectrum. The chemical shifts in the ^{13}C spectra of the colored carbon atoms (green and blue) and the chemical shift of the attached protons in the ^{1}H spectra (black) are depicted in ppm. Reagents and conditions: (i) sodium methanethiolate, CH_3OH, 0 °C → rt; (ii) NBS, DBO, CCl_4, 110 °C, sealed pressure tube.

Apparently, the sulfur atom stabilizes a more favorable transition state at the attached methyl group upon the radical bromination reaction. As a result of that observation, the introduction of a bromine substituent at the methyl group in 4-position needs to take place in an earlier stage of the synthesis route. Hence, compound **29** was used as starting material for the radical bromination reaction. This allowed the isolation of pyridine intermediate **59** (Scheme 18). Predictably, the conversion of **59** with one equivalent of sodium methanethiolate led to an undesired nucleophilic displacement reaction at the bromine substituent. Consequently, the latter represents the most favorable position for the attack of a nucleophile in **59**. Thus, **59** was reacted with sodium benzoate to give the intermediate **60** in a nucleophilic displacement reaction. Next, **60** was treated with one equivalent of sodium methanethiolate under different reaction conditions. At room temperature, an unselective nucleophilic aromatic substitution of

the chlorine residues in 2- and 6-position of the pyridine ring was observed as well as a hydrolysis of the benzoic acid ester. This led to a mixture of several species. Cooling of the reaction mixture in an ice bath delivered the same result. When the reaction temperature was reduced to -78 °C, the nucleophilic attack took place increasingly in 6-position of the pyridine ring and at the benzoic acid ester. However, the reaction rate was very slow and the obtained yields only increased slightly. Since it was intended to cleave the ester in the next step of the synthesis route anyway, two equivalents of sodium methanethiolate were added in a further approach, but this led to an undesired nucleophilic attack in 2-position once again. A possible source of error in this context represents the concentration of sodium methanethiolate. Since air-contact decreases the purity of sodium methanethiolate, the yield of the reaction varied significantly when either a fresh or an old bottle of sodium methanthiolate was used. As the reaction proved to be difficult to reproduce, only small amounts of the desired intermediate **61** could be isolated.

Scheme 18: Attempted synthesis of thienopyridine carboxylic acid scaffold **58**.



Reagents and conditions: (i) NBS, DBO, CCl_4, 110 °C, sealed pressure tube; (ii) sodium methanethiolate, CH_3OH, -50 °C → rt; (iii) sodium benzoate, DMF, -50 °C → rt; (iv) sodium methanethiolate, CH_3OH, -78 °C → rt; (v) **42**, Na_2CO_3, CH_3CH_2OH, reflux.

Remarkably, such problems were not observed during the preparation of the very similar compound **44** in chapter 3.4.1. The main problem herein is the stronger nucleophilic character of a thiolate compared to its respective alkoxide. The negative charge of a thiolate is distributed

over a greater volume than within an alkoxide. Furthermore, sulfur valence electrons are bound more loosely than oxygen valence electrons. This causes the higher tendence of thiolates to donate electron pairs. Nevertheless, the small amounts of compound **61** that have been isolated were used for the following step of the planned synthetic route. To obtain thienopyridine ring **58**, compound **61** was reacted with thiol **42** and sodium carbonate under the same conditions as for the preparation of compound **47** in chapter 3.4.6. In the synthesis of **47**, it was observed that a reaction temperature of 78 °C was required to cause ring closure of the thienopyridine ring. Lower temperatures still led to a nucleophilic attack of thiol **42** in 2-position of the pyridine ring but did not produce closure of the thienopyridine ring. However, when compound **61** was heated in an ethanolic solution under reflux, analysis by TLC as well as LC/MS did not indicate any product formation but showed the presence of several different undefinable species. As the small stock of compound **61** was consumed by this approach and because of the poor reproducibility of **61**, no further attempts to synthesize key intermediate **58** were examined.

In conclusion, a synthetic pathway towards **LY20-MA5-TMA** was designed and evaluated. The presence of a thiomethyl substituent in 6-position of the pyridine ring instead of a methoxy group during the first steps of the synthesis foreclosed a simple transmission of the reaction parameters that have been applied for the preparation of compound **47**. The difficulties to reproduce the synthesis of intermediate **61** and the unsolved question of how to perform closure of the thienopyridine ring to obtain key intermediate **58** hereby represent the main problems.

4 Pharmacological investigations of LY-hybrid ligands

The pharmacological studies of LY-hybrid ligands were performed at the Institute for Molecular Cell Biology of the Friedrich Schiller University of Jena in the research group of Prof. Dr. Carsten Hoffmann by Dr. Michael Kauk and Carolin Grosse. The substances were investigated by Förster resonance energy transfer (FRET) assays and mini-G protein bioluminescence resonance energy transfer (BRET) assays. The theoretical principles of both test systems are described in the following.

4.1 Theoretical background of the assays

4.1.1 FRET assay

A fluorophore can transmit energy via nonradiative dipole-dipole coupling to a second fluorophore that is located in a distance between 1-10 nm. FRET transmission requires an overlap of the donor emission spectrum and the acceptor absorption spectrum of at least 30 % and, additionally, a parallel orientation of the dipole moments. The FRET efficiency correlates with the distance $\boldsymbol{r}$ of the fluorophores and the Förster distance $\boldsymbol{R_0}$ with an inverse 6th-power law according to equation 1.[125, 126]

$$E = \frac{1}{1+(r/R_0)^6} \qquad (1)$$

The strong correlation between FRET efficiency and spatial distance of the involved fluorophores has been widely used to study numerous biological systems, including GPCRs.[127, 128, 129, 130, 131] For the herein used sensors, cells stably expressing modified M_2 or M_4 receptors were utilized. The C-terminal ending of the respective receptor was tagged to a cyan fluorescent protein (CFP). Furthermore, a tetracysteine binding motif was inserted at the *N*-terminal part of IL3 near TM5. After application of a so-called FlAsH (fluorescein arsenical hairpin binder) sensor, the latter binds to the tetracysteine motif and thereby becomes the acceptor fluorophore. (Figure 22a+b). Due to a reduced influence on G protein coupling properties and the relatively small size of FlAsH, this represents an improvement compared to earlier muscarinic FRET sensors with a tetracysteine motif at the opposite end of IL3.[132, 133] When FlAsH is bound to the tetracysteine motif, the emission of a green-yellow light can be observed.[134] Upon ligand binding, a conformational switch is induced within the sensor, thus changing the relative distance between CFP and FlAsH. Afterwards, the variation within the ratio of CFP fluorescence and FlAsH fluorescence can be read out as normalized FRET signal. Moreover,

analysis by confocal microscopy shows an amplification of green-yellow fluorescence after addition of the endogenous neurotransmitter acetylcholine (Figure 22c).

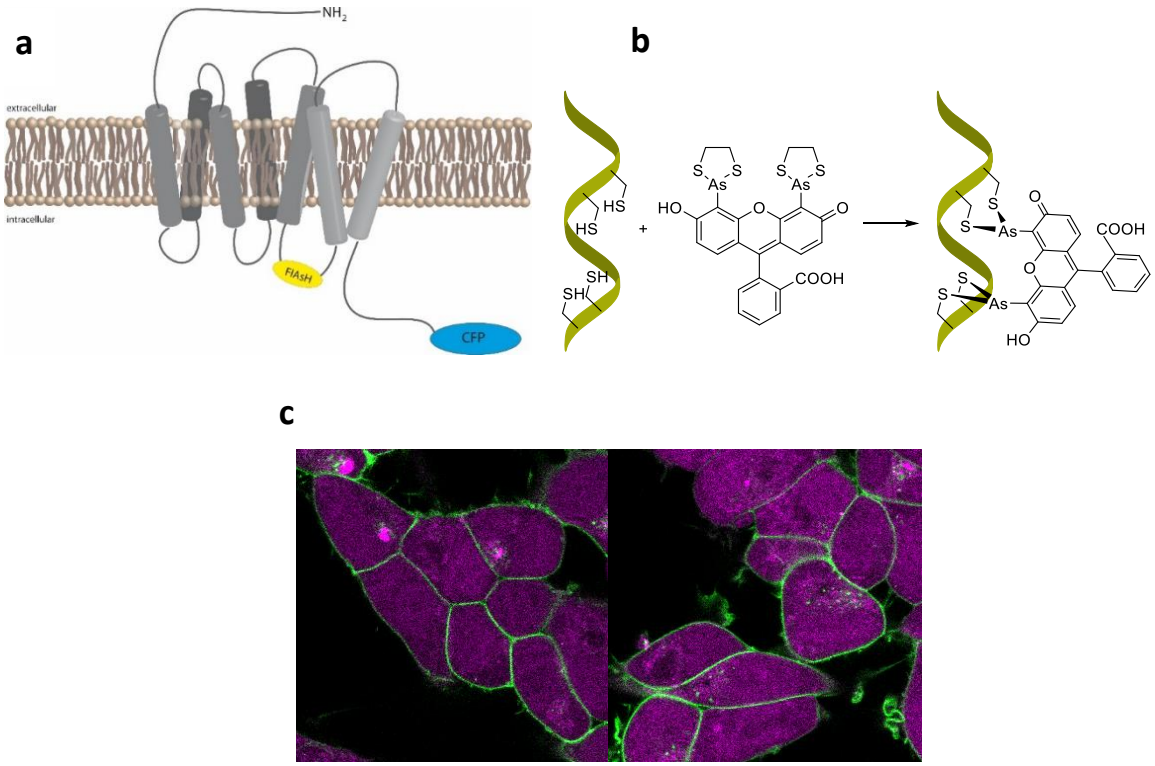


Figure 22: (a) Simplified depiction of CFP/FlAsH FRET sensor. Reprinted with permission of Elsevier; Copyright © 2011.[135] (b) Binding of FlAsH to the tetracysteine motif. (c) Picture of M_2 CFP/FlAsH FRET sensor obtained by confocal microscopy before (left) and after (right) the addition of endogenous neurotransmitter acetylcholine (provided by Prof. Dr. Carsten Hoffmann).

Before the FRET analysis data of the prepared hybrid ligands were analyzed, FRET responses of orthosteric ligands iperoxo and xanomeline at M_2 and M_4 receptor sensors were compared. (Figure 23). The obtained results show a concentration-dependent FRET response for iperoxo at both subtypes, thus confirming the expected conformational change. In contrast, the application of xanomeline did not result in a detectable conformational change. Furthermore, standard deviations for mean values of xanomeline were elevated especially at the M_4 receptor. Notably, the reduced number of xanomeline data points compared to iperoxo contributes to this high standard deviation. Since FlAsH is not able to rotate freely because of its bidentate character, the absence of a FRET signal for xanomeline might be explained by changes in fluorophore orientation.[133] In conclusion, FRET measurements give information about the extent of conformational changes within the intracellular receptor region released by an applied ligand. However, this does not allow to draw direct conclusions towards G protein recruitment or binding of β-arrestin.

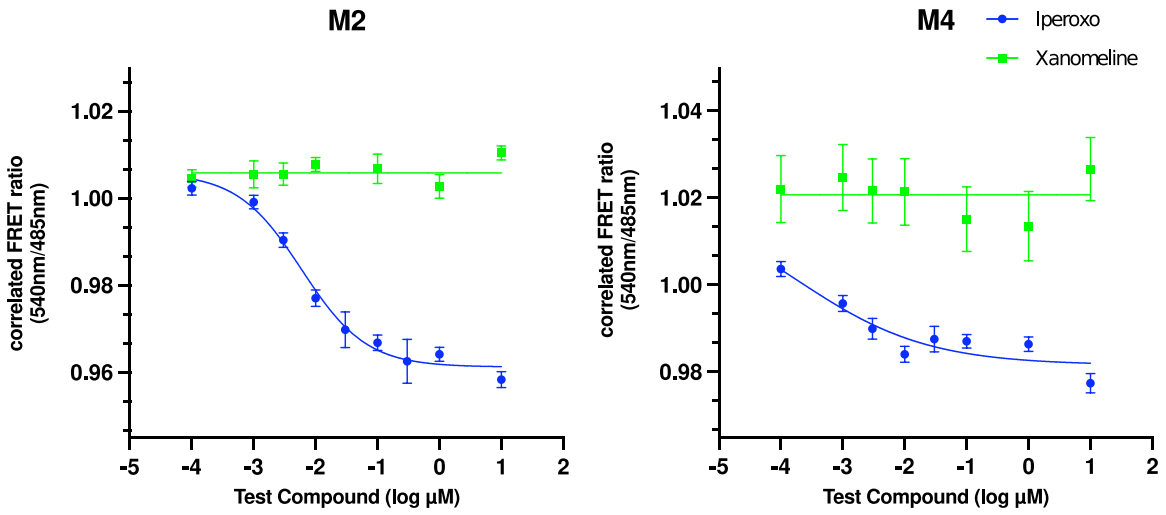


Figure 23: Concentration-response curves in a FRET assay at M_2 and M_4 receptors. The variation of the FRET ratio was determined by stimulation of the sensors with various concentrations of iperoxo or xanomeline. Data are expressed as the means ± standard error of mean (SEM).

4.1.2 Mini-G protein BRET assay

To find out whether the investigated hybrids lead to receptor activation, a mini-G protein BRET assay was implemented. Unlike heterotrimeric G proteins, mini-G proteins are not associated to the cell membrane. Hence, the formation of intensive background signals which would otherwise appear during FRET or BRET measurements of G protein complexes can be circumvented.[136, 137] Furthermore, G protein complexes only stick together for a span that is too short for the generation of stable signals.[138] Mini G proteins, though, consist of a Gα subunit with a shortened *N* terminus to avoid recruitment of Gβ- and Gγ-subunits. In addition, several mutations lead to an enhanced *in vitro* stability of the G protein and the formation of a more stable binding to the respective receptor. Further mutations also allow the retention of receptor-coupling preferences, thus enabling the study of G_s-, G_i-, or G_q-coupling behavior.[139] The herein applied Gα proteins were attached to a yellow fluorescent protein (YFP), also referred to as venus-group. The C-terminal ending of the muscarinic receptors were modified with a luciferase (NanoLuc). Unlike fluorescent proteins, this class of enzymes can generate bioluminescence in absence of external light irradiation. However, the addition of luciferin substrate is necessary. In a mini-G protein BRET assay, YFP and NanoLuc give a FRET signal upon changes in their relative distance. This process is called BRET since the fluorescence donor is replaced by a bioluminescence-producing enzyme. G protein binding can thus be measured through determination of the change in relative distance between the NanoLuc-attached receptors and the YFP-attached mini-G proteins (Figure 24).

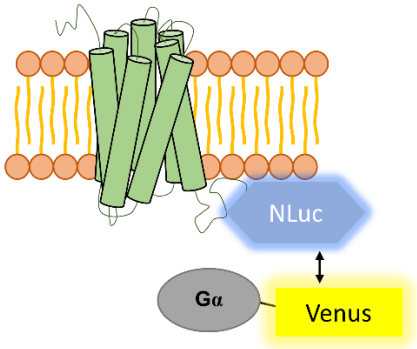


Figure 24: Simplified depiction of the interaction between a mini-G protein attached to a Venus group and a C-terminally bioluminescent NanoLuc-modified muscarinic receptor during a mini-G protein BRET assay.

To examine the reproducibility of coupling preferences at the applied receptors, mini $G\alpha_i$- and $G\alpha_q$-proteins were tested at a M_2-NanoLuc construct, respectively. The preferential recruitment of $G\alpha_i$-proteins as well as the expected weak coupling with $G\alpha_q$-proteins were reproduced, thus demonstrating the reliability of the herein used construct (Figure 25).[18, 20]

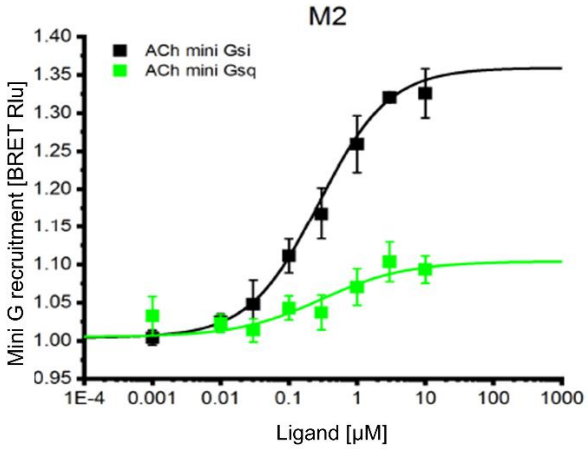


Figure 25: Concentration-response curves in a mini-G protein BRET assay at a M_2 receptor construct under the use of either $G\alpha_i$-proteins or $G\alpha_q$-proteins. Acetylcholine was used for receptor stimulation at various concentrations. For ligand concentrations, a logarithmic scale was used. G-protein recruitment at very low concentrations was set to 1.00. Data are expressed as the means ± SD (standard deviation) (provided by Dr. Michael Kauk).

Moreover, the effects of the orthosteric ligands iperoxo and xanomeline at the M_1, M_2, and M_4 subtypes were compared (Figure 26). At the M_1 and M_4 receptor, recruitment of mini-G proteins was observed for both orthosteric ligands. The effect of xanomeline was significantly lower at both subtypes. However, at the M_2 receptor almost no recruitment of mini-G proteins was detected for xanomeline. This is in accordance with the literature, since xanomeline is subtype selective for M_1 and M_4 receptors.[51]

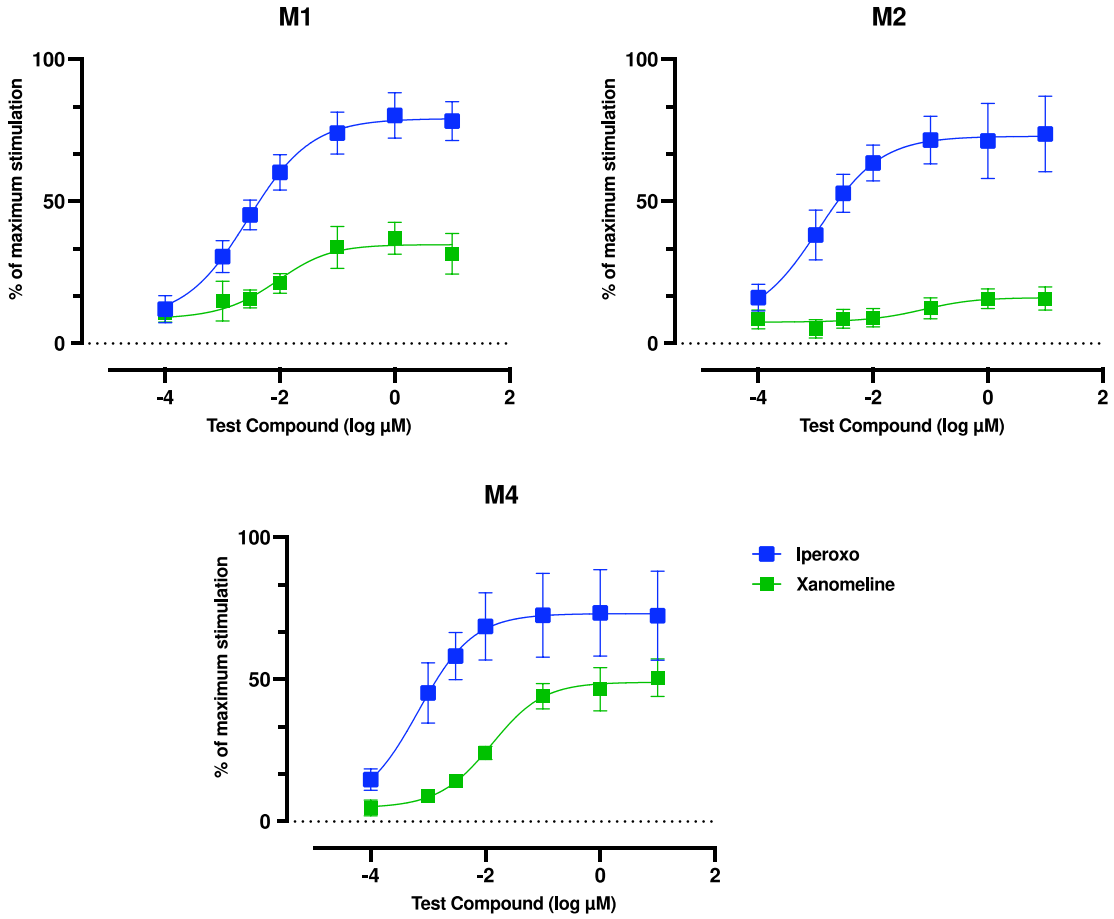


Figure 26: Concentration-response curves in a mini-G protein BRET assay at M_1, M_2, and M_4 receptors. The recruitment of mini-Gα_q proteins (M_1) or mini-Gα_i proteins (M_2 and M_4) was determined by stimulation of the receptors with various concentrations of iperoxo or xanomeline. The highest observed G-protein recruitment upon stimulation with iperoxo was set to 100%. Data are expressed as the means ± SD.

4.2 Investigation of amide functionalized LY2033298- and LY2119620-hybrid ligands

4.2.1 Mini-G protein BRET assay of LY20-An-iper ligands

To determine the degree of receptor activation of **LY20-MAn-iper** hybrids as well as to find out the adequate linker chain length, a mini-G protein BRET assay was performed. The ligands were tested at all five muscarinic subtypes to see whether the desired preference for M_2 and M_4 receptors can be observed. Within this assay, the endogenous neurotransmitter acetylcholine was used as reference substance. The obtained concentration-response curves are depicted in Figure 27. Additionally, maximum responses of **LY20-An-iper** hybrids at all five muscarinic receptor subtypes are shown in Figure 28. Values for standard deviations within this assay were generally moderate and are given in the appendix. **LY20-An-iper** hybrids only differ in terms of linker length.

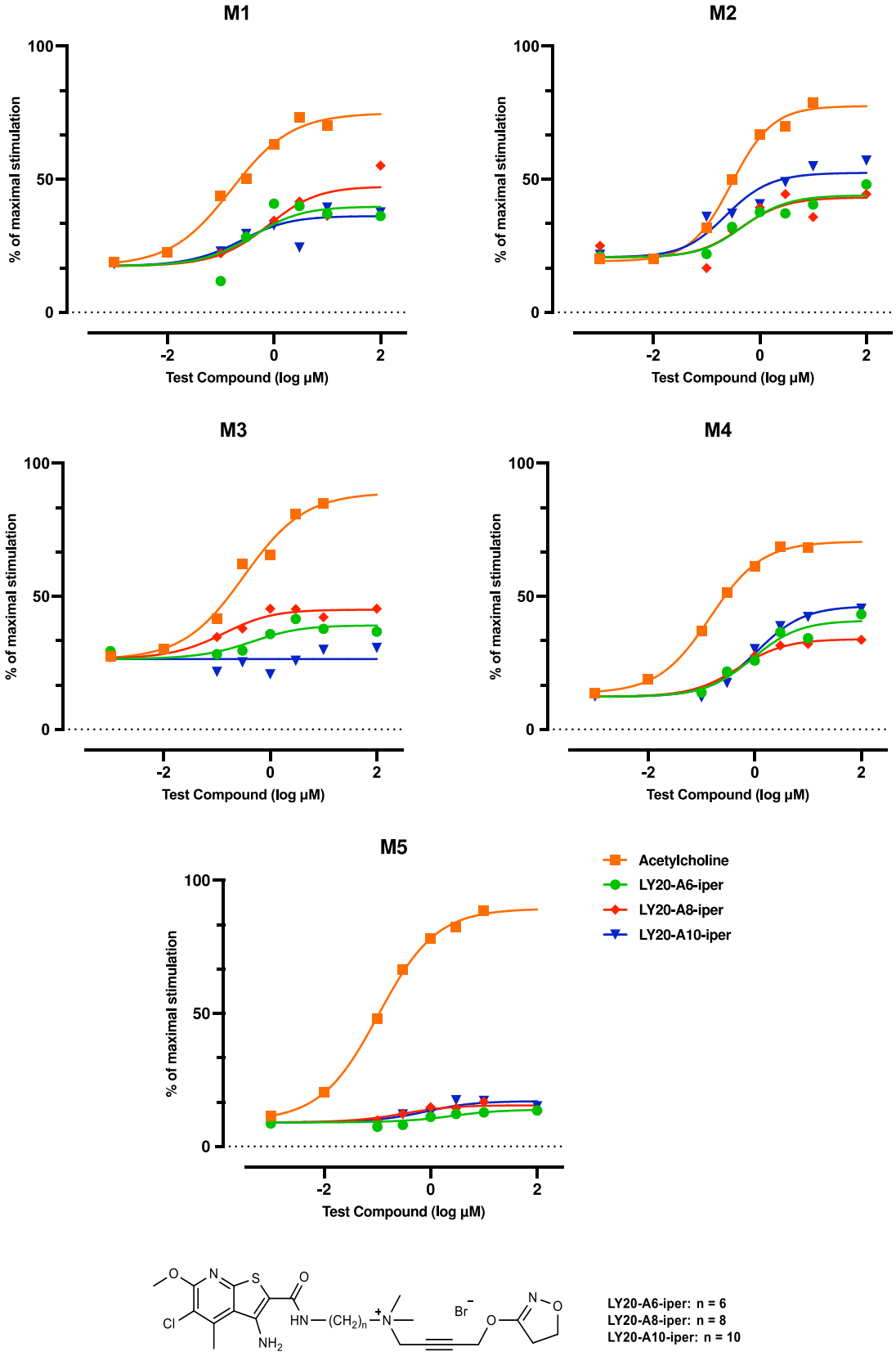


Figure 27: Concentration-response curves of **LY20-An-iper** hybrids in a mini-G protein BRET assay at all five muscarinic receptor subtypes. The recruitment of mini-Gα_q proteins (M_1/M_3/M_5) or mini-Gα_i proteins (M_2/M_4) was determined. Acetylcholine is depicted as reference substance. The highest observed mini-G protein recruitment upon stimulation with acetylcholine was set to 100%. Data are expressed as mean values. Values for standard deviations of measurement points are given in the appendix.

At the M_2 and M_4 subtypes, all ligands of this series released G-protein recruitment. In both cases, **LY20-A10-iper** gave the highest maximum effect and showed a slight left shift of the concentration-response curve. The obtained curves at the M_1 receptor are comparable to the results at the M_2 and M_4 subtypes. However, the maximum response was best for **LY20-A8-iper**. The latter also represents the most effective ligand at the M_3 receptor. Notably, **LY20-A10-iper** solely shows activity at the M_3 subtype at very high concentrations. The M_5 receptor represents the only subtype that does not respond to any of the investigated hybrid ligands.

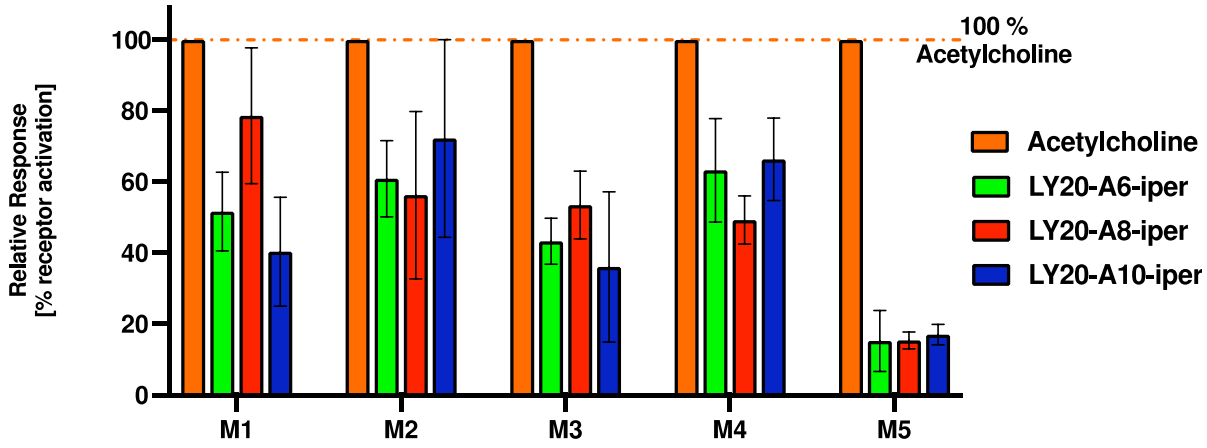


Figure 28: Observed responses for mini-G protein recruitment after stimulation of muscarinic receptor subtypes with 100 µM of the respective **LY20-An-iper** hybrid. Mini-G protein recruitment upon stimulation with 100 µM acetylcholine was set to 100%. Data are expressed as mean values ± SD.

In summary, **LY20-A10-iper** is the most favorable hybrid of this series since it barely activates M_3 and M_5 receptors and shows the highest maximum effect at M_2/M_4 subtypes. However, **LY20-A10-iper** gives an undesired effect at the M_1 receptor. Interestingly, **LY20-A8-iper** triggers binding of mini-G proteins at M_1/M_3 subtypes, thus indicating a possible alternative binding mode at these receptors. As seen in former studies on hybrid ligands for muscarinic receptors, subtype selectivity, binding affinity, and maximum receptor response were once again demonstrated to be dependent of the linker chain length.[95] Therefore, the following investigation of amide functionalized LY2033298- and LY2119620-hybrid ligands focused on derivatives with ten methylene units.

4.2.2 FRET studies of LY20-A10 hybrids and LY21-A10 hybrids

4.2.2.1 FRET measurements of LY20- and LY21-hybrids with Iperoxo or TMA as orthosteric units

Based on the findings of above-described mini-G-protein BRET assay, amide functionalized hybrid ligands with a linker length of ten methylene units were investigated by FRET studies. To find out whether the desired conformational changes at $G\alpha_i$-coupled muscarinic subtypes can be observed, FRET studies were performed at M_2 and M_4 receptor sensors. First, the influence of an additional piperazine moiety within the allosteric part of the molecule was investigated. Therefore, LY20-A10 hybrids were compared to LY21-A10 hybrid ligands which contained the additional piperazine moiety. Furthermore, the orthosteric part was varied between iperoxo and TMA to evaluate whether the quaternary amine is sufficient to release a conformational change. Within these assays, iperoxo was used as reference substance. Notably, the supraphysiological properties of iperoxo at the M_2 receptor that have already been mentioned in the introduction of this work must be taken into account.[140] The four investigated hybrid ligands show similar results at the M_2 receptor (Figure 29). A clear conformational change could only be detected at high ligand concentrations in the micromolar concentration range. At the M_4 receptor, high concentrations of **LY20-A10-iper** and **LY20-A10-TMA** were required to give a FRET signal. Remarkably, the two hybrids which are attached to a piperazine moiety released a detectable conformational change at lower concentrations. This represents an interesting issue since LY2119620-hybrids were supposed to bind M_2 receptors preferentially.[71] However, the required concentrations were still relatively high in comparison to iperoxo. Interestingly, the obtained maximum FRET signal was stronger for all four hybrid ligands than the response of iperoxo. Hybrids containing iperoxo as orthosteric unit gave the highest FRET signal (Figure 29). Notably, the standard deviations for measurement points of hybrid ligands were elevated. The values for these standard deviations are given in the appendix of this work. To sum up, the four investigated hybrid ligands only showed conformational changes at $G\alpha_i$-coupled muscarinic receptors at high ligand concentrations. Solely **LY21-A10-iper** and **LY21-A10-TMA** showed a FRET response at the M_4 receptor at submicromolar concentrations.

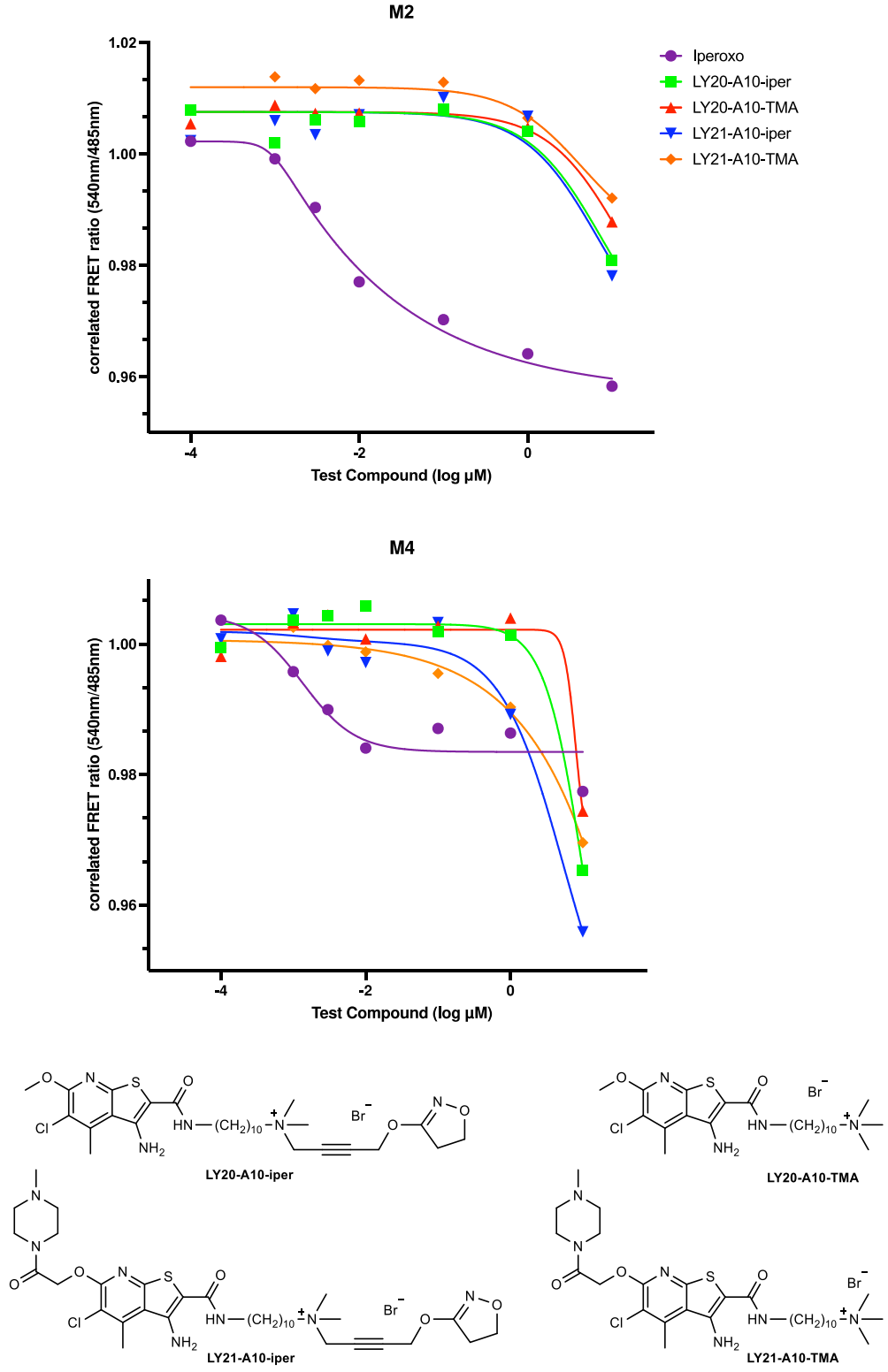


Figure 29: Concentration-response curves in a FRET assay at M_2 and M_4 receptors. The variation of the FRET ratio was determined by stimulation of the sensors with various concentrations of LY2033298- and LY2119620-hybrids. Iperoxo was used as reference substance. Data are expressed as mean values. Values for standard deviations of measurement points are given in the appendix.

4.2.2.2 FRET measurements of LY20-hybrids with xanomeline as orthosteric unit

Subsequently, FRET measurements of **LY20-A10-XanA** and **LY20-A10-XanB** which differ in terms of the chosen attachment point at xanomeline were analyzed. Though, an insufficient solubility of especially **LY20-A10-XanB** in DMSO and methanol led to results with only little significance. Since **LY20-A10-XanA** showed poor solubility as well, the reliability of the obtained measurement points at high concentrations remains questionable. Nevertheless, the concentration-response curves at the M_2 receptor indicate a small conformational change released by **LY20-A10-XanA** in the submicromolar range, whereas no clear FRET signal was obtained for **L20-A10-XanB** (Figure 30). At the M_4 subtype, **LY20-A10-XanB** delivered contradictory results at high ligand concentrations, whereas **LY20-A10-XanA** shows at least a slight FRET response. In conclusion, FRET studies of hybrid ligands containing xanomeline as orthosteric unit were accompanied by solubility problems of the ligands studied. However, the results indicate **LY20-A10-XanA** to be the more promising hybrid ligand among these two, even though the aimed M_4-selectivity caused by the M_4-preference of xanomeline could not be observed.[51, 71] Furthermore, **LY20-A10-XanA** bears a positively charged nitrogen atom and, therefore, is assumed to show a better solubility in polar solvents than the uncharged **LY20-A10-XanB**.

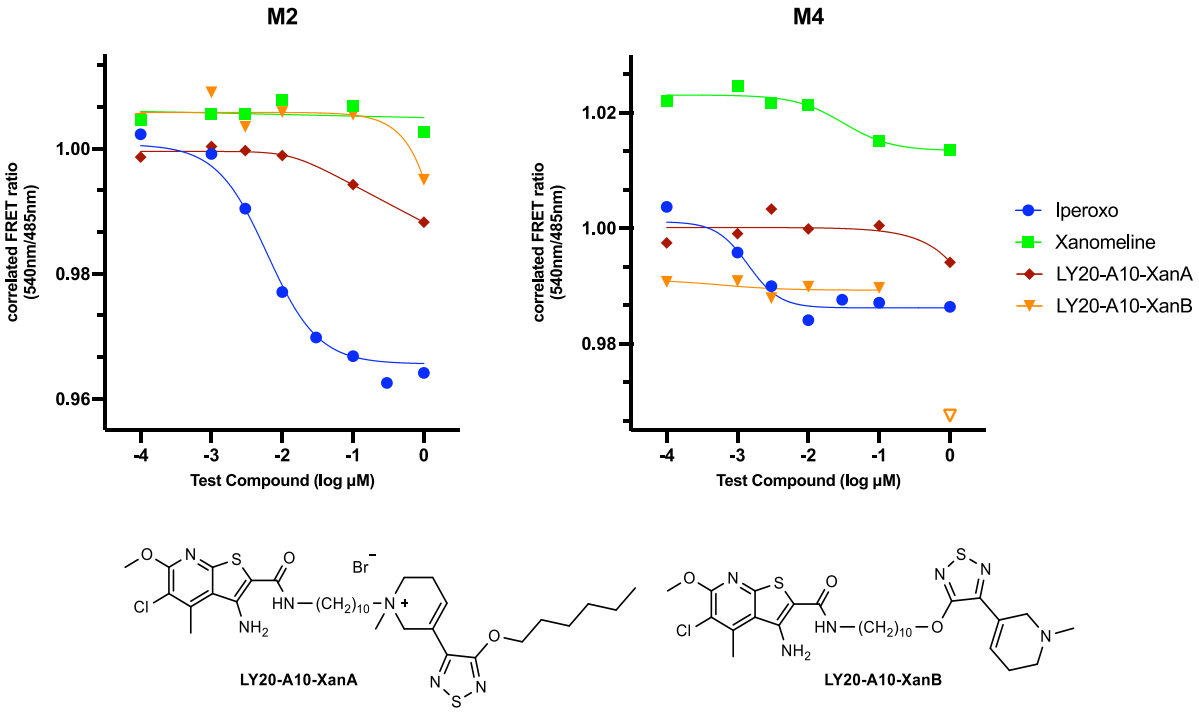


Figure 30: Concentration-response curves of **LY20-A10-XanA** and **LY20-A10-XanB** in a FRET assay at M_2 and M_4 receptors. Iperoxo and xanomeline are depicted as reference substances. Data are expressed as mean values. Values for standard deviations of measurement points are given in the appendix. At the M_4 receptor sensor, the mean value of **LY20-A10-XanB** measurement point at a concentration of 1 µM was not integrated into the curve.

4.2.3 Mini-G protein BRET assay of LY20-A10 hybrids and LY21-A10 hybrids

In order to find out whether the observed FRET signals in chapter 4.2.2 also lead to the recruitment of G-proteins, LY20-A10- and LY21-A10 hybrids were further investigated in a mini-G protein BRET assay. Since a significant level of mini-G protein recruitment was detected for **LY20-An-iper** hybrids at the M_1 subtype (see chapter 4.2.1), the latter was examined within this study along with M_2 and M_4 subtypes. In contrast to the mini-G protein assay in chapter 4.2.1, iperoxo was used as reference substance instead of acetylcholine.

4.2.3.1 Mini-G protein assays of LY20-A10 hybrids and LY21-A10 hybrids containing iperoxo or TMA as orthosteric units

Initially, BRET assay results of compounds **LY20-A10-iper**, **LY20-A10-TMA**, **LY21-A10-iper**, and **LY21-A10-TMA** at the M_1, M_2, and M_4 receptors were analyzed. At the M_1 subtype, all applied hybrid ligands except for **LY20-A10-TMA** displayed a low level of mini-G protein recruitment with a similar maximum effect. Comparable results were obtained at the M_2 subtype, even though neither of the two TMA linked hybrids showed G protein recruitment. At the M_4 receptor, **LY21-A10-iper** proved to be the most active ligand by giving a maximum receptor response almost half of iperoxo. Still, the concentration-response curve showed a clear right-shift compared to iperoxo. The respective hybrid ligand without piperazine moiety also released mini-G protein recruitment at the M_4 subtype. However, **LY20-A10-iper** revealed a reduced maximum effect and a right-shift of the concentration-response curve compared to **LY21-A10-iper**. Hybrid ligands attached to TMA as orthosteric unit showed no recruitment of mini-$G\alpha_i$ proteins at the M_4 subtype (Figure 31).

A comparison of the obtained BRET assay results and the FRET assay findings of subchapter 4.2.2.1 suggests that even if a conformational change is released by all four hybrid ligands at high ligand concentrations, recruitment of G-proteins requires the presence of iperoxo as orthosteric unit. This observation contrasts with former studies describing the implementation of TMA as orthosteric part within BQCA-hybrid molecules to be sufficient for G-protein recruitment at M_1 receptors.[96] Interestingly, **LY21-A10-TMA** was able to recruit mini-G proteins at the M_1 receptor, thus indicating differences in biased signaling between $G\alpha_q$-coupled M_1 receptors and $G\alpha_i$-coupled M_2/M_4 receptors. Another explanation might be a general suboptimal binding mode of amide functionalized LY-hybrids at $G\alpha_i$ coupled muscarinic receptors, hence leading to insufficient interactions between the orthosteric part of the hybrid molecule and the orthosteric binding site.

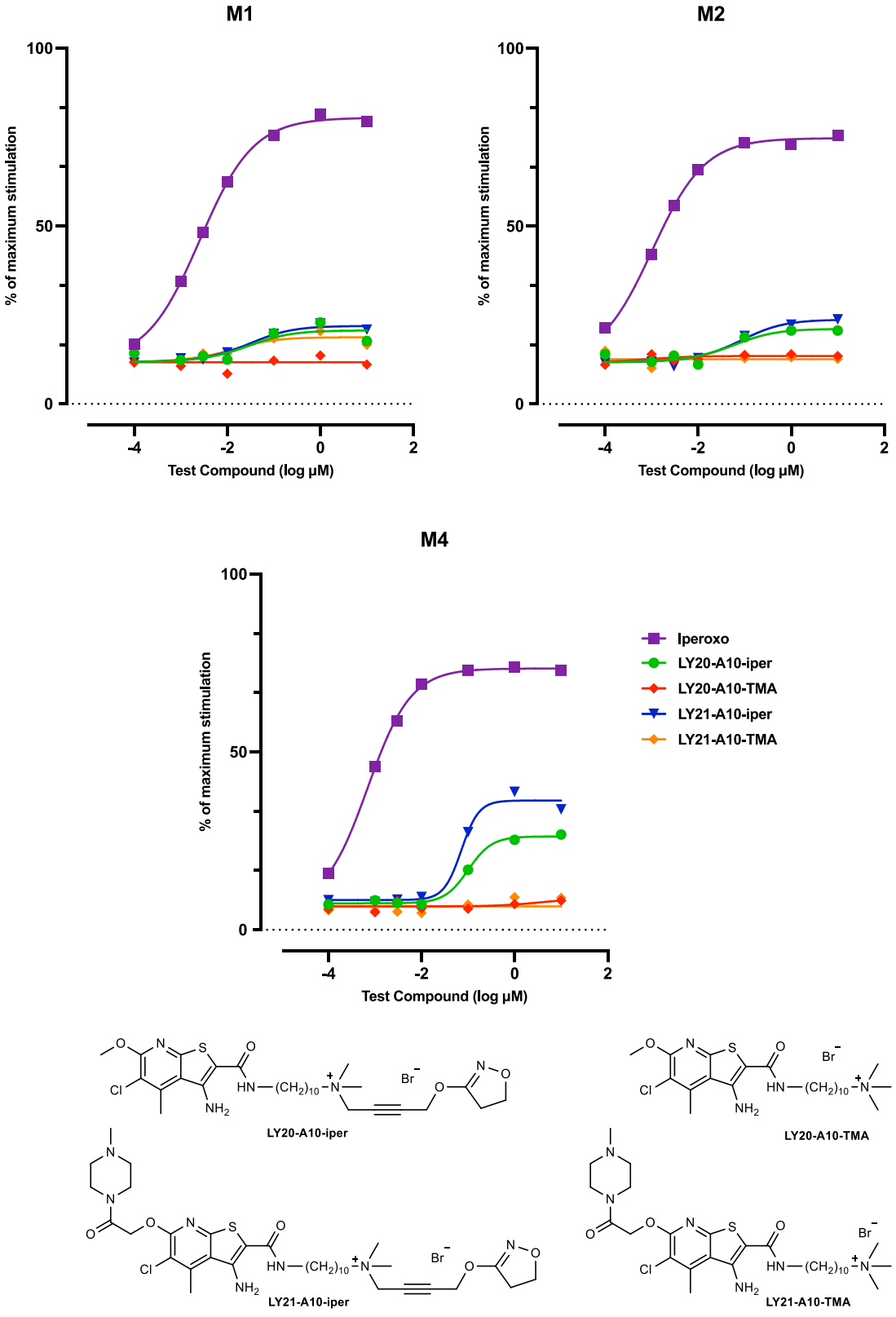


Figure 31: Concentration-response curves of **LY20-A10-iper**, **LY20-A10-TMA**, **LY21-A10-iper**, and **LY21-A10-TMA** in a mini-G protein BRET assay at M_1, M_2, and M_4 receptors. The recruitment of mini-$G\alpha_q$ (M_1) proteins or mini-$G\alpha_i$ (M_2 and M_4) was determined. Iperoxo is depicted as reference substance. The highest observed mini-G protein recruitment upon stimulation with iperoxo was set to 100 %. Data are expressed as mean values. Standard deviations of measurement points are given in the appendix.

Furthermore, **LY21-A10-iper** showed stronger FRET and BRET responses than **LY20-A10-iper** at the M_4 subtype. Consequently, the presence of the piperazine moiety did not lead to an enhanced selectivity towards M_2 subtypes for amide functionalized LY-hybrids. Unlike expected, the piperazine ring even contributed to G protein recruitment at the M_4 subtype.

4.2.3.2 Mini-G protein assays of LY20-A10 hybrids with xanomeline as orthosteric unit

Hybrid ligands **LY20-A10-XanA** and **LY20-A10-XanB** were additionally investigated by a mini-G protein assay at M_1, M_2, and M_4 receptors. For the interpretation of the obtained concentration-response curves, xanomeline and iperoxo were used as reference substances (Figure 32). **LY20-A10-XanA** and **LY20-A10-XanB** were not able to stimulate G protein recruitment at any of the three applied receptor subtypes to a significant extent. A small BRET signal was solely observed for **LY20-A10-XanB** at M_1 and M_4 receptors. However, the depicted results remain uncertain due to the solubility problems of both hybrid ligands (see chapter 4.2.2.2). Nevertheless, neither FRET nor BRET assay results indicate notable activity of amide functionalized LY-hybrids with xanomeline as orthosteric unit at the tested receptors. Hence, the desired M_4 selectivity by application of xanomeline could also not be proven with these types of hybrid molecules. Moreover, the obtained results do not allow any reliable conclusion concerning the appropriate connecting point at xanomeline for the composition of hybrid ligands.

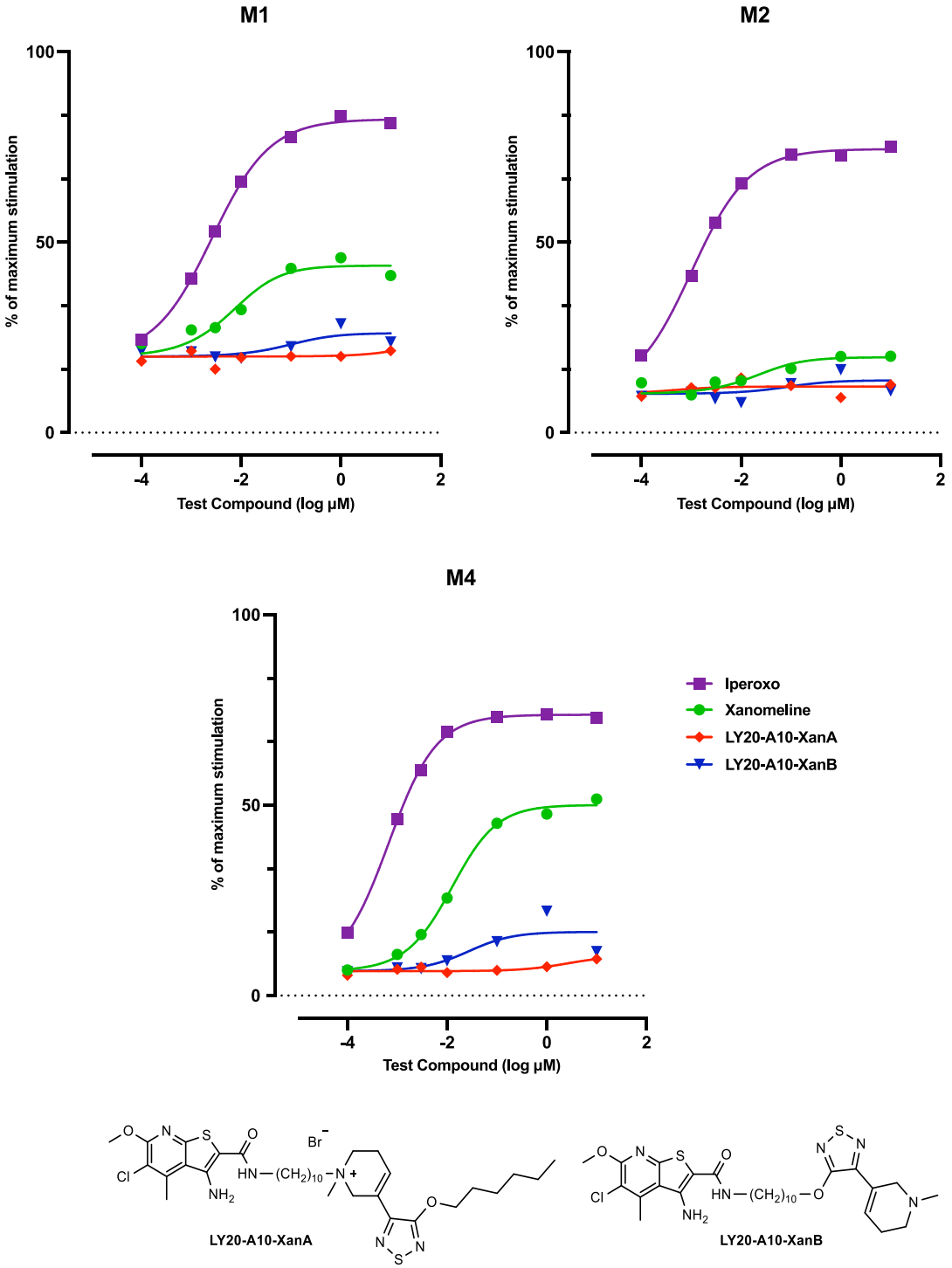


Figure 32: Concentration-response curves of **LY20-A10-XanA** and **LY20-A10-XanB** in a mini-G protein BRET assay at M_1, M_2, and M_4 receptors. The recruitment of mini-Gα_q (M_1) proteins or mini-Gα_i (M_2 and M_4) was determined. Additionally, iperoxo and xanomeline are depicted as reference substances. The highest observed mini-G protein recruitment upon stimulation with iperoxo was set to 100%. Data are expressed as mean values. Standard deviations of measurement points are given in the appendix.

4.3 Investigation of LY20-MQn-iper hybrid ligands

In a further approach, hybrid compounds with a linker attached to the methyl group in 4-position of LY2033298 were explored by FRET and BRET studies. In the following, the obtained results for the bisquaternary **LY20-MQn-iper** compounds are discussed. Iperoxo is depicted as reference substance, respectively.

4.3.1 FRET assay analysis of LY20-MQn-iper hybrids

Analysis of **LY20-MQn-iper** FRET studies at the M_2 receptor revealed that all three hybrid ligands only released conformational changes at high ligand concentrations. At the M_4 receptor, fluctuating measurement values and high standard deviations complicated adequate curve fitting. However, **LY20-MQ6-iper** manifested the clearest variation of FRET ratios. Notably, **LY20-MQ4-iper** and **LY20-MQ8-iper** seemed to release a detectable conformational change at submicromolar concentrations, but to a much smaller extent (Figure 33). In accordance with FRET results of amide functionalized LY-hybrids (see subchapter 4.2.2.1), the observed switch of the correlated FRET ratio in relation to reference substance iperoxo was clearer at the M_4 receptor than at the M_2 subtype. To sum up, the analyzed FRET data in this series indicate **LY20-MQ6-iper** to be the most promising hybrid ligand within the **LY20-MQn-iper** series.

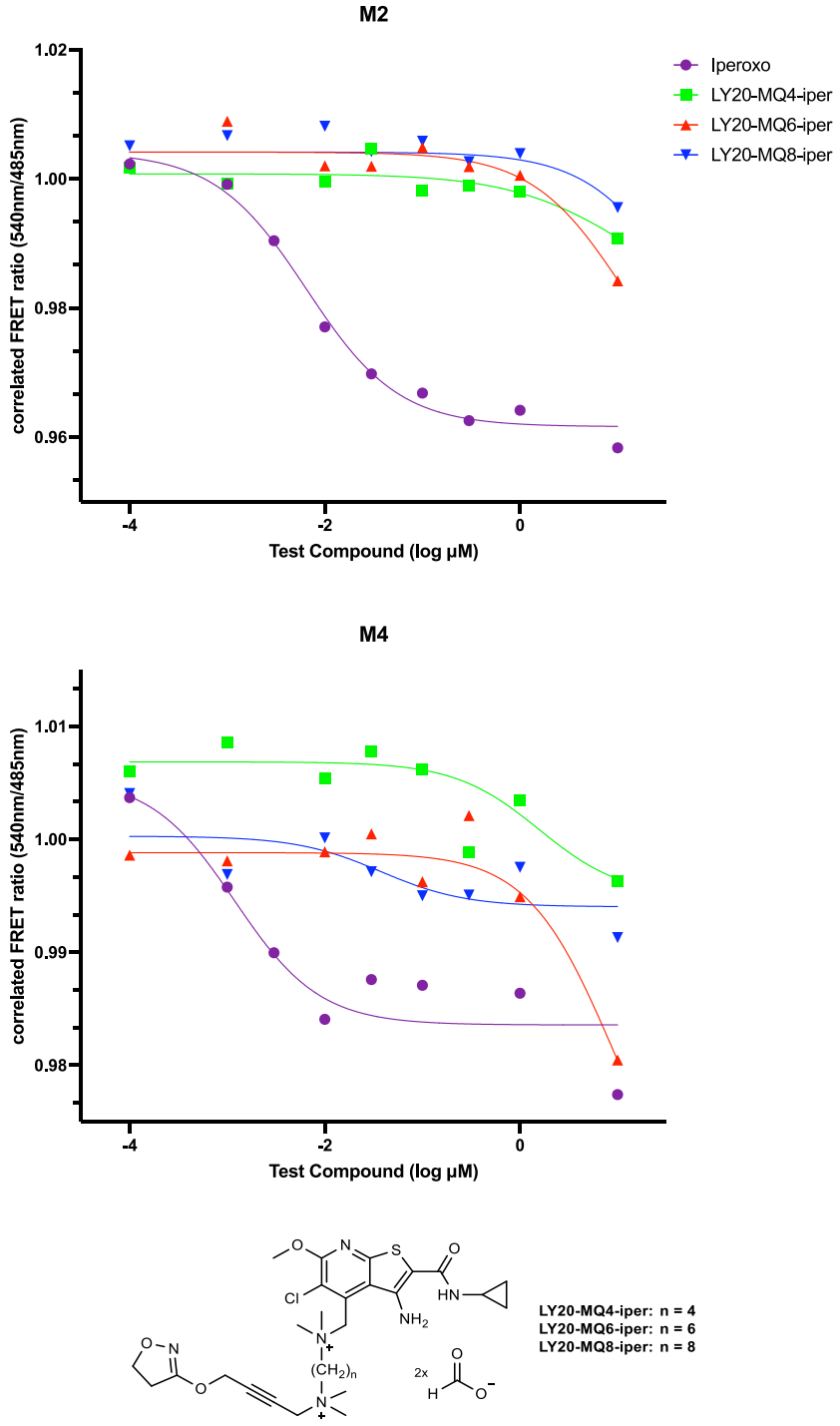


Figure 33: Concentration-response curves of **LY20-MQn-iper** hybrids in a FRET assay at M_2 and M_4 receptors. Iperoxo was used as reference substance. Data are expressed as mean values. Standard deviations of measurement points are given in the appendix.

4.3.2 Mini-G protein assay analysis of LY20-MQn-iper hybrids

Subsequently, BRET analysis data of **LY20-MQn-iper** hybrids at M_1, M_2, and M_4 receptors were examined (Figure 34). At the M_1 and M_1 subtypes, **LY20-MQ6-iper** showed a slight left-

shift of the concentration-response curve compared to **LY20-MQ4-iper** and **LY20-MQ8-iper**. However, the observed mini-G protein recruitment remained on a low level at both receptors.

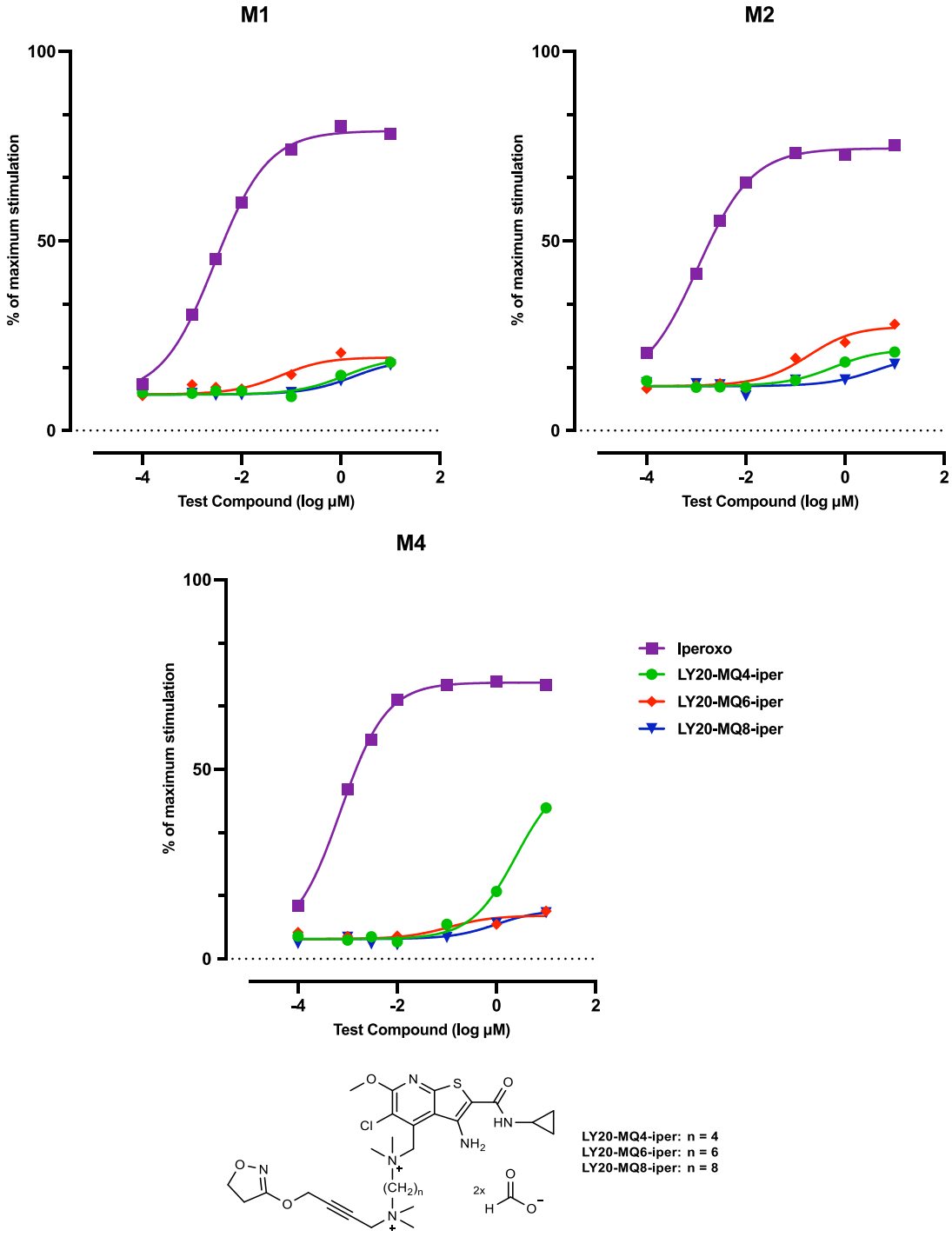


Figure 34: Concentration-response curves of **LY20-MQn-iper** hybrids in a mini-G protein BRET assay at M_1, M_2, and M_4 receptors. The recruitment of mini-$G\alpha_q$ (M_1) proteins or mini-$G\alpha_i$ (M_2 and M_4) was determined. Iperoxo is depicted as reference substance. The highest observed mini-G protein recruitment upon stimulation with iperoxo was set to 100 %. Data are expressed as mean values. Standard deviations of measurement points are given in the appendix.

At the M_4 receptor, **LY20-MQ6-iper** and **LY20-MQ8-iper** solely released a slight BRET signal. Interestingly, **LY20-MQ4-iper** caused an intensive BRET signal at high ligand

concentrations. However, the corresponding concentration-response curve did not reach a plateau within this investigation, thus impeding definite conclusions concerning the maximum effect. Under consideration of the relatively weak response of **LY20-MQ4-iper** at the M_2 subtype, the design of hybrid ligands preferring M_4 over M_2 receptors seems feasible. Noteworthy, even though FRET measurements at the M_4 receptor showed the clearest conformational switch for **LY20-MQ6-iper**, the most intensive effect for mini-G protein recruitment was obtained for **LY20-MQ4-iper**. Regarding the significant differences for G-protein recruitment at the M_4 subtype within the **LY20-MQn-iper** series, the importance of the chain length of the applied linker moiety must be pointed out once again. Moreover, other than observed for amide functionalized LY20-An hybrids, a shorter linker chain length is beneficial to detect activity of **LY20-MQn-iper** hybrids at muscarinic receptors, thus hinting to a different binding pose. Despite the discussed effect of **LY20-MQ4-iper** on $G\alpha_i$ protein recruitment, the investigated **LY20-MQn-iper** hybrids generally required relatively high ligand concentrations to deliver a response at muscarinic M_1, M_2, or M_4 receptors. Since the methyl group in 4-position of the thienopyridine ring was supposed to be the more promising attachment point for the synthesis of LY-hybrid molecules, this represents an unexpected finding. A possible explanation therefore might be the voluminous character of the additional tetrahedral quaternary amine which might lead to an incomplete contraction of the slot-like allosteric binding site. Moreover, the presence of positively charged nitrogen atoms at both ends of **LY20-MQn-iper** linker moieties could be accompanied by an unbeneficial electrostatic repulsion, thus reducing the desired mutual influence of allosteric and orthosteric part. A similar correlation has already been described for the combination of a positively charged allosteric modulator with a positively charged orthosteric antagonist at the M_2 receptor.[74] In this context, the pharmacological investigation of LY20-MAn hybrids which bear only one positively charged nitrogen atom at the orthosteric ending of the linker might lead to further conclusions.

4.4 Comparison of most active hybrid ligands

To evaluate the quality of the two distinct attachment points at the thienopyridine ring, the BRET assay concentration response curves of the most active ligands of chapters 4.2 and 4.3 were compared directly (Figure 35). **LY20-A10-iper** and **LY21-A10-iper** were chosen as amide functionalized hybrids, whereas **LY20-MQ4-iper** and **LY20-MQ6-iper** represented dualsteric ligands with the methyl side chain as functionalization unit. At the M_1 and M_2 receptors, maximum effects were relatively low for all four hybrid ligands. At the M_4 subtype, **LY20-MQ4-iper** gave the clearest BRET response at high ligand concentrations. The measured

maximum effect exceeded the half-maximum BRET response of iperoxo (Figure 36). However, the associated concentration-response curve showed a clear right-shift compared to both amide functionalized hybrid ligands. The maximum effect of **LY21-A10-iper** was a bit lower than **LY20-MQ4-iper**. Nevertheless, submicromolar concentrations of **LY21-A10-iper** were sufficient to release a receptor signal (pEC_{50} = 7.145 ± 0.113). **LY20-A10-iper** was also superior to **LY20-MQ4-iper** in terms of mini-G protein recruitment at lower concentrations (pEC_{50} = 6.998 ± 0.059).

In summary, the M_4 receptor represented the most promising target for three of the four investigated hybrid ligands. Thereby, the amide functionalized hybrid ligands of the **LY20-An-iper** series generally released BRET signals at lower ligand concentrations than **LY20-MQn-iper** hybrids. Nonetheless, this does not necessarily lead to the conclusion that the methyl group in 4-position of the thienopyridine ring does not represent an adequate linkage point. In contrary, the preference for shorter linker chain length within the **LY20-MQn-iper** series serves as proof for a better relative position of the allosteric and orthosteric attachment points since a long and flexible linker is not required for the construction of hybrid ligands. Though, functional groups other than quaternary amines should be incorporated at the methyl group.

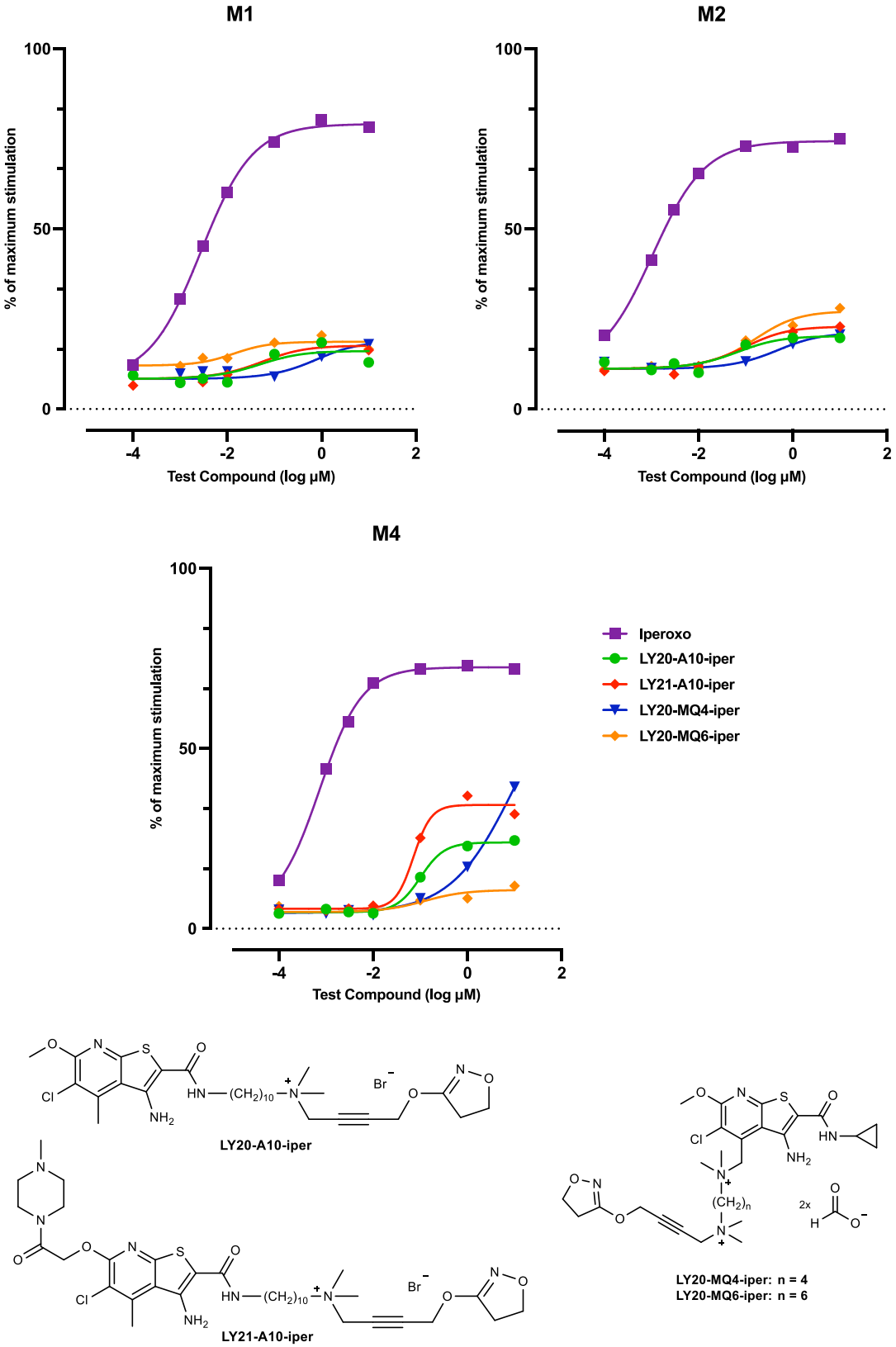


Figure 35: Concentration-response curves of **LY20-A10-iper**, **LY21-A10-iper**, **LY20-MQ4-iper**, **LY20-MQ6-iper**, and reference substance iperoxo in a mini-G protein BRET assay at M_1, M_2, and M_4 receptors. The recruitment of mini-$G\alpha_q$ proteins (M_1) or mini-$G\alpha_i$ proteins (M_2 and M_4) was determined, respectively. The highest observed mini-G protein recruitment upon stimulation with iperoxo was set to 100 %. Data are expressed as mean values. Standard deviations of measurement points are given in the appendix.

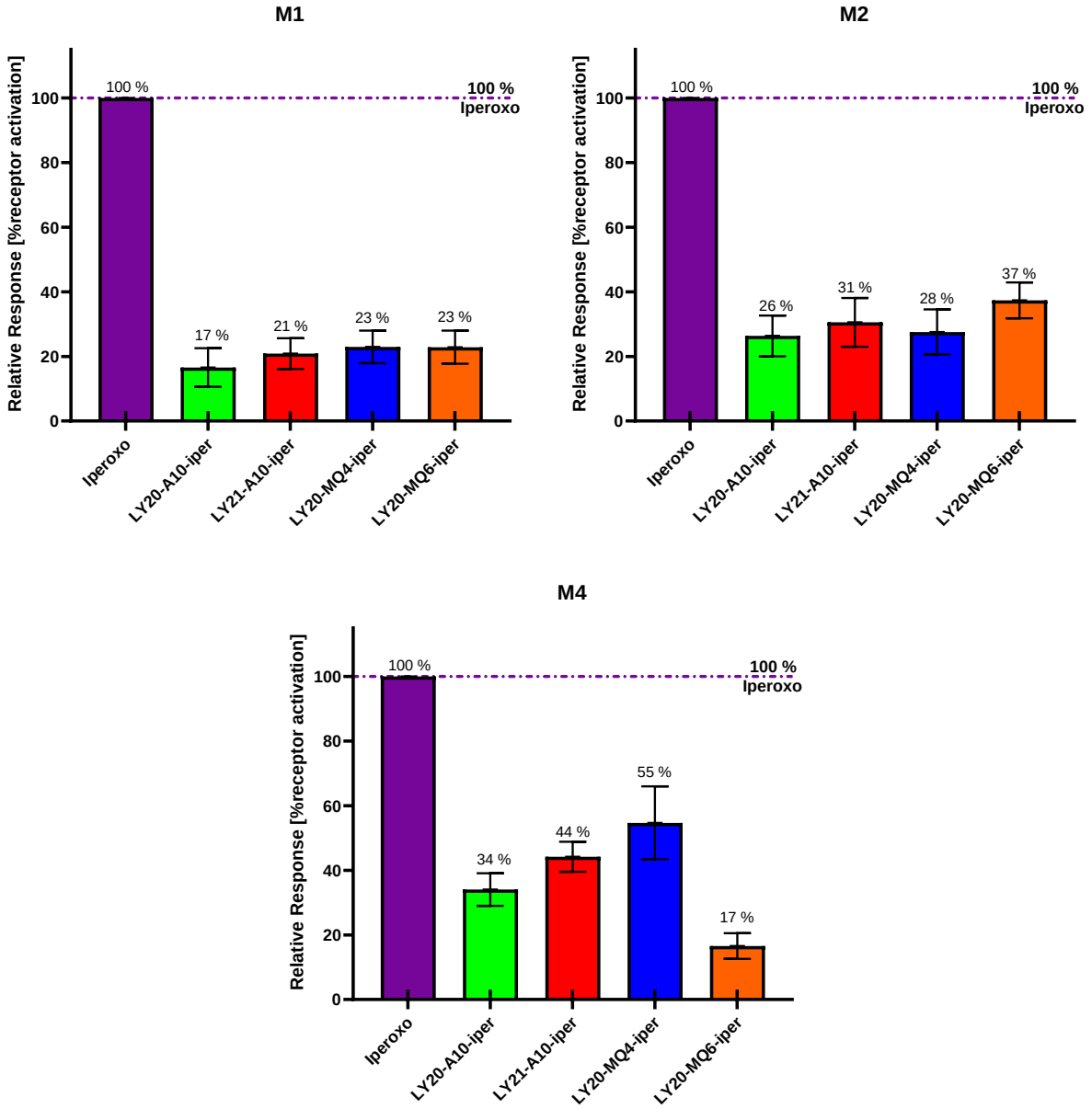


Figure 36: Observed responses for mini-G protein recruitment after stimulation of M_1, M_2, or M_4 receptors with 10 µM of **LY20-A10-iper**, **LY21-A10-iper**, **LY20-MQ4-iper**, or **LY20-MQ6-iper**. Mini-G protein recruitment upon stimulation with 10 µM iperoxo was set to 100 %. Data are expressed as mean values ± SD.

4.5 Preliminary results of LY20-MAn series and β-arrestin measurements

LY20-MAn hybrids were investigated in a mini-G protein BRET assay and, additionally, recruitment of β-arrestin was determined for the most active substances of this work. Since these results were preliminary at the time of writing, first data are only shown in the appendix of this work. For hybrid ligands of the LY20-MAn series, results of G-protein recruitment at M_1, M_2, and M_4 receptors via BRET assay measurements point towards partial agonism combined with a clear M_4 selectivity for **LY20-MA5-iper**. Furthermore, a receptor affinity at submicromolar ligand concentrations was observed. **LY20-MA3-iper** showed activity at both

M_2 as well as M_4 subtypes, whereas **LY20-MA7-iper** and **LY20-MA5-XanA** were not able to release G-protein recruitment at any of the investigated receptor subtypes. Moreover, determination of β-arrestin mediated signaling for **LY20-A10-TMA**, **LY21-A10-TMA**, **LY21-A10-iper**, **LY20-MQ4-iper**, **LY20-MQ6-iper**, and the **LY20-MAn-iper** series at M_2 and M_4 receptors was performed. Preliminary results indicated that none of these ligands causes recruitment of β-arrestin. Conclusively, these data suggest that **LY20-MA5-iper** represents an M_4 selective hybrid ligand which shows biased signaling for $G\alpha_{i/o}$ proteins.

4.6 Overall discussion of pharmacological results

The pharmacological investigation of LY-hybrid ligands revealed several key findings. Concerning the orthosteric unit, solely hybrids that contain iperoxo released a significant effect at M_1-, M_2-, or M_4-subtypes when using BRET assays. Consequently, xanomeline and TMA do not represent adequate orthosteric parts for G-protein recruitment in combination with LY substances. Furthermore, the expected M_2 affinity shift that was supposed to be induced by the attached piperazine moiety at the allosteric unit was not observed for LY21-An-hybrid compounds. This observation combined with the unsolved synthetic accessibility of such compounds makes further investigations of LY21-hybrid ligands less promising for future approaches. Regarding the attachment points, the results of this work point towards the methyl substituent to be more suitable for linker introduction since shorter chain length ranging between three and six methylene units were required to release an effect in FRET and BRET assays. However, good results in terms of ligand binding were also obtained for some hybrids with the amide group as functionalization unit and a chain length of ten methylene units.

Among the active LY-hybrid ligands, all compounds showed a distinct selectivity pattern at mAChR-subtypes compared to iperoxo or acetylcholine. This observation is in accordance with earlier studies and underlines the capability of hybrid ligands to induce subtype-selectivity.[84, 95, 108] **LY20-A10-iper**, **LY21-A10-iper**, and **LY20-MQ4-iper** were identified as the most active fully investigated compounds. They all preferentially stimulated the M_4-receptor and proved that even between the structurally closely related M_2- and M_4- subtypes binding selectivity can be achieved by LY-hybrid ligands. With a maximum effect of 55 % in comparison to iperoxo, **LY20-MQ4-iper** was able to release a strong receptor response at the M_4-subtype. Furthermore, preliminary results of LY20-MAn hybrids indicated the identification of an M_4 selective ligand. Within this series, **LY20-MA5-iper** not only showed a clear subtype selectivity but also biased signaling by preferring $G\alpha_{i/o}$ protein activation over recruitment of β-arrestin.

5 Summary

As part of the parasympathetic nervous system, muscarinic receptors are involved in the regulation of numerous functions in the human body. However, targeting a specific subtype of muscarinic receptors is challenging due to the high degree of similarity within the binding site of the endogenous neurotransmitter acetylcholine.[9, 19] Therefore, this study focused on the investigation of dualsteric ligands. Such hybrid ligands target the orthosteric acetylcholine binding site and, simultaneously, a distinct allosteric binding site. Since allosteric binding regions show significant structural differences throughout muscarinic receptor subtypes, it was aimed to produce selective ligands by means of combination of two pharmacophores in one molecule.[8, 60, 61]

Herein, the thienopyridine derivatives LY2033298 and LY2119620 which have shown an enhanced selectivity towards $G\alpha_i$ coupled M_2/M_4 receptors were chosen as allosteric moieties.[7, 68, 69, 70, 71] Based on literature studies, the investigated allosteric modulators were analyzed in terms of adequate attachment points for the combination with an orthosteric agonist. To this end, the amide group at the thienopyridine ring and the methyl substituent were selected as promising attachment points for dualsteric ligand design (Figure 37).

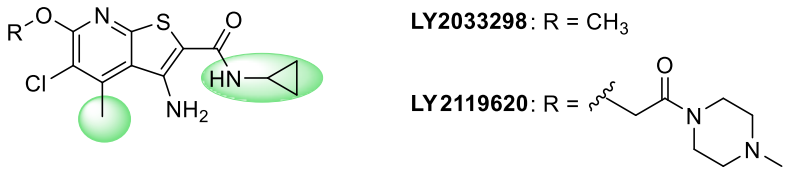


Figure 37: Identified attachment points at the thienopyridine scaffold of LY2033298 and LY2119620.

As orthosteric units, muscarinic superagonist iperoxo, xanomeline, and TMA were applied in this work. Since the distance between orthosteric and allosteric moieties plays a crucial role for dualsteric ligand binding, the linker chain length was also varied.[95]

At first, several amide functionalized thienopyridine hybrids were synthesized. Combination of a carbonylic acid precursor of LY2033298 with different types of orthosteric amine linkers resulted in four series of LY20-An hybrid compounds. Moreover, a different synthetic pathway was developed to produce two series of LY21-An hybrid molecules. Each of these series consists of three hybrid molecules with different linker chain length. Pharmacological investigations of LY20-An- and LY21-An hybrids by FRET- and BRET-assay measurements in the research group of Prof. Dr. Carsten Hoffmann showed best results for a linker chain length of ten methylene units. Concerning the orthosteric units, solely hybrid ligands with

iperoxo released G protein recruitment at M_2/M_4 receptors. In general, the measured maximum responses of amide functionalized hybrid ligands were higher at the M_4 subtype than at the M_2 receptor. After all, **LY21-A10-iper** and **LY20-A10-iper** were identified as the most active amide functionalized hybrid compounds (Figure 38). A selectivity shift of **LY21-A10-iper** towards M_2 receptors was not detected. However, the attachment of a piperazine ring to the thienopyridine moiety enhanced maximum response as well as binding affinity at the M_4 receptor.

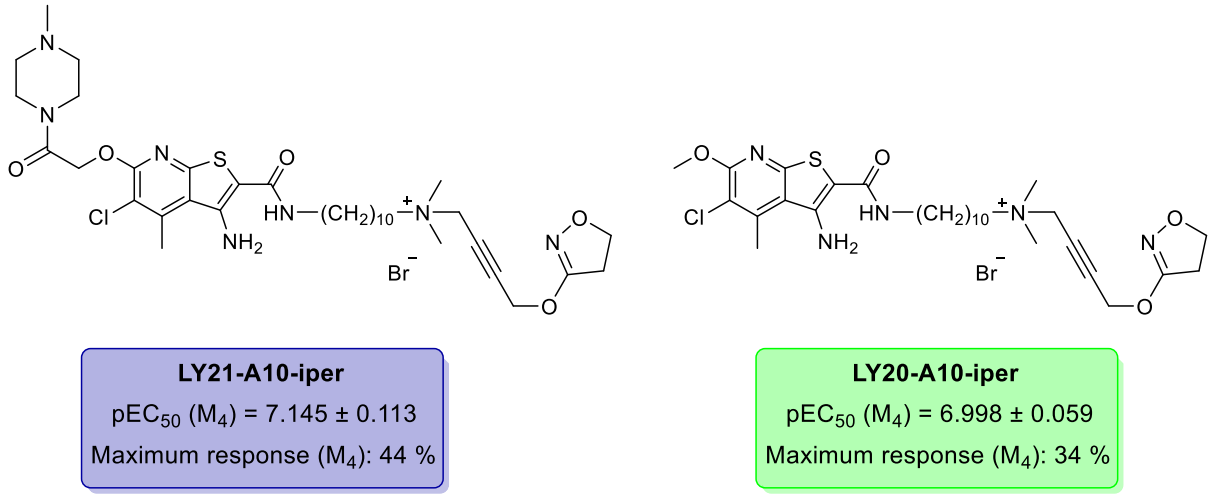


Figure 38: Structures of most promising amide functionalized LY-hybrid ligands. pEC_{50} values at the M_4 receptor were determined in a mini-G protein BRET assay. Maximum responses were measured at ligand concentrations of 10 µM and are referenced to the response of 10 µM iperoxo.

Furthermore, two series of LY-hybrid ligands were prepared by using the methyl side chain as functionalization unit. Since comparable modifications at the thienopyridine scaffold have not been reported so far, a new synthetic route towards this type of hybrid ligands was designed. A key step during this synthesis represented a radical bromination of the methyl side chain. In the further course of the synthetic route, the linker moieties were introduced via a quaternary amine (LY20-MQn series) or an amide function (LY20-MAn series) (Figure 39). In total, nine hybrid molecules with the methyl group as functionalization unit were synthesized. Thereof, hybrids containing a quaternary amine at the attachment point were investigated by FRET and BRET studies as well. The results for **LY20-MQn-iper** hybrids showed an M_4 preference when a chain length of four methylene units was applied. However, relatively high ligand concentrations were required in both assays to trigger an effect at the respective receptor sensor. Notably, **LY20-MQ4-iper** showed the highest response for G protein recruitment at the M_4 receptor among all investigated hybrid ligands. Furthermore, preliminary results of LY20-MAn hybrids indicated the identification of **LY20-MA5-iper** as an M_4 selective ligand which,

additionally, showed biased signaling properties by preferring $G\alpha_{i/o}$ protein activation over recruitment of β-arrestin.

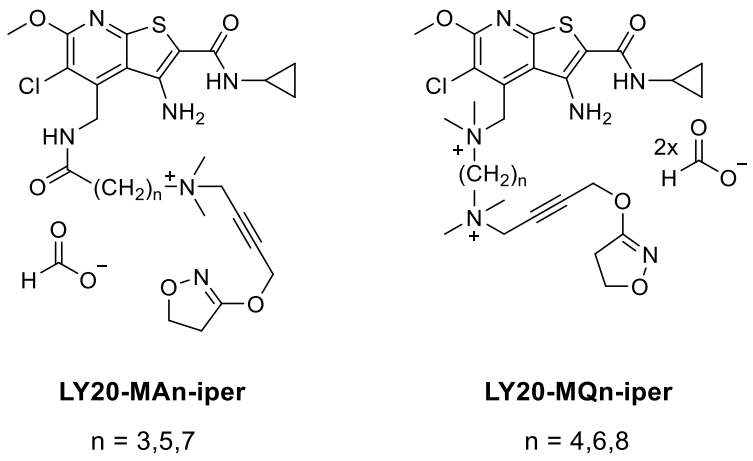


Figure 39: Structures of **LY20-MAn-iper** and **LY20-MQn-iper** hybrid ligands.

An overall comparison among the most active hybrid ligands with a methyl- or amide group as functionalization unit showed preferential properties of the former since the preference for shorter linker chain length indicates a better relative position of the orthosteric unit towards the allosteric modulator. The promising preliminary results of **LY20-MA5-iper** underline this conclusion.

6 Zusammenfassung

Als Teil des parasympathischen Nervensystems sind muskarinische Acetylcholinrezeptoren an der Regulation einer Vielzahl von Prozessen des menschlichen Körpers beteiligt. Die fünf unterschiedlichen Subtypen der Muskarinrezeptoren weisen allerdings kaum Abweichungen innerhalb der Bindestelle für den körpereigenen Neurotransmitter Acetylcholin auf, sodass eine subtypspezifische Rezeptoraktivierung immer noch eine große Herausforderung darstellt.[9, 19] Die vorliegende Arbeit beschäftigte sich deswegen mit dem Design dualsterischer Liganden, welche gleichzeitig die orthosterische Bindestelle für Acetylcholin sowie eine weitere, allosterische Bindestelle adressieren. Da sich die allosterischen Bindestellen der verschiedenen Muskarinrezeptoren teilweise deutlich voneinander unterscheiden, war das Ziel dieser Studie die Herstellung selektiver Liganden durch die Kombination zweier Pharmakophore.[8, 60, 61]

Als allosterische Einheiten wurden die Thienopyridinderivate LY2033298 und LY2119620 ausgewählt, da diese eine erhöhte Affinität zu den $G\alpha_i$-gekoppelten Subtypen M_2 und M_4 besitzen.[7, 68, 69, 70, 71] Durch Literaturrecherche wurden zunächst geeignete Verknüpfungspunkte an den allosterischen Modulatoren identifiziert. Hierbei erwiesen sich die Amidgruppe am Thienopyridinring und der Methylrest als vielversprechend (Abbildung 1).

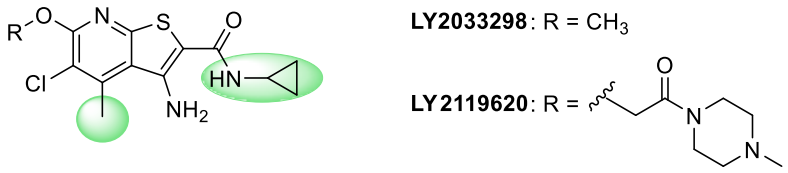


Abbildung 1: Identifizierte Verknüpfungspunkte an den Thienopyridinderivaten LY2033298 und LY2119620.

Als orthosterische Einheiten wurden Iperoxo, Xanomelin und TMA in dieser Arbeit untersucht. Da der korrekte Abstand zwischen orthosterischen und allosterischen Bausteinen einen entscheidenden Einfluss auf das Bindungsverhalten von dualsterischen Liganden hat, wurde die Länge des Alkyllinkers variiert.[95] Zunächst wurden mehrere Hybride hergestellt, welche über die Amidgruppe mit den jeweiligen Linkern verbunden sind. Die Kombination einer Carbonsäure-Vorstufe von LY2033298 mit verschiedenen orthosterischen Linkermolekülen ergab vier Serien an LY20-An-Hybridmolekülen. Außerdem führte ein weiterer Syntheseweg zu zwei Serien von LY21-An-Hybridmolekülen. Jede dieser Serien besteht aus drei dualsterischen Liganden mit unterschiedlichen Kettenlängen des Linkers. Pharmakologische Untersuchungen dieser Hybride mittels FRET- und BRET-Assay-Messungen in der Arbeitsgruppe von Prof. Dr. Carsten Hoffmann zeigten die besten Resultate bei einer

Alkylkettenlänge von zehn Methyleneinheiten. Bezüglich der orthosterischen Bausteine konnte nur bei der Verwendung von Iperoxo eine Rekrutierung des G-Proteins an den M_2/M_4-Rezeptoren beobachtet werden. Allgemein erzeugten die über das Amid modifizierten Hybride am M_4-Rezeptor eine stärkere Rezeptorantwort als am M_2-Rezeptor. Letztlich wurden die beiden Verbindungen **LY21-A10-iper** und **LY20-A10-iper** als die aktivsten Hybride innerhalb dieser Serie identifiziert (Abbildung 2). Interessanterweise zeigte dabei auch **LY21-A10-iper**, trotz des enthaltenen Piperazin-Rings, eine deutlichere Rezeptorantwort am M_4-Rezeptor als am M_2-Rezeptor.

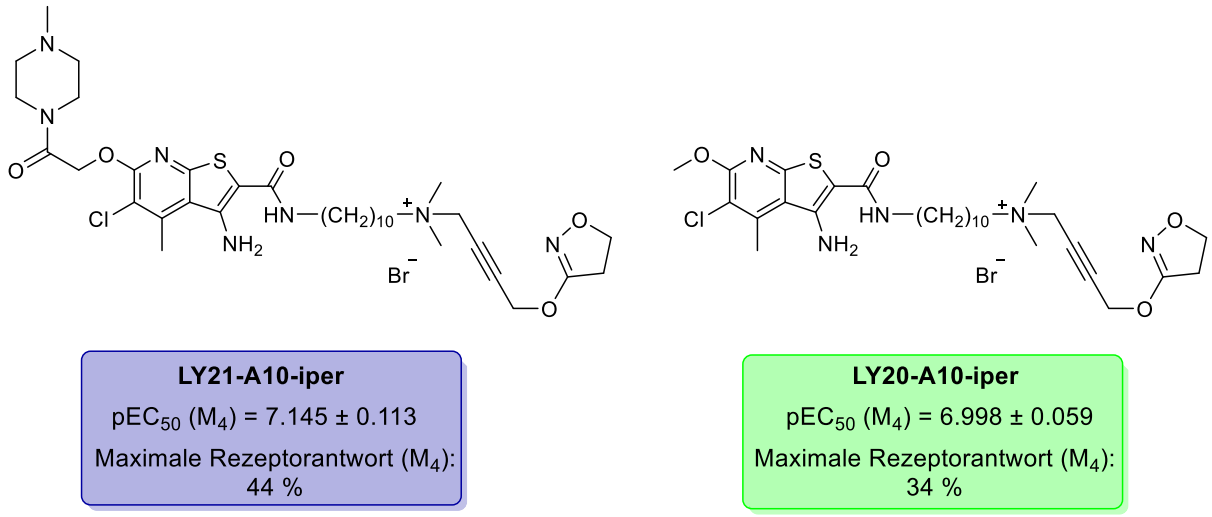


Abbildung 2: Strukturen der zwei aktivsten Verbindungen aus der Reihe der an der Amidgruppe funktionalisierten Hybridliganden. Die pEC_{50}-Werte am M_4-Rezeptor beziehen sich auf den mini-G-Protein-BRET-Assay. Die maximalen Rezeptorantworten wurden bei einer Konzentration des jeweiligen Liganden von 10 µM gemessen und in Relation zu der von 10 µM Iperoxo ausgelösten Rezeptorantwort gesetzt.

Des Weiteren wurden zwei Serien an LY-Hybriden hergestellt, welche über den Methylrest mit der Alkylkette verbunden sind. Da eine derartige Modifikation des Thienopyridin-Bausteins noch nicht bekannt war, musste hierfür ein neuer Syntheseweg entworfen werden. Hierbei besteht das Schlüsselelement der Syntheseroute aus einer radikalischen Bromierung des Methylrests. Im weiteren Verlauf der Synthese erfolgte die Verknüpfung mit dem orthosterischen Teil entweder über ein quartäres Amin oder über eine Amidbindung (Abbildung 3). Insgesamt wurden neun derartige Hybridmoleküle hergestellt. Davon wurden die über das quartäre Amin verknüpften Hybridliganden mittels FRET- und BRET-Assays untersucht. Die Ergebnisse zeigten eine bevorzugte Aktivierung des M_4-Subtyps, wenn eine Alkylkettenlänge von vier Methyleneinheiten gewählt wurde. In beiden Assays waren jedoch hohe Konzentration des Liganden notwendig, um eine Rezeptorantwort auszulösen. Allerdings zeigte **LY20-MQ4-iper** das höchste Level an G-Protein-Rekrutierung am M_4-Rezeptor von allen in dieser Arbeit pharmakologisch untersuchten Substanzen. Vorläufige Ergebnisse der

LY20-MAn-Serie deuten außerdem darauf hin, dass mit **LY20-MA5-iper** die Identifizierung eines M_4-selektiven Hybridliganden gelungen ist. Dieser führte darüber hinaus zu einer bevorzugten Aktivierung von $G\alpha_{i/o}$ Proteinen gegenüber der Rekrutierung von β-Arrestin.

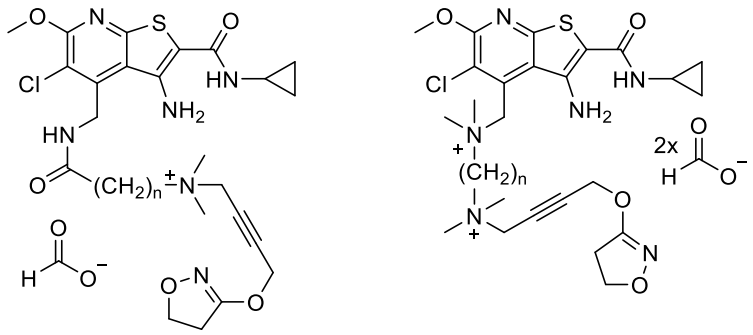


Abbildung 3: Strukturen der **LY20-MAn-iper** sowie der **LY20-MQn-iper**-Hybridliganden. Die Verknüpfung erfolgte jeweils über den Methylrest des Thienopyridin-Gerüsts.

Die aktivsten, vollständig untersuchten Hybridliganden an den jeweiligen Verknüpfungspunkten wurden abschließend miteinander verglichen. Hierbei stellt der Methylrest den attraktiveren Verknüpfungspunkt für das Design dualsterischer Liganden dar, da die Bevorzugung kurzer Kettenlängen innerhalb der **LY20-MQn-iper**-Serie auf eine bessere relative Positionierung der orthosteren zur allosteren Einheit hindeutet. Die bislang vorläufigen Ergebnisse der LY-MAn Serie unterstreichen diese Schlussfolgerung zusätzlich.

7 Experimental section

7.1 General experimental procedures and equipment

Chemicals

Common reagents used for the synthesis in this work were obtained from *Merck* (Darmstadt, Germany), *Avantor* (Darmstadt, Germany), *TCI Deutschland GmbH* (Eschborn, Germany), *Fisher Scientific* (Schwerte, Germany), and *ABCR* (Karlsruhe, Germany). The purchased chemicals were used without further purification.

Chromatography

- For gravity-driven column chromatography, silica gel 60 (0.063-0.200 nm) purchased from *Merck* (Darmstadt, Germany) or basic alumina 90 (0.050-0.200 nm) purchased from *Macherey-Nagel* (Düren, Germany) were used.
- For purification by medium pressure liquid chromatography (MPLC, flash chromatography), a puriFlash®430 system of *Interchim* (Montluçon, France) was used. The applied pre-packed columns contained endcapped C-18 silica and were purchased from *Macherey-Nagel* (Düren, Germany). For flash chromatography, only deionized water purified by a Milli-Q®-System from *Merck* (Darmstadt, Germany) and methanol (HPLC gradient, *Merck*, Darmstadt, Germany) were used.
- HPLC (high performance liquid chromatography) method **I** for purity analysis and determination of solubility:

instrument	*Agilent* HPLC system (1100 series) (*Agilent Technologies*, Böblingen, Germany) with an integrated degasser, binary pump, column thermostat, and a DAD detector.
column	Synergi C18-Fusion-RP (5 µm, 250 mm x 4.6 mm) (*Phenomenex*, Aschaffenburg, Germany)
eluent	water + 0.1 % TFA (A), methanol + 0.1 % TFA (B) (ratios as indicated in experimental section)
detection	λ = 280 nm
temperature	25 °C
injection volume	10 µl
flow	1 ml/min

- HPLC method **II** for purity analysis:

instrument	*Shimadzu* HPLC system (*Shimadzu Scientific instruments*, Kyoto,

	Japan), equipped with a DGU-20A3R controller, an LC20AB liquid chromatograph, and an SPD-20 UV/Vis detector.	
column	Synergi C18-Fusion-RP (4 µm, 150 mm x 4.6 mm) (*Phenomenex*, Aschaffenburg, Germany)	
eluent	water + 0.1 % FA (A), methanol + 0.1 % FA (B)	
gradient elution	0-8 min	5 % → 100 % (B)
	8-12 min	100 % (B)
	12-16 min	100 % → 5 % (B)
	16-18 min	5 % (B)
detection	**a**: λ = 254 nm	
	b: λ = 280 nm	
temperature	rt	
flow	1 ml/min	

For purity determination, deionized water purified by a Milli-Q®-System from *Merck* (Darmstadt, Germany), and methanol (HPLC gradient, *Merck*, Darmstadt, Germany) of appropriate purity were used.

- Thin layer chromatography was performed on pre-coated silica gel glass plates SIL G-25 (*Macherey-Nagel*, Düren, Germany) or on pre-coated basic alumina glass plates (*Merck*, Darmstadt, Germany). Spots were evidenced by quenching at 254 nm, intrinsic fluorescence at 366 nm, or with Dragendorff reagent.[141]

Infrared Spectroscopy (IR)

IR spectra were recorded on a Jasco-FT-IR-6100 system (*Jasco Deutschland GmbH*, Groß-Umstadt, Germany) or on a Jasco FT-IR-4700 system (*Jasco Deutschland GmbH*, Groß-Umstadt, Germany) in combination with a diamond ATR accessory, respectively.

Mass spectrometry

Electrospray ionization (ESI) mass spectra were measured with a Shimadzu LCMS-2020 (*Shimadzu Scientific instruments*, Kyoto, Japan). Data are reported as mass-to-charge ratio (m/z) of the respective positively charged molecular ions.

Melting points

For determination of melting points, a MP70 Melting Point System (*Mettler-Toledo GmbH*, Gießen, Germany) or an MPM-H2 melting point meter (*Schorpp Gerätetechnik*, Überlingen, Germany) were used.

Microwave System

Reactions under microwave irradiation were performed in an MLS-Ethos-CFR 1600 system (*MLS-GmbH*, Leutkirch, Germany).

Nuclear Magnetic Resonance Spectroscopy

^{1}H (400.132 MHz) and ^{13}C (100.613) NMR spectra were recorded on a Bruker AV 400 instrument (*Bruker Biospin*, Ettlingen, Germany). ^{13}C spectrum of **LY20-MQ8-iper** was recorded on Bruker Avance III HD 600 instrument (*Bruker Biospin*, Ettlingen, Germany). The signal of the respective deuterated solvent was used as internal standard ($CDCl_3$ ^{1}H 7.24 ppm ^{13}C 77.23 ppm; CD_3OD ^{1}H 3.31 ppm ^{13}C 49.15 ppm; CD_3CN ^{1}H 1.94 ppm ^{13}C 118.69 ppm; DMSO-d_6 ^{1}H 2.50 ppm ^{13}C 39.51 ppm).[142] Abbreviations for multiplicity are: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad; dd, doublet of doublets; dt, doublet of triplets. Topspin® (version 3.2-pl7) software (*Bruker Biospin*, Ettlingen, Germany) was applied for processing of NMR spectra.

Solvents

Dry solvents for organic synthesis were produced and stored according to general procedures.[143] CCl_4 was pre-dried over barium carbonate prior to use.

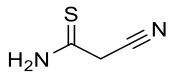
Thermomixer

For the determination of solubility, 1 mg of compound **LY20-A6-iper** was dissolved in PBS of pH = 7.4, tempered at 37 °C and shaken (800 rpm) in a thermomixer for 72 h (*Eppendorf*, Hamburg, Germany). Samples were then centrifuged (13000 rpm, 1 min) and the supernatant was analyzed by HPLC method **I** (B: 65 %).

7.2 Synthesis of LY20-An hybrids

7.2.1 Preparation of thienopyridine carboxylic acid scaffold 1

*Cyanothioacetamide, **2** (FG_B_22)*



$C_3H_4N_2S$

M_r = 100.14 g/mol

According to Dotsenko *et al.*[110], a solution of malononitril (32.0 g, 484.4 mmol) in ethanol (100 ml) was treated with TEA (0.4 ml, 2.8 ml). A current of H_2S, generated by adding 6 M hydrochloric acid (120 ml) slowly to sodium sulfide (60 g) in a separate flask, was passed through the solution for 2 h. Reaction temperature was maintained between 15-20 °C. The reaction mixture was cooled to -20 °C and the sandy-yellow precipitate was filtered and washed with cooled ethanol, diethylether, and petroleum ether to yield compound **2**. Product must be stored at 4 °C.

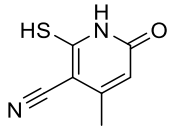
appearance:	Sandy-yellow crystals
yield:	34.8 g (71 %, Lit.:[110] 86 %)
melting point [°C]:	119-120 (Lit.:[110] 117-120)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3354, 3280, 3154, 2920, 2887, 2259, 1622

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 9.82 (s, 1H, N**H**$_2$), 9.46 (s, 1H, N**H**$_2$), 3.97 (s, 2H, C**H**$_2$).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 194.5 (**C**(S)NH$_2$), 116.4 (**C**N), 33.8 (**C**H$_2$).

The obtained physical and spectroscopic data are consistent with that found in literature. Dotsenko *et al.* did not provide ^{13}C NMR.[110]

*2-Mercapto-4-methyl-6-oxo-1,6-dihydropyridine-3-carbonitrile, **3** (FG_B_24)*



$C_7H_6N_2OS$

M_r = 166.20 g/mol

According to Szabo *et al.*[109], compound **2** (12.0 g, 119.8 mmol) and morpholine (10.3 ml, 119.8 mmol) were dissolved in ethanol (100 ml) under heating. Methyl acetoacetate was added and the mixture was heated for 14 h under reflux. After cooling to rt, the precipitate was filtered and washed with cold DCM. The thiolate formed was dissolved in a minimum amount of water.

Addition of 6 M hydrochloric acid (25 ml) and subsequent cooling to 0 °C led to precipitation of **3** as yellowish solid. The product was filtered and dried *in vacuo*.

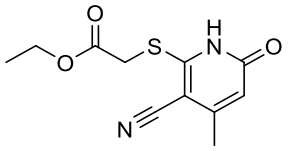
appearance:	yellowish powder
yield:	8.1 g (40 %, Lit.:[109] 51 %)
melting point [°C]:	262-264 (Lit.:[109] 267-269)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2847, 2749, 2229, 1587, 1305, 1201

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 10.72 (br s, 1H, S**H**), 8.70 (s, 1H, C**H**), 2.00 (s, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 175.1 (**C**$_q$), 161.0 (**C**$_q$), 150.5 (**C**$_q$), 119.1 (**C**H), 106.9 (**C**$_q$), 93.0 (**C**$_q$), 21.2 (**C**H$_3$).

The obtained physical and spectroscopic data are consistent with literature data.[109, 144]

Ethyl 2-((3-cyano-4-methyl-6-oxo-1,6-dihydropyridin-2-yl)thio)acetate, ***4*** *(FG_B_26)*



$C_{11}H_{12}N_2O_3S$

M_r = 252.29 g/mol

According to Szabo *et al.*[109], a solution of **3** (3.4 g, 20.3 mmol) and TEA (2.7 ml, 20.3 mmol) in DMF (160 ml) was cooled to 0 °C in an ice bath. A solution of ethyl-2-chloroacetate (2.2 ml, 20.3 mmol) in DMF (30 ml) was added dropwise. The reaction mixture was allowed to warm to rt and stirred overnight. After removal of solvent *in vacuo*, the residue was dissolved in DCM (75 ml). The solution was treated with water (70 ml) and, after phase separation, the aqueous phase was extracted with DCM (2x50 ml). The combined organic layers were dried over sodium sulfate. The crude product was purified by column chromatography (silica gel, ethyl acetate (EtOAc)/*n*-hexane: 2/1) to give **4**.

appearance:	white solid
yield:	3.5 g (68 %, Lit.:[109] 86 %)
reaction control:	R_f = 0.50 (silica gel; EtOAc/Cy: 1/1)
melting point [°C]:	100-102 (Lit.:[109] 103-104)

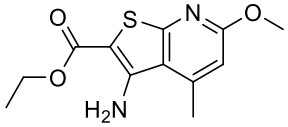
IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3003, 2951, 2848, 2224, 1734, 1636, 1264

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.32-6.31 (m, 1H, C**H**), 4.20 (q, ^{3}J = 7.1 Hz, 2H, C**H$_2$**CH$_3$), 3.89 (s, 3H, C**H$_2$**), 2.35 (s, 2H, C**H$_3$**), 1.25 (t, ^{3}J = 7.1 Hz, 3H, CH$_2$C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 169.8 (**C**$_q$), 163.6 (**C**$_q$), 154.8 (**C**$_q$), 153.5 (**C**$_q$), 114.5 (**C**$_q$), 113.8 (**C**H), 98.3 (**C**$_q$), 62.9 (**C**H$_2$), 34.2 (**C**H$_2$), 20.9 (**C**H$_3$), 14.2 (**C**H$_3$).

The obtained physical and spectroscopic data are consistent with that found in literature.[109]

*Ethyl 2-((3-cyano-6-methoxy-4-methylpyridin-2-yl)thio)acetate, **5** (FG_B_27)*



$C_{12}H_{14}N_2O_3S$

M_r = 266.32 g/mol

According to Szabo *et al.*[109], compound **4** (3.5 g, 13.9 mmol) was dissolved in DMF (60 ml). After addition of potassium carbonate (2.9 g, 20.8 mmol) and methyl iodide (1.4 ml, 22.2 mmol), the reaction mixture was stirred 6 h at rt. A solution of potassium hydroxide (1 M, 50 ml) was added, followed by further stirring for 30 min. Addition of water (60 ml) to the reaction mixture and cooling to 0 °C led to precipitation of **5** as a white solid which was filtered and dried under high vacuum.

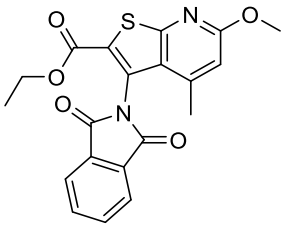
appearance:	white solid
yield:	3.3 g (89 %, Lit.:[109] 58 %)
melting point [°C]:	146-148 (Lit.:[109] 150)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3501, 3361, 2979, 2850, 1664, 1557, 1266

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.42-6.40 (m, 1H, C**H**), 4.31 (q, ^{3}J = 7.1 Hz, 2H, C**H$_2$**CH$_3$), 3.96 (s, 3H, OC**H$_3$**), 2.67 (s, 3H, C**H$_3$**), 1.35 (t, ^{3}J = 7.1 Hz, 3H, CH$_2$C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.0 (**C**$_q$), 164.7 (**C**$_q$), 160.6 (**C**$_q$), 149.6 (**C**$_q$), 146.0 (**C**$_q$), 119.5 (**C**$_q$), 110.1 (**C**H), 96.3 (**C**$_q$), 60.5 (**C**H$_2$CH$_3$), 54.1 (O**C**H$_3$), 20.5 (**C**H$_3$), 14.7 (CH$_2$**C**H$_3$).

The obtained physical and spectroscopic data are consistent with that found in literature.[109]

Ethyl-3-(1,3-dioxoisoindolin-2-yl)-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxylate, ***6*** *(FG_B_28)*



$C_{20}H_{16}N_2O_5S$

M_r = 396.42 g/mol

According to Szabo *et al.*[109], compound **5** (3.5 g, 13.1 mmol) was dissolved in acetic acid (20 ml). The mixture was heated under reflux and phthalic anhydride (7.8 g, 52.6 mmol) was added portionswise. In the meantime, the mixture was heated under reflux. Cooling of the reaction mixture in an ice bath led to precipitation of a white solid which was filtered, washed with cooled ethanol, and air-dried to yield compound **6**.

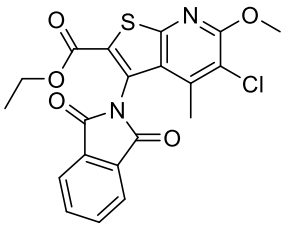
appearance:	white solid
yield:	3.9 g (75 %, Lit.:[109] 55 %)
reaction control:	R_f = 0.31 (silica gel; EtOAc/Cy: 1/4)
melting point [°C]:	216
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2984, 2935, 1702, 1550, 1367, 1243

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 8.11-8.07 (m, 2H, indolinyl-C**H**), 8.04-7.99 (m, 2H, indolinyl-C**H**), 6.89 (s, 1H, thienopyridinyl-C**H**), 4.15 (q, ^{3}J = 7.1 Hz, 2H, C**H$_2$**CH$_3$), 3.99 (s, 3H, OC**H$_3$**), 2.34 (s, 3H, C**H$_3$**), 0.96 (t, ^{3}J = 7.1 Hz, 3H, CH$_2$C**H$_3$**).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 167.0 (**C**$_q$), 164.3 (**C**$_q$), 160.2 (**C**$_q$), 157.7 (**C**$_q$), 146.6 (**C**$_q$), 135.6 (indolinyl-**C**H), 131.4 (**C**$_q$), 127.9 (**C**$_q$), 124.3 (**C**$_q$), 124.2 (indolinyl-**C**H), 124.1 (**C**$_q$), 112.2 (thienopyridinyl-**C**H), 61.8 (**C**H$_2$CH$_3$), 54.1 (O**C**H$_3$), 17.5 (**C**H$_3$), 13.5 (CH$_2$**C**H$_3$).

The obtained physical and spectroscopic data are consistent with that found in literature.[109] Szabo *et al.*[109] did not provide a melting point.

Ethyl-5-chloro-3-(1,3-dioxoisoindolin-2-yl)-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxylate, ***7*** *(FG_B_29)*



$C_{20}H_{15}ClN_2O_5S$

M_r = 430.86 g/mol

According to Szabo *et al.*[109], compound **6** (3.9 g, 9.7 mmol) was suspended in acetonitrile (80 ml), followed by the addition of *N*-chlorosuccinimide (2.6 g, 19.5 mmol) and two drops of concentrated hydrochloric acid. The reaction mixture was heated under reflux for 4 h. After solvent removal *in vacuo*, the residue was dissolved in DCM (80 ml) and treated with water (60 ml). After phase separation, the organic layer was treated with brine (1x40 ml). The layers were again separated and the organic layer was dried over sodium sulfate. Subsequent solvent removal yielded compound **7**.

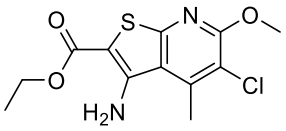
appearance:	white solid
yield:	3.6 g (87 %, Lit.:[109] 93 %)
reaction control:	R_f = 0.37 (silica gel; EtOAc/Cy: 1/4)

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 8.01-7.97 (m, 2H, indolinyl-C**H**), 7.84-7.81 (m, 2H, indolinyl-C**H**), 4.19 (q, ^3J = 7.1 Hz, 2H, C**H$_2$**CH$_3$), 4.11 (s, 3H, OC**H$_3$**), 2.47 (s, 3H, C**H$_3$**), 1.10 (t, ^3J = 7.1 Hz, 3H, CH$_2$C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 167.6 (**C**$_q$), 160.9 (**C**$_q$), 159.6 (**C**$_q$), 156.0 (**C**$_q$), 143.0 (**C**$_q$), 134.9 (indolinyl-**C**H), 132.5 (**C**$_q$), 127.4 (**C**$_q$), 127.1 (**C**$_q$), 125.2 (**C**$_q$), 124.4 (indolinyl-**C**H), 118.9 (**C**$_q$), 62.0 (**C**H$_2$CH$_3$), 55.5 (O**C**H$_3$), 14.8 (**C**H$_3$), 14.0 (CH$_2$**C**H$_3$).

The obtained physical and spectroscopic data are consistent with that found in literature.[109]

Ethyl-3-amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxylate, ***8*** *(FG_B_31)*



$C_{12}H_{13}ClN_2O_3S$

M_r = 300.76 g/mol

According to Szabo *et al.*[109], compound **7** (3.6 g, 8.2 mmol) was dissolved in ethanol (150 ml). After addition of hydrazine monohydrate (1.6 ml, 33.0 mmol), the mixture was heated under

reflux for 3 h. The mixture was then cooled to rt. The resulting white precipitate was filtered off and washed with cold chloroform. The filtrate was further diluted with chloroform (60 ml) and washed with water (3x20 ml). After phase separation, the organic layer was dried over sodium sulfate. Solvent removal yielded compound **8**.

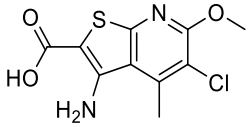
appearance:	pale yellow solid
yield:	2.1 g (85 %, Lit.:[109] 84 %)
reaction control:	R_f = 0.60 (silica gel; EtOAc/Cy: 1/1)
melting point [°C]:	213
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3483, 3356, 1658, 1552, 1446, 1365, 1259

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.31 (q, ^3J = 7.1 Hz, 2H, C**H$_2$**CH_3), 4.06 (s, 3H, OC**H$_3$**), 2.80 (s, 3H, C**H$_3$**), 1.36 (t, ^3J = 7.1 Hz, 3H, CH_2C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 170.4 (**C**$_q$), 165.8 (**C**$_q$), 159.6 (**C**$_q$), 157.4 (**C**$_q$), 149.1 (**C**$_q$), 143.0 (**C**$_q$), 120.2 (**C**$_q$), 116.4 (**C**$_q$), 60.7 (**C**H_2CH_3), 55.3 (O**C**H_3), 16.5 (**C**H_3), 14.7 (CH_2**C**H_3).

The obtained physical and spectroscopic data are consistent with that found in literature.[109] Szabo *et al.*[109] did not provide a melting point.

3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxylic acid, ***1*** *(FG_B_32)*



$C_{10}H_9ClN_2O_3S$

M_r = 272.70 g/mol

According to Szabo *et al.*[109], compound **8** (250.0 mg, 831.2 µmol) was suspended in a 1/1 mixture of ethanol and 2 M sodium hydroxide (50 ml) and heated under reflux for 2 h. After cooling to rt, an excess of 6 M hydrochloric acid was added to cause precipitation of compound **1**. For complete precipitation, the mixture was cooled in an ice bath. The precipitate was filtered, washed with cold water, and dried *in vacuo***.**

appearance:	beige solid
yield:	207.0 mg (91 %, Lit.:[109] 84 %)
reaction control:	R_f = 0.08 (silica gel; EtOAc/Cy: 2/3)

melting point [°C]:	>300 °C
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3497, 3352, 2961, 1602, 1546, 1361, 1259, 1071

^{1}H NMR (400 MHz, DMSO-d^{6}, δ [ppm]): 6.42 (bs, 2H, N**H$_2$**), 3.95 (s, 3H, C**H$_3$**), 2.77 (s, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, DMSO-d^{6}, δ [ppm]): 168.3 (**C$_q$**), 156.8 (**C$_q$**), 154.0 (**C$_q$**), 143.2 (**C$_q$**), 142.4 (**C$_q$**), 122.1 (**C$_q$**), 113.7 (**C$_q$**), 96.4 (**C$_q$**), 54.4 (O**C**H$_3$), 15.7 (**C**H$_3$).

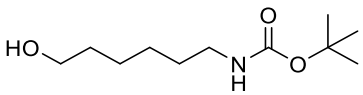
The obtained physical and spectroscopic data are consistent with that found in literature.[109] Szabo *et al.*[109] did not provide a melting point.

7.2.2 Preparation of orthosteric iperoxo linkers 1-Ln and iperoxo

*General synthesis procedure **G.1** for Boc-protection of primary aminoalkanols*

According to Szabo *et al*.[109], the appropriate aminoalkanol (1 equiv) was dissolved in DCM, followed by the addition of a 30 % solution of Boc anhydride (Boc$_2$O) in THF (1 equiv) and TEA (1.1 equiv). After the reaction mixture was stirred for 2 h at rt, the solvent was removed and the crude product was purified by column chromatography (silica gel, DCM/CH$_3$OH: 10/1).

*tert-Butyl(6-hydroxyhexyl)carbamate, **9a** (FG_B_30)*



C$_{11}$H$_{23}$NO$_3$

M_r = 217.31 g/mol

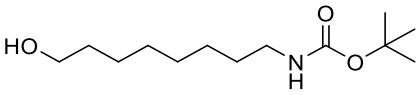
A solution of 6-aminohexanol (1.05 g, 9.0 mmol) in DCM (20 ml) was treated with a 30 % solution of Boc$_2$O in THF (6.42 ml, 9.0 mmol) and TEA (1.38 ml, 9.9 mmol) and stirred at rt for 2 h. The reaction mixture was worked up as described in general procedure **G.1** to give **9a** as a white solid.

yield:	1.9 g (98 %)
reaction control:	R_f = 0.55 (silica gel; DCM/CH$_3$OH: 10/1; 4-anisaldehyde)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3364, 2932, 2856, 1684, 1518, 1246, 1167

^{1}H NMR (400 MHz, CDCl$_3$, δ [ppm]): 4.51 (br s, 1H, N**H**), 3.60 (t, ^{3}J = 6.5 Hz, 2H, C**H$_2$**OH), 3.12-3.04 (m, 2H, C**H$_2$**NH), 1.57-1.47 (m, 4H, C**H$_2$**), 1.41 (s, 9H, C**H$_3$**), 1.37-1.28 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.3 (**C**(O)NH), 79.3 (**C**$_q$), 62.9 (**C**H_2OH), 40.6 (**C**H_2NH), 32.8 (**C**H_2), 30.3 (**C**H_2), 28.6 (**C**H_3), 26.6 (**C**H_2), 25.5 (**C**H_2).

*tert-Butyl(8-hydroxyoctyl)carbamate, **9b** (FG_B_40)*



$C_{13}H_{27}NO_3$

M_r = 245.36 g/mol

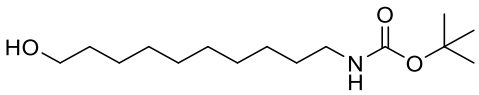
A solution of 8-aminooctanol (560 mg, 3.86 mmol) in DCM (10 ml) was treated with a 30 % solution of Boc_2O in THF (2.75 ml, 3.86 mmol) and TEA (591.1 µl, 4.2 mmol) and stirred at rt for 2 h. The reaction mixture was worked up as described in general procedure **G.1** to give **9b** as a white solid.

yield:	0.9 g (95 %)
reaction control:	R_f = 0.57 (silica gel; DCM/CH_3OH: 10/1; 4-anisaldehyde)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3421, 3365, 2929, 2852, 1685, 1518, 1364, 1165

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.48 (br s, 1H, N**H**), 3.59 (t, ^{3}J = 6.6 Hz, 2H, C**H$_2$**OH), 3.06 (t, ^{3}J = 6.6 Hz, 2H, C**H$_2$**NH), 1.57-1.48 (m, 2H, C**H$_2$**), 1.46-1.37 (m, 11H, C**H$_2$**, C**H$_3$**), 1.33-1.23 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.2 (**C**(O)NH), 79.3 (**C**$_q$), 63.1 (**C**H_2OH), 40.9 (**C**H_2-NH), 32.9 (**C**H_2), 30.2 (**C**H_2), 29.5 (**C**H_2), 29.4 (**C**H_2), 28.6 (**C**H_3), 26.6 (**C**H_2), 25.5 (**C**H_2).

*tert-Butyl(10-hydroxydecyl)carbamate, **9c** (FG_B_46)*



$C_{15}H_{31}NO_3$

M_r = 273.42 g/mol

A solution of 10-aminodecanol (630 mg, 3.64 mmol) in DCM (10 ml) was treated with a 30 % solution of Boc_2O in THF (2.59 ml, 3.64 mmol) and TEA (557.4 µl, 4.0 mmol) and stirred at rt for 2 h. The reaction mixture was worked up as described in general procedure **G.1** to give **9c** as a white solid.

yield:	0.9 g (95 %)
reaction control:	R_f = 0.58 (silica gel; DCM/CH_3OH: 10/1; 4-anisaldehyde)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3421, 3366, 2919, 2851, 1685, 1519, 1364, 1169

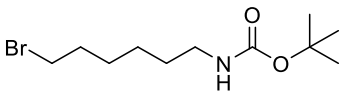
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.54 (br s, 1H, N**H**), 3.58 (t, ^{3}J = 6.5 Hz, 2H, C**H$_2$**OH), 3.10-3.02 (m, 2H, C**H$_2$**NH), 1.71 (s, 1H, O**H**), 1.56-1.45 (m, 2H, C**H$_2$**), 1.43-1.37 (m, 11H, C**H$_2$**, C**H$_3$**), 1.31-1.21 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.2 (**C**(O)NH), 79.2 (**C**$_q$), 63.1 (**C**H_2OH), 40.8 (**C**H_2-NH), 32.9 (**C**H_2), 30.2 (**C**H_2), 29.6 (**C**H_2), 29.6 (**C**H_2), 29.5 (**C**H_2), 29.4 (**C**H_2), 28.6 (**C**H_3), 26.9 (**C**H_2), 25.9 (**C**H_2).

*General synthesis procedure **G.2** for formation of Boc protected bromoalkanamines **10a-c***

The respective Boc protected aminoalkanol was added to dry DCM under argon atmosphere and treated with TEA (3 equiv). The mixture was cooled to 0 °C before methanesulfonyl chloride (2 equiv) was added dropwise. After further stirring for 1 h at 0 °C and 12 h at rt, DCM (30 ml) was added and the mixture was washed with water (3x40 ml). After phase separation, the organic layer was dried over sodium sulfate and the solvent was removed to afford slightly red methanesulfonate as intermediate compound. The methanesulfonate was subsequently dissolved in dry THF under argon atmosphere, followed by addition of lithium bromide (2 equiv) and heating at reflux for 1 h. After cooling to rt, the reaction mixture was quenched with water (15 ml) and extracted with diethyl ether (3x20 ml). The phases were separated and the combined organic layers were dried over sodium sulfate. Subsequently, the solvent was removed to give the crude product. Further purification was achieved by column chromatography (silica gel, Cy/EtOAc: 100/1 to 10/1) to give compounds **10a-c**.

*tert-Butyl(6-bromohexyl)carbamate, **10a** (FG_B_35)*



$C_{11}H_{22}BrNO_2$

M_r = 280.21 g/mol

According to general procedure **G.2**, methanesulfonyl chloride (570 µl, 7.36 mmol) was added dropwise to a solution of **9a** (800 mg, 3.68 mmol) and TEA (1.54 ml, 11.0 mmol) in dry DCM (10 ml) at 0 °C under argon atmosphere and stirred at rt for 12 h. The sulfonate ester was reacted with lithium bromide (639.2 mg, 7.36 mmol) in dry THF (20 ml) under reflux for 1 h. The reaction mixture was worked up as described in **G.2** to give **10a** as a colorless oil.

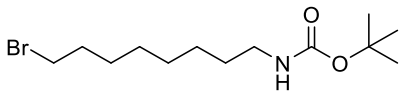
yield: 784 mg (76 %)

reaction control:	R_f = 0.48 (silica gel; Cy/EtOAc: 10/1; 4-anisaldehyde)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3350, 2974, 2932, 2859, 1688, 1512, 1365, 1166

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.47 (s, 1H, N**H**), 3.38 (t, ^3J = 6.8 Hz, 2H, C**H$_2$**Br), 3.12-3.05 (m, 2H, NHC**H$_2$**), 1.87-1.80 (m, 2H, C**H$_2$**CH$_2$Br), 1.51-1.40 (m, 13H, C**H$_3$**, C**H$_2$**), 1.36-1.29 (m, 2H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.2 (**C**(O)NH), 79.4 (**C$_q$**), 40.7 (NH**C**H$_2$), 34.0 (**C**H$_2$Br), 32.9 (**C**H$_2$CH$_2$Br), 30.2 (**C**H$_2$), 28.6 (**C**H$_3$), 28.0 (**C**H$_2$), 26.2 (**C**H$_2$).

tert-Butyl(8-bromooctyl)carbamate, ***10b*** *(FG_B_42)*



$C_{13}H_{26}BrNO_2$

M_r = 308.26 g/mol

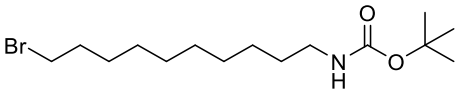
According to general procedure **G.2**, methanesulfonyl chloride (505 µl, 6.52 mmol) was added dropwise to a solution of **9b** (800 mg, 3.26 mmol) and TEA (1.36 ml, 9.78 mmol) in dry DCM (10 ml) at 0 °C under argon atmosphere and stirred at rt for 12 h. The sulfonate ester was reacted with lithium bromide (566 mg, 6.52 mmol) in dry THF (20 ml) under reflux for 1 h. The reaction mixture was worked up as described in **G.2** to give **10b** as a colorless oil.

yield:	462 mg (46 %)
reaction control:	R_f = 0.53 (silica gel; Cy/EtOAc: 10/1; 4-anisaldehyde)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3351, 2976, 2929, 2856, 1690, 1511, 1247, 1167

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.46 (s, 1H, N**H**), 3.38 (t, ^3J = 6.8 Hz, 2H, C**H$_2$**Br), 3.11-3.04 (m, 2H, NHC**H$_2$**), 1.87-1.78 (m, 2H, C**H$_2$**CH$_2$Br), 1.49-1.36 (m, 13H, C**H$_3$**, C**H$_2$**), 1.34-1.29 (m, 6H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.2 (**C**(O)NH), 79.2 (**C$_q$**), 40.8 (NH**C**H$_2$), 34.2 (**C**H$_2$Br), 33.0 (**C**H$_2$CH$_2$Br), 30.3 (**C**H$_2$), 29.3 (**C**H$_2$), 28.9 (**C**H$_2$), 28.6 (**C**H$_3$), 28.3 (**C**H$_2$), 26.9 (**C**H$_2$).

*tert-Butyl(10-bromodecyl)carbamate, **10c** (FG_B_48)*



$C_{15}H_{30}BrNO_2$

M_r = 336.31 g/mol

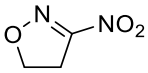
According to general procedure **G.2**, methanesulfonyl chloride (159 µl, 2.05 mmol) was added dropwise to a solution of **9c** (280 mg, 1.02 mmol) and TEA (428 µl, 3.07 mmol) in dry DCM (10 ml) at 0 °C under argon atmosphere and stirred at rt for 12 h. The sulfonate ester was reacted with lithium bromide (177 mg, 2.04 mmol) in dry THF (10 ml) under reflux for 1 h. The reaction mixture was worked up as described in **G.2** to give **10c** as a white solid.

yield:	148 mg (43 %)
reaction control:	R_f = 0.57 (silica gel; Cy/EtOAc: 10/1; 4-anisaldehyde)
melting point [°C]:	45-46
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3373, 2981, 2920, 2849, 1680, 1510, 1239, 1159

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.48 (s, 1H, N**H**), 3.37 (t, 2H, ^3J = 6.9 Hz, C**H$_2$**Br), 3.11-3.02 (m, 2H, NHC**H$_2$**), 1.86-1.77 (m, 2H, C**H$_2$**CH$_2$Br), 1.47-1.34 (m, 13H, C**H$_3$**, C**H$_2$**), 1.30-1.21 (m, 10H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.2 (**C**(O)NH), 79.2 (**C$_q$**), 40.8 (NH**C**H$_2$), 34.2 (**C**H$_2$Br), 33.0 (**C**H$_2$CH$_2$Br), 30.3 (**C**H$_2$), 29.6 (**C**H$_2$), 29.5 (**C**H$_2$), 29.4 (**C**H$_2$), 28.9 (**C**H$_2$), 28.6 (**C**H$_3$), 28.3 (**C**H$_2$), 27.0 (**C**H$_2$).

*3-Nitro-Δ^2-isoxazolin, **11** (FG_IP_1)*



$C_3H_4N_2O_3$

M_r = 116.08 g/mol

According to Klöckner *et al.*[111], 1-bromo-3-chloropropane (25.1 ml, 254.1 mmol) was added dropwise to a solution of sodium nitrite (36.5 g, 528.5 mmol) and isopentyl nitrite (35.5 ml, 264.2 mmol) in DMSO (250 ml) at rt. After 48 h of stirring at rt, the reaction mixture was poured into water (250 ml) and extracted with DCM (5x75 ml). The phases were separated and the solvent of the combined organic layers was removed. **11** was obtained by fractional distillation under high vacuum (58 °C, 1x10^{-3} bar). Product must be stored at 4 °C.

appearance:	yellow oil
yield:	13.0 g (44 %, Lit.:[145] 65 %)

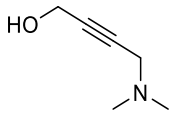
IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 2976, 2899, 1606, 1526, 1362

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.89 (t, ^{3}J = 10.9 Hz, 2H, OC**H$_2$**), 3.51 (t, ^{3}J = 10.9 Hz, 2H, OCH_2C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 164.2 (**C$_q$**), 75.7 (O**C**H_2), 30.8 (OCH_2**C**H_2).

The obtained physical and spectroscopic data are consistent with that found in literature.[146]

4-(Dimethylamino)but-2-yn-1-ol, ***12*** *(FG_IP_2)*



$C_6H_{11}NO$

M_r = 113.16 g/mol

According to Klöckner *et al.*[111], a solution of dimethylammonium chloride (18.2 g, 223.0 mmol) in water (50 ml) was adjusted to pH 9 by addition of 2 M sodium hydroxide solution. An aqueous formaldehyde solution (40 %, 12.3 ml, 178.4 mmol), 2-propyn-1-ol (10.4 ml, 178.4 mmol), and an aqueous solution of $CuSO_4 \cdot 5H_2O$ (10 ml, 1.4 g, 5.6 mmol) were added subsequently. The pH was adjusted to 8 by addition of 2 M sodium hydroxide solution and the reaction mixture was stirred at 80 °C for 2 h. After cooling to rt, an aqueous ammonia solution (25 %, 60 ml) was added. The mixture was extracted with diethyl ether by using a liquid-liquid continuous extractor for 40 h. The organic layer was dried over $MgSO_4$ and **12** was obtained after solvent removal.

appearance: yellowish oil

yield: 10.7 g (53 %, Lit.:[145] 80 %)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3167, 2943, 2825, 2780, 1458

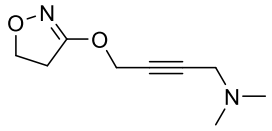
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.58 (s, 1H, O**H**), 4.21 (t, ^{5}J = 1.9 Hz, 2H, OC**H$_2$**), 3.22 (t, ^{5}J = 1.8 Hz, 2H, NC**H$_2$**), 2.25 (s, 6H, N(C**H$_3$**)$_2$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 84.5 (NCH_2**C**), 79.5 (OCH_2**C**), 50.4 (N**C**H_2), 48.0 (**C**H_2OH), 44.0 (N(**C**H_3)$_2$).

MS (ESI) m/z [M^+] Calcd for $C_6H_{12}NO^+$: 114.1. Found: 114.1.

The obtained physical and spectroscopic data are consistent with that found in literature.[145]

*Iperoxo base, **13** (FG_IP_3)*



$C_9H_{14}N_2O_2$

M_r = 182.22 g/mol

According to Klöckner *et al.*[111], compound **12** (2.5 g, 22.1 mmol) was dissolved in dry THF (30 ml) under argon atmosphere. After addition of sodium hydride (1.1 g, 44.2 mmol), the reaction mixture was stirred at rt for 1 h. A solution of **11** (2.6 g, 22.1 mmol) in dry THF (20 ml) was added dropwise, followed by stirring of the reaction mixture for 3 h under reflux. After cooling to rt, the mixture was poured into water (100 ml) and extracted with chloroform (3x60 ml). The phases were separated and the combined organic layers were dried over Na_2SO_4 before solvent removal. The crude product was purified by column chromatography (silica gel, $CHCl_3$/CH_3OH 100/1 to 10/1) to give **13**.

appearance:	yellowish oil
yield:	3.5 g (87 %, Lit.:[145] 80 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2940, 2875, 2778, 1732, 1624

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.79 (t, ^5J = 1.9 Hz, 2H, OC**H$_2$**C≡C), 4.40 (t, ^3J = 9.6 Hz, 2H, NOC**H$_2$**), 3.29 (t, ^5J = 1.9 Hz, 2H, NC**H$_2$**), 2.97 (t, ^3J = 9.6 Hz, 2H, OCH_2C**H$_2$**), 2.28 (s, 6H, $N(C$**H$_3$**$)_2$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 167.1 (N**C**O), 82.7 (NCH_2**C**), 79.4 (OCH_2**C**), 70.0 (NO**C**H_2), 58.2 (N**C**H_2), 48.0 (O**C**H_2C), 44.1 (N(**C**$H_3)_2$), 33.2 (OCH_2**C**H_2).

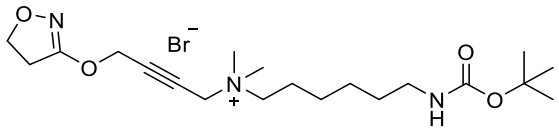
MS (ESI) m/z [M^+] Calcd for $C_9H_{15}N_2O_2^+$: 183.1. Found: 183.2.

The obtained physical and spectroscopic data are consistent with that found in literature.[122]

*General synthesis procedure **G.3** for formation of Boc protected amine iperoxo linkers **14a-c** and tert-butyl protected ester iperoxo linkers **53a-c***

The required Boc protected amine or *tert*-butyl ester (1 equiv) was dissolved in acetonitrile and treated with iperoxo base (1.5 equiv). The reaction mixture was heated under microwave irradiation (500 W, 78 °C) for 7-8 h. After cooling to rt, the solvent was removed and dissolved in only a few drops of acetonitrile. The addition of diethyl ether (10 ml) caused the precipitation of product. The solvent was decanted and the product was dried *in vacuo*.

*6-((tert-Butoxycarbonyl)amino)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethylhexan-1-aminium bromide, **14a** (FG_B_36)*



$C_{20}H_{36}BrN_3O_4$

M_r = 462.43 g/mol

A solution of **10a** (190 mg, 678 µmol) and **13** (247 mg, 1.36 mmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 7 h. The reaction mixture was worked up as described in general procedure **G.3** to give **14a** as a viscous, yellowish oil.

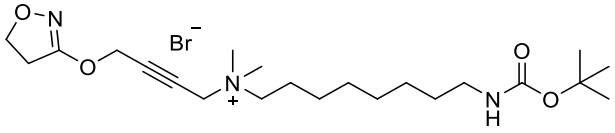
yield:	188 mg (60 %)
reaction control:	R_f = 0.40 (silica gel; $CHCl_3$/CH_3OH: 4/1; Dragendorff-reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3371, 2976, 2930, 2864, 1688, 1516, 1338, 1165

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.83 (s, 2H, OC**H$_2$**C≡C), 4.78-4.73 (m, 3H, $N^+(CH_3)_2C$**H$_2$**C≡C, N**H**), 4.33 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 3.64-3.55 (m, 2H, C**H$_2$**$N^+(CH_3)_2$), 3.36 (s, 6H, $N^+(C$**H$_3$**$)_2$), 3.02-2.97 (m, 2H, NHC**H$_2$**), 2.93 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 1.74-1.65 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.45-1.37 (m 2H, $NHCH_2C$**H$_2$**), 1.34-1.26 (m, 13H, $NH(CH_2)_2(C$**H$_2$**$)_2(CH_2)_2N^+(CH_3)_2$, $C(C$**H$_3$**$)_3$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 164.8 (isoxazolinyl- **C$_q$**), 154.2 (**C**(O)NH), 84.5 (C≡**C**-CH_2O), 77.0 (**C**$(CH_3)_3$), 74.1 (C≡**C**$CH_2N^+(CH_3)_2$), 68.1 (isoxazolinyl-O**C**H_2), 62.0 ($N^+(CH_3)_2$**C**H_2), 55.4 ($N^+(CH_3)_2$**C**H_2C≡C), 52.8 (O**C**H_2C≡C), 48.6 ($N^+($**C**$H_3)_2$), 38.3 (NH**C**H_2), 31.0 (isoxazolinyl-OCH_2**C**H_2), 27.7 (**C**H_2), 26.5 (C(**C**$H_3)_3$), 24.1 (**C**H_2), 23.8 (**C**H_2), 20.7 (**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{20}H_{36}N_3O_4^+$: 382.3. Found: 382.5.

*8-((tert-Butoxycarbonyl)amino)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyloctan-1-aminium, **14b** (FG_B_43)*



$C_{22}H_{40}BrN_3O_4$

M_r = 490.48 g/mol

A solution of **10b** (390 mg, 1.27 mmol) and **13** (461 mg, 2.53 mmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 8 h. The reaction mixture was worked up as described in general procedure **G.3** to give **14a** as a viscous, yellowish oil.

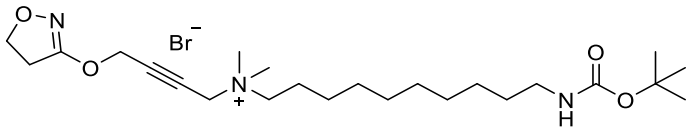
yield:	430 mg (69 %)
reaction control:	R_f = 0.45 (silica gel; $CHCl_3/CH_3OH$: 4/1; Dragendorff-reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3367, 2911, 2855, 1708, 1629, 1521, 1338

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.93 (s, 2H, OC**H$_2$**C≡C), 4.80 (s, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 4.53 (s, 1H, N**H**), 4.40 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 3.63-3.55 (m, 2H, C**H$_2$**$N^+(CH_3)_2$), 3.42 (s, 6H, $N^+(CH_3)_2$), 3.10-3.03 (m, 2H, NHC**H$_2$**), 2.99 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 1.78-1.68 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.48-1.22 (m, 19H, C**H$_2$**, $C(CH_3)_3$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.9 (isoxazolinyl-**C$_q$**), 156.2 (**C**(O)NH), 86.7 (C≡**C**-CH_2O), 79.2 (**C**$(CH_3)_3$), 76.3 (C≡**C**$CH_2N^+(CH_3)_2$), 70.3 (isoxazolinyl-O**C**H_2), 64.5 ($N^+(CH_3)_2$**C**H_2), 57.6 ($N^+(CH_3)_2$**C**H_2C≡C), 55.4 (O**C**H_2C≡C), 50.9 (N^+(**C**$H_3)_2$), 40.7 (NH**C**H_2), 33.2 (isoxazolinyl-OCH_2**C**H_2), 30.1 (**C**H_2), 29.1 (**C**H_2), 29.1 (**C**H_2), 28.6 (C(**C**$H_3)_3$), 26.7 (**C**H_2), 26.3 (**C**H_2), 23.1 (**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{22}H_{40}N_3O_4^+$: 410.3. Found: 410.6.

10-((tert-Butoxycarbonyl)amino)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyldecan-1-aminium, ***14c*** *(FG_B_50)*



$C_{24}H_{44}BrN_3O_4$

M_r = 518.54 g/mol

A solution of **10c** (370 mg, 1.10 mmol) and **13** (401 mg, 2.20 mmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 8 h. The reaction mixture was worked up as described in general procedure **G.3** to give **14c** as a viscous, yellowish oil.

yield:	404 mg (71 %)
reaction control:	R_f = 0.46 (silica gel; $CHCl_3/CH_3OH$: 4/1; Dragendorff-reagent)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3327, 2913, 2853, 1685, 1627, 1339

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.94 (s, 2H, OC**H$_2$**C≡C), 4.79 (s, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 4.50 (s, 1H, N**H**), 4.40 (t, ^3J = 9.6 Hz, isoxazolinyl-OC**H$_2$**), 3.62-3.54 (m, 2H, C**H$_2$**$N^+(CH_3)_2$), 3.43 (s, 6H, $N^+(CH_3)_2$), 3.10-3.03 (m, 2H, NHC**H$_2$**), 2.98 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 1.76-1.64 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.47-1.22 (m, 23H, C**H$_2$**, $C(CH_3)_3$).

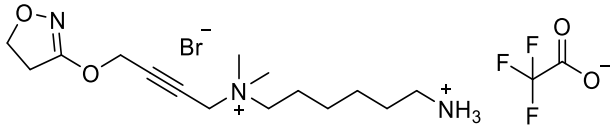
^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.9 (isoxazolinyl-**C$_q$**), 156.2 (**C**(O)NH), 86.8 (C≡**C**-CH_2O), 79.2 (**C**$(CH_3)_3$), 76.2 (C≡**C**$CH_2N^+(CH_3)_2$), 70.2 (isoxazolinyl-O**C**H_2), 64.4 ($N^+(CH_3)_2$**C**H_2), 57.4 ($N^+(CH_3)_2$**C**H_2C≡C), 55.0 (O**C**H_2C≡C), 50.7 (N^+(**C**$H_3)_2$), 40.8 (NH**C**H_2), 33.1 (isoxazolinyl-OCH_2**C**H_2), 31.8 (**C**H_2), 29.5 (**C**H_2), 29.4 (**C**H_2), 29.3 (**C**H_2), 29.3 (**C**H_2), 28.6 (C(**C**$H_3)_3$), 26.9 (**C**H_2), 26.3 (**C**H_2), 23.1 (**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{24}H_{44}N_3O_4^+$: 438.3. Found: 438.6

*General synthesis procedure **G.4** for Boc deprotection and removal of tert-butyl groups*

The required Boc protected amine or *tert*-butyl protected ester (1 equiv) was dissolved in dry DCM under argon atmosphere and cooled to -20 °C in a sodium chloride ice bath. TFA (6 equiv) was added dropwise and the reaction mixture was stirred at -20 °C for 30 min before it was allowed to warm to rt and stirred for further 2-14 h. The solvent was evaporated and remaining TFA was removed by dissolving the product in a few drops of acetonitrile, subsequent addition of diethyl ether (5 ml), and decantation of the solvent from the precipitated oil. The latter was obtained as product after drying *in vacuo*.

*N^1-(4-((4,5-Dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N^1,N^1-dimethylhexane-1,6-diaminium 2,2,2-trifluoroacetate bromide, **1-L6** (FG_B_38)*



$C_{17}H_{29}BrF_3N_3O_4$

M_r = 476.34 g/mol

TFA (545 µl, 7.07 mmol) was added dropwise to a solution of **14a** (545 mg, 1.18 mmol) in dry DCM (5 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 2 h at rt, the reaction mixture was worked up according to **G.4** to give **1-L6** as orange oil.

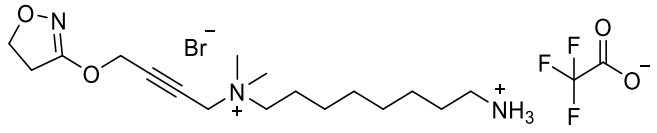
yield: 489 mg (87 %)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3430, 3026, 2941, 2876, 1778, 1675, 1342, 1134

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 4.91 (t, ^{5}J = 1.7 Hz, 2H, OC**H$_2$**C≡C), 4.43-4.37 (m, 4H, isoxazolinyl-OC**H$_2$**, $N^+(CH_3)_2$C**H$_2$**C≡C), 3.48-3.43 (m, 2H, C**H$_2$**$N^+(CH_3)_2$), 3.18 (s, 6H, N^+(C**H$_3$**)$_2$), 3.03 (t, ^{3}J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.98-2.92 (m, 2H, C**H$_2$**NH_3^+), 1.88-1.76 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.74-1.66 (m 2H, C**H$_2$**$CH_2NH_3^+$), 1.55-1.40 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 168.9 (isoxazolinyl-**C$_q$**), 87.9 (OCH_2**C**≡C), 76.7 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 65.4 ($N^+(CH_3)_2$**C**H_2), 58.4 (O**C**H_2C≡C), 55.3 ($N^+(CH_3)_2$**C**H_2C≡C), 51.3 (N^+(**C**H_3)$_2$), 40.7 (**C**$H_2NH_3^+$), 33.8 (isoxazolinyl-OCH_2**C**H_2), 28.4 (**C**$H_2CH_2NH_3^+$), 27.0 (**C**H_2), 26.8 (**C**H_2), 23.6 (**C**$H_2CH_2N^+(CH_3)_2$).

*N^1-(4-((4,5-Dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N^1,N^1-dimethyloctane-1,8-diaminium 2,2,2-trifluoroacetate bromide, **1-L8** (FG_B_44)*



$C_{19}H_{33}BrF_3N_3O_4$

M_r = 504.39 g/mol

TFA (356 µl, 4.65 mmol) was added dropwise to a solution of **14b** (380 mg, 775 µmol) in dry DCM (5 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 3 h at rt, the reaction mixture was worked up according to **G.4** to give **1-L8** as orange oil.

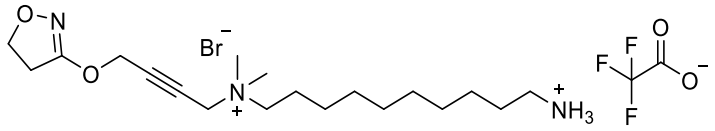
yield: 283 mg (94 %)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3432, 3026, 2940, 2878, 1777, 1671, 1340, 1141

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 4.91 (t, ^{5}J = 1.7 Hz, 2H, OC**H$_2$**C≡C), 4.42-4.37 (m, 4H, isoxazolinyl-OC**H$_2$**, $N^+(CH_3)_2$C**H$_2$**C≡C), 3.45-3.41 (m, 2H, C**H$_2$**$N^+(CH_3)_2$), 3.17 (s, 6H, N^+(C**H$_3$**)$_2$), 3.03 (t, ^{3}J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.95-2.89 (m, 2H, C**H$_2$**NH_3^+), 1.84-1.74 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.71-1.61 (m, 2H, C**H$_2$**$CH_2NH_3^+$), 1.48-1.38 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 168.9 (isoxazolinyl-**C$_q$**), 87.9 (OCH_2**C**≡C), 76.7 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 65.6 ($N^+(CH_3)_2$**C**H_2), 58.4 (O**C**H_2C≡C), 55.3 ($N^+(CH_3)_2$**C**H_2C≡C), 51.3 (N^+(**C**H_3)$_2$), 40.9 (**C**$H_2NH_3^+$), 33.8 (isoxazolinyl-OCH_2**C**H_2), 31.1 (**C**H_2), 30.1 (**C**H_2), 28.4 (**C**$H_2CH_2NH_3^+$), 27.0 (**C**H_2), 26.8 (**C**H_2), 23.6 (**C**$H_2CH_2N^+(CH_3)_2$).

*N^1-(4-((4,5-Dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N^1,N^1-dimethyldecane-1,10-diaminium 2,2,2-trifluoroacetate bromide, **1-L10** (FG_B_51)*



$C_{21}H_{37}BrF_3N_3O_4$

M_r = 532.44 g/mol

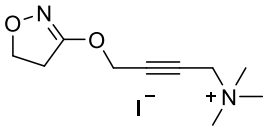
TFA (336 µl, 4.60 mmol) was added dropwise to a solution of **14c** (380 mg, 733 µmol) in dry DCM (5 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 2 h at rt, the reaction mixture was worked up according to **G.4** to give **1-L10** as orange oil.

yield:	223 mg (73 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3430, 3028, 2942, 2873, 1777, 1676, 1342, 1140

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]):]): 4.94 (t, ^5J = 1.7 Hz, 2H, OC**H$_2$**C≡C), 4.45-4.38 (m, 4H, isoxazolinyl-OC**H$_2$**, $N^+(CH_3)_2C$**H$_2$**C≡C), 3.48-3.43 (m, 2H, C**H$_2$**$N^+(CH_3)_2$), 3.19 (s, 6H, $N^+(C$**H$_3$**$)_2$), 3.06 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.96-2.92 (m, 2H, C**H$_2$**NH_3^+), 1.85-1.76 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.72-1.63 (m, 2H, C**H$_2$**$CH_2NH_3^+$), 1.46-1.38 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 168.8 (isoxazolinyl-**C$_q$**), 87.9 (OCH_2**C**≡C), 76.7 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 65.6 ($N^+(CH_3)_2$**C**H_2), 58.4 (O**C**H_2C≡C), 55.2 ($N^+(CH_3)_2$**C**H_2C≡C), 51.3 (N^+(**C**$H_3)_2$), 40.9 (**C**$H_2NH_3^+$), 33.8 (isoxazolinyl-OCH_2**C**H_2), 30.6 (**C**H_2), 30.6 (**C**H_2), 30.4 (**C**H_2), 30.4 (**C**H_2), 28.8 (**C**$H_2CH_2NH_3^+$), 27.6 (**C**H_2), 27.5 (**C**H_2), 23.8 (**C**$H_2CH_2N^+(CH_3)_2$).

*Iperoxo, **15** (FG_IP_4)*



$C_{10}H_{17}IN_2O_2$

M_r = 324.16 g/mol

According to Klöckner *et al.*[111], **13** (150.0 mg, 823.2 µmol) was dissolved in dry $CHCl_3$ (10 ml) under argon atmosphere. After addition of methyl iodide (292.1 mg, 2.1 mmol), the reaction mixture was stirred at rt for 1 d. The formed precipitate was filtered, washed with cold diethyl ether, and dried under vacuum to give **15**.

appearance:	white crystals

yield:	264 mg (99 %, Lit.:[145] 99 %))
melting point [°C]:	decomposition > 185 °C
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3009, 2919, 1625, 1413, 1336, 1247, 912

^{1}H NMR (400 MHz, DMSO-d$_6$, δ [ppm]): 4.94 (s, 2H, OC**H$_2$**C), 4.48 (s, 2H, C**H$_2$**N$^+$(CH$_3$)$_3$), 4.32 (t, ^{3}J = 9.5 Hz, 2H, NOC**H$_2$**), 3.02 (t, ^{3}J = 9.5 Hz, 2H, OCH$_2$C**H$_2$**), 3.14 (s, 9H, N$^+$(C**H$_3$**)$_3$).

^{13}C NMR (100 MHz, DMSO-d$_6$, δ [ppm]): 166.7 (N**C**O), 86.0 (NCH$_2$**C**), 76.2 (OCH$_2$**C**), 69.6 (NO**C**H$_2$), 57.2 (O**C**H$_2$C), 55.1 (**C**H$_2$N$^+$(CH$_3$)$_3$), 52.0 (N$^+$(**C**H$_3$)$_3$), 32.2 (OCH$_2$**C**H$_2$).

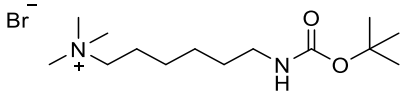
The obtained physical and spectroscopic data are consistent with that found in the literature.[122]

7.2.3 Preparation of orthosteric TMA linkers 2-Ln

*General synthesis procedure **G.5** for formation of Boc protected TMA linkers **16a-c***

The required Boc protected amine (1 equiv) was dissolved in ethanol in a sealed pressure tube and treated with a 4.2 M solution of trimethylamine in ethanol (15 equiv). The reaction mixture was heated at 100 °C for 13-15 h. After cooling to rt, the solvent was removed and the crude product was purified by column chromatography (alox basic, DCM/CH$_3$OH: 10/1) to give **16a-c**.

*6-((tert-Butoxycarbonyl)amino)-N,N,N-trimethylhexan-1-aminium bromide, **16a** (FG_B_108)*



$C_{14}H_{31}BrN_2O_2$

M_r = 339.32 g/mol

An ethanolic solution of trimethylamine (4.2 M, 5.1 ml, 21.4 mmol) was added to a solution of **10a** (400 mg, 1.43 mmol) in ethanol (5 ml). After heating at 100 °C for 15 h, the reaction mixture was worked up as described in **G.5** to give **16a** as a colorless oil.

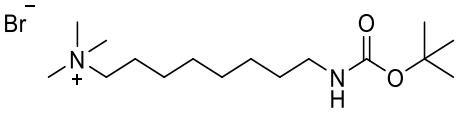
yield:	384 mg (79 %)
reaction control:	R_f = 0.35 (alox basic; DCM/CH$_3$OH: 10/1; Dragendorff-reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3394, 2930, 2861, 1688, 1516, 1251, 1169

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.76 (s, 1H, N**H**), 3.58-3.51 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.34 (s, 9H, $N^+(C$**H$_3$**$)_3$), 3.01-2.93 (m, 2H, C**H$_2$**NH), 1.73-1.62 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.43-1.25 (m, 15H, C**H$_2$**, $C(C$**H$_3$**$)_3$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.1 (**C**(O)NH), 79.0 (**C**$(CH_3)_3$), 66.6 (**C**$H_2N^+(CH_3)_3$), 53.4 (N^+(**C**$H_3)_3$), 40.2 (**C**H_2NH), 29.7 (**C**H_2), 28.5 ($C($**C**$H_3)_3$), 26.1 (**C**H_2), 25.7 (**C**H_2), 23.0 (**C**$H_2CH_2N^+(CH_3)_3$).

MS (ESI) m/z [M^+] Calcd for $C_{14}H_{31}N_2O_2^+$: 259.2. Found: 259.1

8-((tert-Butoxycarbonyl)amino)-N,N,N-trimethyloctan-1-aminium bromide, ***16b*** *(FG_B_112)*



$C_{16}H_{35}BrN_2O_2$

M_r = 367.37 g/mol

An ethanolic solution of trimethylamine (4.2 M, 5.2 ml, 21.9 mmol) was added to a solution of **10b** (450 mg, 1.46 mmol) in ethanol (5 ml). After heating at 100 °C for 13 h, the reaction mixture was worked up as described in **G.5** to give **16b** as a colorless oil.

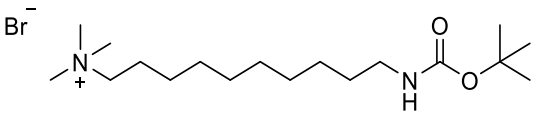
yield:	518 mg (97 %)
reaction control:	R_f = 0.38 (alox basic; DCM/CH_3OH: 10/1; Dragendorff-reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3362, 2927, 2857, 1687, 1518, 1250, 1170

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.54 (s, 1H, N**H**), 3.60-3.54 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.44 (s, 9H, $N^+(C$**H$_3$**$)_3$), 3.10-3.01 (m, 2H, C**H$_2$**NH), 1.77-1.68 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.48-1.25 (m, 19H, C**H$_2$**, $C(C$**H$_3$**$)_3$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.3 (**C**(O)NH), 79.2 (**C**$(CH_3)_3$), 67.2 (**C**$H_2N^+(CH_3)_3$), 53.6 (N^+(**C**$H_3)_3$), 40.6 (**C**H_2NH), 30.1 (**C**H_2), 29.1 (**C**H_2), 29.0 (**C**H_2), 28.6 ($C($**C**$H_3)_3$), 26.2 (**C**H_2), 23.2 (**C**$H_2CH_2N^+(CH_3)_3$).

MS (ESI) m/z [M^+] Calcd for $C_{16}H_{35}N_2O_2^+$: 287.3. Found: 287.2.

10-((tert-Butoxycarbonyl)amino)-N,N,N-trimethyldecan-1-aminium bromide, ***16c*** *(FG_B_117)*



$C_{18}H_{39}BrN_2O_2$

M_r = 395.43 g/mol

An ethanolic solution of trimethylamine (4.2 M, 2.2 ml, 9.4 mmol) was added to a solution of **10c** (211 mg, 627 µmol) in ethanol (5 ml). After heating at 100 °C for 15 h, the reaction mixture was worked up as described in **G.5** to give **16c** as a colorless oil.

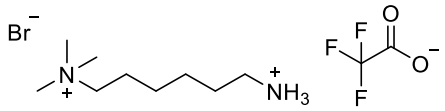
yield:	204 mg (82 %)
reaction control:	R_f = 0.39 (alox basic; DCM/CH_3OH: 10/1; Dragendorff-reagent).
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3366, 2925, 2854, 1688, 1520, 1391, 1171

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.51 (s, 1H, N**H**), 3.57-3.52 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.42 (s, 9H, $N^+(C\mathbf{H_3})_3$), 3.07-3.02 (m, 2H, C**H$_2$**NH), 1.77-1.64 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.44-1.37 (m, 11H, C**H$_2$**, $C(C\mathbf{H_3})_3$), 1.35-1.27 (m, 4H, C**H$_2$**), 1.27-1.19 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 156.2 (**C**(O)NH), 79.2 (**C**$(CH_3)_3$), 67.2 (**C**$H_2N^+(CH_3)_3$), 53.6 ($N^+(\mathbf{C}H_3)_3$), 40.8 (**C**H_2NH), 30.2 (**C**H_2), 29.4 (**C**H_2), 29.4 (**C**H_2), 29.3 (**C**H_2), 29.3 (**C**H_2), 28.6 ($C(\mathbf{C}H_3)_3$), 26.9 (**C**H_2), 26.3 (**C**H_2), 23.3 (**C**$H_2CH_2N^+(CH_3)_3$).

MS (ESI) m/z [M^+] Calcd for $C_{18}H_{39}N_2O_2^+$: 315.3. Found: 315.2.

N^1,N^1,N^1-Trimethylhexane-1,6-diaminium 2,2,2-trifluoroacetate bromide, ***2-L6*** *(FG_B_109)*



$C_{11}H_{24}BrF_3N_2O_2$

M_r = 353.22 g/mol

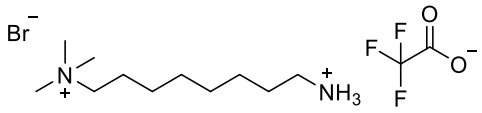
TFA (204 µl, 2.67 mmol) was added dropwise to a solution of **16a** (151 mg, 445 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 14 h at rt, the reaction mixture was worked up according to **G.4** to give **2-L6** as orange oil.

yield:	150 mg (79 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3416, 2942, 2865, 1673, 1482, 1174, 1125

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 3.39-3.36 (m, 2H, C**H$_2$**NH_3^+), 3.16 (s, 9H, $N^+(C\mathbf{H_3})_3$), 2.97 (t, ^3J = 7.7 Hz, 2H, C**H$_2$**$N^+(CH_3)_3$), 1.89-1.80 (m, 2H, C**H$_2$**$CH_2NH_3^+$), 1.77-1.66 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.57-1.40 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 162.1 (**C**(O)O^-), 115.6 (**C**F_3), 67.8 (**C**$H_2N^+(CH_3)_3$), 53.7 ($N^+(\mathbf{C}H_3)_3$), 40.7 (**C**$H_2NH_3^+$), 28.5 (**C**$H_2CH_2N^+(CH_3)_3$), 27.1 (**C**H_2), 27.0 (**C**H_2), 23.9 (**C**$H_2CH_2NH_3^+$).

N^1,N^1,N^1-Trimethyloctane-1,8-diaminium 2,2,2-trifluoroacetate bromide, ***2-L8*** *(FG_B_113)*



$C_{13}H_{28}BrF_3N_2O_2$

M_r = 381.28 g/mol

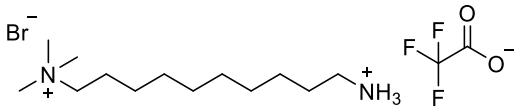
TFA (187 µl, 2.45 mmol) was added dropwise to a solution of **16b** (150 mg, 408 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 14 h at rt, the reaction mixture was worked up according to **G.4** to give **2-L8** as orange oil.

yield:	135 mg (87 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3441, 2935, 2862, 1674, 1481, 1172, 1129

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 3.38-3.33 (m, 2H, C**H$_2$**NH_3^+), 3.15 (s, 9H, N^+(C**H$_3$**)$_3$), 2.94 (t, ^3J = 7.8 Hz, 2H, C**H$_2$**$N^+(CH_3)_3$), 1.87-1.77 (m, 2H, C**H$_2$**$CH_2NH_3^+$), 1.73-1.65 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.50-1.32 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 162.2 (**C**$(O)O^-$), 116.4 (**C**F_3), 68.0 (**C**$H_2N^+(CH_3)_3$), 53.7 (N^+(**C**H_3)$_3$), 40.9 (**C**$H_2NH_3^+$), 30.1 (**C**H_2), 30.1 (**C**H_2), 28.7 (**C**$H_2CH_2N^+(CH_3)_3$), 27.5 (**C**H_2), 27.4 (**C**H_2), 24.0 (**C**$H_2CH_2NH_3^+$).

N^1,N^1,N^1-Trimethyldecane-1,10-diaminium 2,2,2-trifluoroacetate bromide, ***2-L10*** *(FG_B_118)*



$C_{15}H_{32}BrF_3N_2O_2$

M_r = 409.33 g/mol

TFA (165 µl, 2.15 mmol) was added dropwise to a solution of **16c** (142 mg, 359 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 14 h at rt, the reaction mixture was worked up according to **G.4** to give **2-L10** as orange oil.

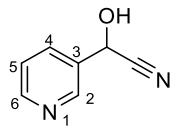
yield:	140 mg (95 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3423, 2929, 2858, 1673, 1481, 1199, 1126

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 3.38-3.31 (m, 2H, C**H$_2$**NH_3^+), 3.15 (s, 9H, N^+(C**H$_3$**)$_3$), 2.93 (t, ^3J = 7.3 Hz, 2H, C**H$_2$**$N^+(CH_3)_3$), 1.85-1.76 (m, 2H, C**H$_2$**$CH_2NH_3^+$), 1.72-1.63 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.48-1.35 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 162.4 (**C**$(O)O^-$), 116.7 (**C**F_3), 68.0 (**C**$H_2N^+(CH_3)_3$), 53.7 (N^+(**C**H_3)$_3$), 40.9 (**C**$H_2NH_3^+$), 30.5 (**C**H_2), 30.5 (**C**H_2), 30.3 (**C**H_2), 30.3 (**C**H_2), 28.7 (**C**$H_2CH_2N^+(CH_3)_3$), 27.6 (**C**H_2), 27.5 (**C**H_2), 24.1 (**C**$H_2CH_2NH_3^+$).

7.2.4 Preparation of quaternary orthosteric xanomeline linkers 3-Ln

2-Hydroxy-2-(pyridin-3-yl)acetonitrile, ***17*** *(FG_XA_10)*



$C_7H_6N_2O$

M_r = 134.14 g/mol

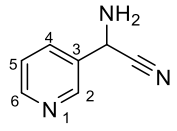
According to Kane *et al.*[114], nicotinaldehyde (9.7 ml, 103.2 mmol) was added at 5 °C to a solution of potassium cyanide (9.1 g, 139.1 mmol) in water (120 ml). Then acetic acid (6.2 ml, 108.3 mmol) was added dropwise over a 1 h period at 5 °C. The yellowish solution was allowed to warm to rt and stirred for 2 h, followed by extraction with EtOAc (6x100 ml). After phase separation, the combined organic layers were dried over magnesium sulfate and the solvent was removed. The product was used without further purification.

appearance:	brown oil
yield:	11.9 g (86 %, Lit.:[114] 66 %)
reaction control:	R_f = 0.41 (silica gel; $CHCl_3$/CH_3OH: 20/1)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3061, 2918, 2850, 1673, 1592, 1423, 1026

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 8.65 (s, 1H, **H**-2), 8.57 (d, ^3J = 4.5 Hz, 1H, **H**-6), 7.92 (d, ^3J = 7.8 Hz, 1H, **H**-4), 7.42-7.37 (m, 1H, **H**-5), 5.62 (s, 1H, C**H**OH).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 150.4 (**C**-6), 147.7 (**C**-2), 135.2 (**C**-4), 132.9 (**C**N), 124.4 (**C**-3), 117.1 (**C**-5), 61.4 (**C**HOH).

2-Amino-2-(pyridin-3-yl)acetonitrile, ***18*** *(FG_XA_11)*



$C_7H_7N_3$

M_r = 133.15 g/mol

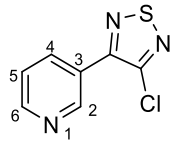
According to Kane *et al.*[114], a solution of ammonium chloride (29.2 g, 545.4 mmol) in water (100 ml) was treated with compound **17** (11.8 g, 88.0 mmol), followed by the addition of aqueous ammonia solution (25 %, 6.7 ml, 88.0 mmol). The reaction mixture was stirred at rt for 24 h and extracted with EtOAc (6x 100 mL). After phase separation, the combined organic layers were dried over magnesium sulfate and the solvent was removed. Product **18** was used without further purification.

appearance:	dark orange oil
yield:	5.4 g (46 %, Lit.:[114] 33 %)
reaction control:	R_f = 0.52 (silica gel; EtOAc/CH_3OH: 4/1)

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 8.76 (d, ^4J = 2.1 Hz, 1H, **H**-2), 8.59 (dd, ^3J = 4.8 Hz, ^4J = 1.3 Hz, 1H, **H**-6), 7.89-7.84 (m, 1H, **H**-4), 7.42-7.37 (dd, ^3J = 7.9 Hz, ^3J = 4.8 Hz, 1H, **H**-5), 4.94 (s, 1H, C**H**NH_2).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 150.4 (**C**-6), 148.4 (**C**-2), 134.6 (**C**-4), 132.3 (**C**N) 123.9 (**C**-3), 120.2 (**C**-5), 45.5 (**C**HNH_2).

3-Chloro-4-(pyridin-3-yl)-1,2,5-thiadiazole, ***19*** *(FG_XA_12)*



$C_7H_4ClN_3S$

M_r = 197.64 g/mol

According to Kane *et al.*[114], a solution of compound **18** (5.3 g, 39.8 mmol) in DMF (10 ml) was added dropwise to a solution of disulfur dichloride (6.4 ml, 79.6 mmol) in DMF (7.5 ml) at 0 °C over a 1 h period. After further stirring at 0 °C for 1 h, the reaction mixture was quenched with ice water (20 ml). The resulting precipitate was filtered off and the filtrate was treated with 5 M NaOH solution until a pH of 9 was reached. The mixture was extracted with EtOAc (3x50 ml). The phases were separated and the combined organics were dried over magnesium sulfate. The solvent was removed and the obtained crude product was purified by column chromatography (silica, EtOAc/Cy 1/1) to give **19**.

appearance:	white solid
yield:	2.2 g (28 %, Lit.:[114] 58 %))
reaction control:	R_f = 0.53 (silica gel; EtOAc/CH_3OH: 1/1)
melting point [°C]:	57-59
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3034, 1591, 1413, 1364, 1167

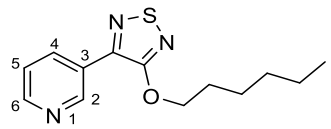
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.13 (d, ^4J = 1.7 Hz, 1H, **H**-2), 8.64 (dd, ^3J = 4.9 Hz, ^4J = 1.6 Hz, 1H, **H**-6), 8.21-8.17 (m, 1H, **H**-4), 7.38-7.33 (m, 1H, **H**-5).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 155.2 (thiadiazolyl-N**C**O), 150.9 (**C**-6), 149.3 (**C**-2), 143.5 (thiadiazolyl-**C$_q$**), 135.8 (**C**-4), 126.9 (**C**-3), 123.4 (**C**-5).

*General procedure **G.6** for nucleophilic substitution at thiadiazole ring*

Following a modified procedure from She *et al.*[113], the appropriate alcohol (1.5 equiv) was dissolved in dry THF under argon atmosphere and cooled to 0 °C in an ice bath. After addition of sodium hydride (3.5 equiv), the ice bath was removed and the reaction mixture was stirred at rt for 2 h, followed by the dropwise addition of a solution of **19** (1 equiv) in dry THF at 0 °C. The mixture was warmed to rt and then heated under reflux for a period of 3-5 h. After cooling to rt, the mixture was concentrated to a few ml *in vacuo*, quenched with ice cold water (10 ml) and extracted with EtOAc (3x50 ml). After phase separation, the combined organic layers were dried over sodium sulfate and concentrated *in vacuo*. Further purification of the product was achieved by column chromatography as indicated.

*3-(Hexyloxy)-4-(pyridin-3-yl)-1,2,5-thiadiazole, **20** (LH_XA_04)*



$C_{13}H_{17}N_3OS$

M_r = 263.36 g/mol

A solution of 1-hexanol (698 mg, 6.83 mmol) in dry THF (15 ml) at 0 °C was treated with sodium hydride (60 % suspension in paraffin oil, 637 mg, 15.9 mmol) and stirred at rt for 2 h under argon atmosphere. A solution of **19** (900 mg, 4.55 mmol) in dry THF (10 ml) was added dropwise at 0 °C and the mixture was heated under reflux for 5 h. The reaction was worked up according to general procedure **G.6** and purified by column chromatography (silica, petroleum ether/acetone 10/3) to give **20** as white solid.

yield:	1.2 g (96 %)
reaction control:	R_f = 0.63 (silica gel; petroleum ether/acetone: 10/3)
melting point [°C]:	55-56
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2952, 2927, 2853, 1513, 1468, 1373, 1259

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.41 (d, ^4J = 2.2 Hz, 1H, **H**-2), 8.65 (dd, ^4J = 4.8 Hz, ^5J = 1.6 Hz, 1H, **H**-6), 8.44.-8.41 (m, 1H, **H**-4), 7.39 (dd, ^3J = 8.0 Hz, ^4J = 4.8 Hz, 1H, **H**-5), 4.52 (t, ^3J = 6.7 Hz, 2H, OC**H$_2$**), 1.89 (quint, ^3J = 7.1 Hz, 2H, OCH$_2$C**H$_2$**), 1.53-1.46 (m, 2H, C**H$_2$**), 1.38-1.34 (m, 4H, C**H$_2$**), 0.93-0.89 (m, 3H, C**H$_3$**).

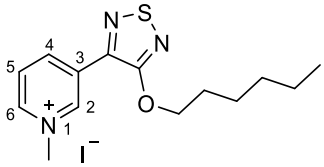
^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 163.0 (thiadiazolyl-N**C**O), 150.3 (**C**-6), 148.8 (**C**-2), 145.1 (thiadiazolyl-**C$_q$**), 134.8 (**C**-4), 127.8 (**C**-3), 123.5 (**C**-5), 71.6 (O**C**H$_2$), 63.2 (**C**H$_2$), 32.9 (**C**H$_2$), 29.0 (**C**H$_2$), 25.8 (**C**H$_2$), 14.8 (**C**H$_3$).

MS (ESI) m/z [M^+] Calcd for $C_{13}H_{18}N_3OS^+$: 264.1. Found: 264.0.

*General procedure **G.7** for N-methylation of pyridine ring*

Following a modified procedure from She *et al.*[113], the required pyridine compound (1 equiv) was dissolved in dry acetone under argon atmosphere and treated with methyl iodide (10 equiv). The reaction mixture was stirred at rt for 24 h, whereupon the solvent was removed, and the obtained crude product was purified by column chromatography as indicated.

*3-(4-(Hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methylpyridin-1-ium iodide, **21** (LH_XA_05)*



$C_{14}H_{20}IN_3OS$

M_r = 405.30 g/mol

Methyl iodide (2.69 ml, 43.3 mmol) was added to a solution of compound **20** (1.20 g, 4.33 mmol) in dry acetone (10 ml) under argon atmosphere and stirred at rt for 24 h. The reaction was worked up according to general procedure **G.7** and purified by column chromatography (silica, DCM/CH_3OH 6/1) to give **21** as yellow solid.

yield:	950 mg (75 %)
reaction control:	R_f = 0.47 (silica gel; DCM/CH_3OH: 6/1)
melting point [°C]:	111
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2949, 2923, 2852, 1515, 1443, 1359, 1273

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.69 (d, ^3J = 5.9 Hz, 1H, **H**-6), 9.40 (s, 1H, **H**-2), 9.14 (d, ^3J = 8.3 Hz, 1H, **H**-4), 8.25 (m, 1H, **H**-5), 4.80 (s, 3H, $N^+C\mathbf{H_3}$), 4.61 (t, ^3J = 6.9 Hz, 2H, $OC\mathbf{H_2}$), 1.94 (quint, ^3J = 7.2 Hz, 2H, $OCH_2C\mathbf{H_2}$), 1.41-1.36 (m, 4H, $C\mathbf{H_2}$), 0.92 (t, ^3J = 7.0 Hz, $C\mathbf{H_3}$).

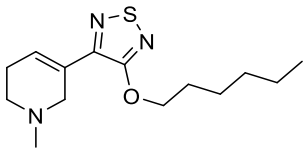
^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 164.9 (thiadiazolyl-N**C**O), 144.4 (**C**-6), 142.2 (**C**-2), 141.9 (**C**-4), 139.7 (thiadiazolyl-$\mathbf{C_q}$), 131.6 (**C**-3), 128.7 (**C**-5), 72.6 (OCH_2), 50.6 (N^+CH_3), 31.4 (CH_2), 28.8 (CH_2), 25.6 (CH_3), 22.5 (CH_2), 14.0 (CH_3).

MS (ESI) m/z [M^+] Calcd for $C_{15}H_{23}N_3OS^+$: 278.1. Found: 278.1.

*General procedure **G.8** for reduction of pyridinium salts*

According to a modified procedure from Kane *et al* [114], the appropriate pyridinium salt (1 equiv) was dissolved in dry ethanol (20 ml) under argon atmosphere and cooled to 0 °C in an ice bath, followed by the addition of sodium borohydride (5 equiv). The ice bath was removed and the mixture was first stirred for 20-24 h at rt and, subsequently, heated under reflux for 2-3 h. After cooling to rt, the solvent was removed and the residue was partitioned between DCM (30 ml) and water (30 ml). The aqueous layer was further extracted with DCM (2x30 ml). After phase separation, the combined organic layers were washed with brine (20 ml) and phases were again separated. The organic layer was dried over magnesium sulfate and concentrated *in vacuo*. The obtained crude product was purified by column chromatography as indicated.

*Xanomeline, **22** (LH_XA_06)*



$C_{14}H_{23}N_3OS$

M_r = 281.42 g/mol

A solution of compound **21** (940 mg, 2.32 mmol) in dry ethanol (10 ml) at 0 °C was treated with sodium borohydride (438 mg, 11.6 mmol) under argon atmosphere. The reaction mixture was stirred for 24 h at rt, heated under reflux for 3 h, and worked up according to **G.8**. Purification of the crude product was achieved by column chromatography (silica, EtOAc/CH_3OH 3/1) to give **22** as dark orange solid.

yield:	466 mg (71 %, Lit.:[114] 83 %)
melting point [°C]:	40
reaction control:	R_f = 0.60 (silica; EtOAc/CH_3OH: 4/1)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2951, 2930, 2790, 1639, 1504, 1434, 1374, 1244, 1135

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.08 (t, ^3J = 4.1 Hz, 1H, tetrahydropyridinyl-C**H**), 4.44 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 3.45-3.43 (m, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 2.55 (t, ^3J = 5.7 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.46-2.39 (m, 5H, tetrahydropyridinyl-NCH_2C**H$_2$**, NC**H$_3$**), 1.85-1.77 (m, 2H, OCH_2C**H$_2$**), 1.47-1.39 (m, 2H, C**H$_2$**), 1.36-1.30 (m, 4H, C**H$_2$**), 0.88 (t, ^3J = 6.9 Hz, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.6 (thiadiazolyl-N**C**O), 146.8 (thiadiazolyl-**C_q**), 129.3 (tetrahydropyridinyl-**C_q**), 129.3 (tetrahydropyridinyl-**C**H), 70.9 (O**C**H_2), 55.0 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.2 (tetrahydropyridinyl-N**C**H_2CH_2), 45.9 (N**C**H_3), 31.4 (**C**H_2), 28.8 (**C**H_2), 26.6 (**C**H_2), 25.6 (**C**H_2), 22.5 (tetrahydropyridinyl-NCH$_2$**C**H_2), 14.0 (**C**H_3).

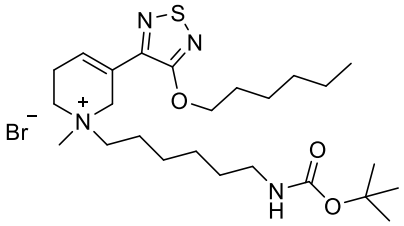
MS (ESI) m/z [M^+] Calcd for $C_{14}H_{24}N_3OS^+$: 282.2. Found: 282.1.

Kane *et al* [114] did not provide a melting point.

*General procedure **G.9** for preparation of Boc protected and tert*-butyl protected *xanomeline linkers*

The appropriate Boc protected or *tert*-butyl protected linker (1.7-2.0 equiv) was dissolved in acetonitrile (40 ml) and treated with **22** (1.0 equiv). The reaction mixture was heated under microwave irridiation (500 W, 78 °C) for 24-32 h. After cooling to rt, the solvent was removed and the crude product was purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 10/1).

*1-(6-((tert-Butoxycarbonyl)amino)hexyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium bromide, **23a** (FG_XA_16)*



$C_{25}H_{45}BrN_4O_3S$

M_r = 561.62 g/mol

A solution of **10a** (169.3 mg, 604 µmol) and **22** (100.0 mg, 355 µmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 32 h. The reaction mixture was worked up as described in general procedure **G.9** to give **23a** as a dark orange oil.

yield:	99 mg (50 %)
reaction control:	R_f = 0.55 (alox basic; $CHCl_3$/CH_3OH 10/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2970, 2929, 2858, 1692, 1509, 1447, 1364, 1245

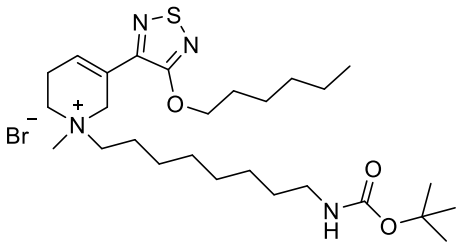
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.23-7.18 (m, 1H, tetrahydropyridinyl-C**H**), 4.67 (s, 1H, N**H**), 4.53 (s, 2H, tetrahydropyridinyl-N$^+$C**H**$_2$C(C)=C), 4.44 (t, ^3J = 6.7 Hz, 2H, OC**H**$_2$), 4.36-4.26 (m, 1H, tetrahydropyridinyl-N$^+$C**H**$_2$CH$_2$), 3.98-3.90 (m, 1H, tetrahydropyridinyl-

$N^+CH_2CH_2$), 3.89-3.80 (m, 1H, $N^+(CH_3)CH_2$), 3.72-3.63 (m, 1H, $N^+(CH_3)CH_2$), 3.46 (s, 3H, N^+CH_3), 3.08-3.02 (m, 2H, $NHCH_2$), 2.87-2.67 (m, 2H, tetrahydropyridinyl-NCH_2CH_2), 1.88-1.78 (m, 4H, $N^+(CH_3)CH_2CH_2$, OCH_2CH_2), 1.49-1.36 (m, 17H, CH_2 , $C(CH_3)_3$), 1.35-1.29 (m, 4H, CH_2), 0.88 (t, ^3J = 6.8 Hz, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.8 (thiadiazolyl-NCO), 156.3 (C(O)NH), 143.8 (thiadiazolyl-C_q), 126.1 (tetrahydropyridinyl-CH), 123.3 (tetrahydropyridinyl-C_q), 79.2 ($C(CH_3)_3$), 71.8 (OCH_2), 63.7 ($N^+(CH_3)CH_2$), 59.0 (tetrahydropyridinyl-$N^+CH_2C(C)=C$), 56.8 (tetrahydropyridinyl-$N^+CH_2CH_2$), 48.5 (N^+CH_3), 40.4 ($NHCH_2$), 31.5 (CH_2), 29.9 (CH_2), 29.0 (CH_2), 28.6 ($C(CH_3)_3$), 26.3 (CH_2), 26.1 (CH_2), 25.8 (CH_2), 22.7 (CH_2), 22.4 (tetrahydropyridinyl-$N^+CH_2CH_2$), 22.1 (CH_2), 14.2 (CH_3).

MS (ESI) m/z [M^+] Calcd for $C_{25}H_{45}N_4O_3S^+$: 481.3. Found: 481.3.

1-(8-((tert-Butoxycarbonyl)amino)octyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium bromide, ***23b*** *(FG_XA_18)*



$C_{27}H_{49}BrN_4O_3S$

M_r = 589.68 g/mol

A solution of **10b** (186.2 mg, 604 µmol) and **22** (100.0 mg, 355 µmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 24 h. The reaction mixture was worked up as described in general procedure **G.9** to give **23b** as a dark orange oil.

yield:	177 mg (85 %)
reaction control:	R_f = 0.56 (alox basic; $CHCl_3$/CH_3OH 10/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2926, 2856, 1691, 1509, 1446, 1364, 1247

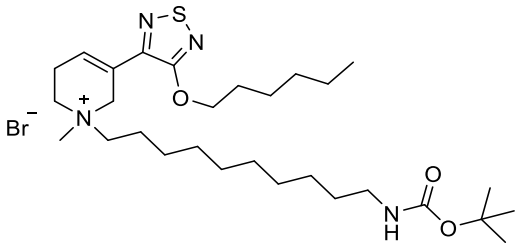
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.21-7.18 (m, 1H, tetrahydropyridinyl-CH), 4.58-4.51 (m, 3H, NH, tetrahydropyridinyl-$N^+CH_2C(C)=C$), 4.43 (t, ^3J = 6.7 Hz, 2H, OCH_2), 4.31 (dt, ^2J = 12.1 Hz, ^3J = 5.9 Hz, 1H, tetrahydropyridinyl-$N^+CH_2CH_2$), 3.92 (dt, ^2J = 12.7 Hz, ^3J = 6.4 Hz, 1H, tetrahydropyridinyl-$N^+CH_2CH_2$), 3.85-3.76 (m, 1H, $N^+(CH_3)CH_2$), 3.67-3.58 (m, 1H, $N^+(CH_3)CH_2$), 3.45 (s, 3H, N^+CH_3), 3.07-3.02 (m, 2H, $NHCH_2$), 2.90-2.67 (m, 2H,

tetrahydropyridinyl-$N^+CH_2C\mathbf{H_2}$), 1.85-1.76 (m, 4H, $N^+(CH_3)CH_2C\mathbf{H_2}$, $OCH_2C\mathbf{H_2}$), 1.45-1.36 (m, 15H, $C\mathbf{H_2}$, $C(C\mathbf{H_3})_3$), 1.35-1.23 (m, 10H, $C\mathbf{H_2}$), 0.87 (m, 3H, $C\mathbf{H_3}$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.8 (thiadiazolyl-N**C**O), 156.2 (**C**(O)NH), 143.8 (thiadiazolyl-$\mathbf{C_q}$), 126.1 (tetrahydropyridinyl-**C**H), 123.2 (tetrahydropyridinyl-$\mathbf{C_q}$), 79.2 ($\mathbf{C}(CH_3)_3$), 71.8 ($O\mathbf{C}H_2$), 63.8 ($N^+(CH_3)\mathbf{C}H_2$), 58.9 (tetrahydropyridinyl-$N^+\mathbf{C}H_2C(C)=C$), 56.8 (tetrahydropyridinyl-$N^+\mathbf{C}H_2CH_2$), 48.6 ($N^+\mathbf{C}H_3$), 40.6 ($NH\mathbf{C}H_2$), 31.5 ($\mathbf{C}H_2$), 30.1 ($\mathbf{C}H_2$), 29.1 ($\mathbf{C}H_2$), 29.0 ($\mathbf{C}H_2$), 28.9 ($\mathbf{C}H_2$), 28.6 ($C(\mathbf{C}H_3)_3$), 26.7 ($\mathbf{C}H_2$), 26.4 ($\mathbf{C}H_2$), 25.8 ($\mathbf{C}H_2$), 22.7 (tetrahydropyridinyl-$N^+CH_2\mathbf{C}H_2$), 22.5 ($\mathbf{C}H_2$), 22.1 ($\mathbf{C}H_2$), 14.2 ($\mathbf{C}H_3$).

MS (ESI) m/z [M^+] Calcd for $C_{27}H_{49}N_4O_3S^+$: 509.4. Found: 509.3.

1-(10-((tert-Butoxycarbonyl)amino)decyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium bromide, ***23c*** *(FG_XA_20)*



$C_{29}H_{53}BrN_4O_3S$

M_r = 617.73 g/mol

A solution of **10c** (203.2 mg, 604 µmol) and **22** (100.0 mg, 355 µmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 26 h. The reaction mixture was worked up as described in general procedure **G.9** to give **23c** as a dark orange oil.

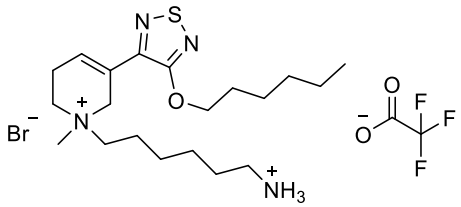
yield:	213 mg (97 %)
reaction control:	R_f = 0.50 (alox basic; $CHCl_3$/CH_3OH 20/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3355, 2926, 2856, 2188, 1692, 1510, 1365, 1171

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.24-7.20 (m, 1H, tetrahydropyridinyl-C**H**), 4.54-4.38 (m, 6H, N**H**, tetrahydropyridinyl-$N^+C\mathbf{H_2}C(C)=C$, $OC\mathbf{H_2}$, tetrahydropyridinyl-$N^+C\mathbf{H_2}CH_2$), 3.94-3.82 (m, 2H, tetrahydropyridinyl-$N^+C\mathbf{H_2}CH_2$, $N^+(CH_3)C\mathbf{H_2}$), 3.65-3.56 (m, 1H, $N^+(CH_3)C\mathbf{H_2}$), 3.46 (s, 3H, $N^+C\mathbf{H_3}$), 3.11-3.02 (m, 2H, $NHC\mathbf{H_2}$), 2.92-2.67 (m, 2H, tetrahydropyridinyl-$N^+CH_2C\mathbf{H_2}$), 1.88-1.77 (m, 4H, $N^+(CH_3)CH_2C\mathbf{H_2}$, $OCH_2C\mathbf{H_2}$), 1.47-1.31 (m, 21H, $C\mathbf{H_2}$, $C(C\mathbf{H_3})_3$), 1.29-1.21 (m, 8H, $C\mathbf{H_2}$), 0.89 (t, ^3J = 6.8 Hz, 3H, $C\mathbf{H_3}$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.7 (thiadiazolyl-N**C**O), 156.1 (**C**(O)NH), 143.8 (thiadiazolyl-**C_q**), 126.0 (tetrahydropyridinyl-**C**H), 123.2 (tetrahydropyridinyl-**C_q**), 79.0 (**C**$(CH_3)_3$), 71.6 (O**C**H_2), 63.5 ($N^+(CH_3)$**C**H_2), 58.7 (tetrahydropyridinyl-N^+**C**$H_2C(C)=C$), 56.6 (tetrahydropyridinyl-N^+**C**H_2CH_2), 48.5 (N^+**C**H_3), 40.7 (NH**C**H_2), 31.4 (**C**H_2), 30.1 (**C**H_2), 29.3 (**C**H_2), 29.3 (**C**H_2), 29.2 (**C**H_2), 29.2 (**C**H_2), 28.8 (**C**H_2), 28.5 (C(**C**$H_3)_3$), 26.8 (**C**H_2), 26.4 (**C**H_2), 25.7 (**C**H_2), 22.6 (**C**H_2), 22.5 (tetrahydropyridinyl-N^+CH_2**C**H_2), 22.0 (**C**H_2), 14.0 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{29}H_{53}N_4O_3S^+$: 537.4. Found: 537.3.

*1-(6-Ammoniohexyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium 2,2,2-trifluoroacetate bromide, **3-L6** (FG_XA_17)*



$C_{22}H_{38}BrF_3N_4O_3S$

M_r = 575.53 g/mol

TFA (57 µl, 748 µmol) was added dropwise to a solution of **23a** (70.0 mg, 125 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 2 h at rt, the reaction mixture was worked up according to **G.4** to give **3-L6** as dark orange oil.

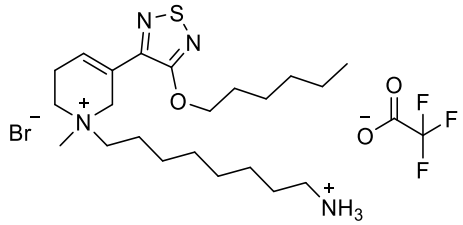
yield:	68 mg (95 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3452, 2932, 2861, 1673, 1511, 1377, 1173

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 8.13 (s, 3H, N**H_3^+**), 7.20 (s, 1H, tetrahydropyridinyl-C**H**), 4.46-4.30 (m, 4H, tetrahydropyridinyl-N^+C**H_2**C(C)=C, OC**H_2**), 3.80-3.50 (m, 4H, tetrahydropyridinyl-N^+C**H_2**CH_2, $N^+(CH_3)$C**H_2**), 3.19 (s, 3H, N^+C**H_3**), 3.00 (bs, 2H, C**H_2**NH_3^+), 2.74 (bs, 2H, tetrahydropyridinyl-N^+C**H_2**CH_2), 1.98-1.65 (m, 6H, C**H_2**), 1.52-1.37 (m, 6H, C**H_2**), 1.34-1.28 (m, 4H, C**H_2**), 0.87 (t, ^3J = 6.7 Hz, 3H, C**H_3**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.8 (thiadiazolyl-N**C**O), 143.7 (thiadiazolyl-**C_q**), 125.9 (tetrahydropyridinyl-**C**H), 123.1 (tetrahydropyridinyl-**C_q**), 71.8 (O**C**H_2), 64.9 ($N^+(CH_3)$**C**H_2), 59.5 (tetrahydropyridinyl-N^+**C**$H_2C(C)=C$), 56.7 (tetrahydropyridinyl-N^+**C**H_2CH_2), 47.8 (N^+**C**H_3), 39.7 (NH**C**H_2), 31.5 (**C**H_2), 28.9 (**C**H_2), 26.6 (**C**H_2), 25.8 (**C**H_2), 25.2 (**C**H_2), 25.2 (**C**H_2), 22.7 (**C**H_2), 21.7 (**C**H_2), 21.5 (**C**H_2), 14.1 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{20}H_{38}N_4OS^{2+}$: 191.1. Found: 191.2.

*1-(8-Ammoniooctyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium 2,2,2-trifluoroacetate bromide, **3-L8** (FG_XA_21)*



$C_{24}H_{42}BrF_3N_4O_3S$

M_r = 603.58 g/mol

TFA (125 µl, 1.63 mmol) was added dropwise to a solution of **23b** (160.0 mg, 271 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 2 h at rt, the reaction mixture was worked up according to **G.4** to give **3-L8** as dark orange oil.

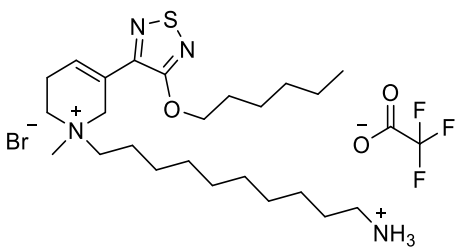
yield:	159 mg (97 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3391, 3037, 2931, 2861, 2360, 1672, 1512, 1437, 1133

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.78 (s, 3H, N**H$_3^+$**), 7.23-7.19 (m, 1H, tetrahydropyridinyl-C**H**), 4.47-4.28 (m, 4H, tetrahydropyridinyl-N$^+$C**H$_2$**C(C)=C, OC**H$_2$**), 3.78-3.45 (m, 4H, tetrahydropyridinyl-N$^+$C**H$_2$**CH$_2$, N$^+$(CH$_3$)C**H$_2$**), 3.20 (s, 3H, N$^+$C**H$_3$**), 2.98-2.85 (m, 2H, C**H$_2$**NH$_3^+$), 2.79-2.70 (m, 2H, tetrahydropyridinyl-N$^+$C**H$_2$**CH$_2$), 1.88-1.79 (m, 4H, C**H$_2$**), 1.72-1.62 (m, 2H, C**H$_2$**), 1.46-1.25 (m, 14H, C**H$_2$**), 0.87 (t, ^3J = 6.7 Hz, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.8 (thiadiazolyl-N**C**O), 143.7 (thiadiazolyl-**C$_q$**), 126.1 (tetrahydropyridinyl-**C**H), 123.1 (tetrahydropyridinyl-**C$_q$**), 71.8 (O**C**H$_2$), 64.9 (N$^+$(CH$_3$)**C**H$_2$), 59.4 (tetrahydropyridinyl-N$^+$**C**H$_2$C(C)=C), 56.9 (tetrahydropyridinyl-N$^+$**C**H$_2$CH$_2$), 48.2 (N$^+$**C**H$_3$), 40.1 (NH**C**H$_2$), 31.5 (**C**H$_2$), 28.9 (**C**H$_2$), 28.1 (**C**H$_2$), 26.9 (**C**H$_2$), 26.9 (**C**H$_2$), 25.8 (**C**H$_2$), 25.8 (**C**H$_2$), 25.2 (**C**H$_2$), 22.7 (**C**H$_2$), 22.0 (**C**H$_2$), 21.8 (**C**H$_2$), 14.1 (**C**H$_3$).

MS (ESI) m/z [M$^+$] Calcd for $C_{22}H_{42}N_4OS^{2+}$: 205.2 Found: 205.2.

*1-(10-Ammoniodecyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium 2,2,2-trifluoroacetate bromide, **3-L10** (FG_XA_22)*



$C_{26}H_{46}BrF_3N_4O_3S$

M_r = 631.64 g/mol

TFA (97 µl, 1.26 mmol) was added dropwise to a solution of **23c** (130.0 mg, 210 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 2 h at rt, the reaction mixture was worked up according to **G.4** to give **3-L10** as dark orange oil.

yield:	79 mg (59 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3402, 2928, 2857, 1675, 1511, 1376, 1173

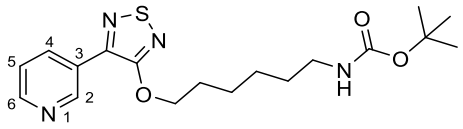
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 8.13 (s, 3H, N**H**$_3^+$), 7.21 (s, 1H, tetrahydropyridinyl-C**H**), 4.48-4.33 (m, 4H, tetrahydropyridinyl-N$^+$C**H**$_2$C(C)=C, OC**H**$_2$), 3.92-3.51 (m, 4H, tetrahydropyridinyl-N$^+$C**H**$_2$CH$_2$, N$^+$(CH$_3$)C**H**$_2$), 3.26 (s, 3H, N$^+$C**H**$_3$), 3.00-2.86 (m, 2H, C**H**$_2$NH$_3^+$), 2.84-2.68 (m, 2H, tetrahydropyridinyl-N$^+$C**H**$_2$CH$_2$), 1.87-1.77 (m, 4H, C**H**$_2$), 1.73-1.62 (m, 2H, C**H**$_2$), 1.47-1.24 (m, 18H, C**H**$_2$), 0.87 (t, ^3J = 6.5 Hz, 3H, C**H**$_3$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.8 (thiadiazolyl-N**C**O), 143.8 (thiadiazolyl-**C$_q$**), 126.1 (tetrahydropyridinyl-**C**H), 123.1 (tetrahydropyridinyl-**C$_q$**), 71.8 (O**C**H$_2$), 64.8 (N$^+$(CH$_3$)**C**H$_2$), 59.4 (tetrahydropyridinyl-N$^+$**C**H$_2$C(C)=C), 56.9 (tetrahydropyridinyl-N$^+$**C**H$_2$CH$_2$), 48.4 (N$^+$**C**H$_3$), 40.1 (NH**C**H$_2$), 31.6 (**C**H$_2$), 28.9 (**C**H$_2$), 28.6 (**C**H$_2$), 28.6 (**C**H$_2$), 28.5 (**C**H$_2$), 28.4 (**C**H$_2$), 27.1 (**C**H$_2$), 26.0 (**C**H$_2$), 25.8 (**C**H$_2$), 25.2 (**C**H$_2$), 22.7 (**C**H$_2$), 22.2 (**C**H$_2$), 21.9 (**C**H$_2$), 14.2 (**C**H$_3$).

MS (ESI) m/z [M$^+$] Calcd for $C_{24}H_{46}N_4OS^{2+}$: 219.2 Found: 219.2.

7.2.5 Preparation of tertiary orthosteric xanomeline linkers 4-Ln

tert-Butyl (6-((4-(pyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)hexyl)carbamate, ***24a*** *(FG_XA_2)*



$C_{18}H_{26}N_4O_3S$

M_r = 378.49 g/mol

A solution of **9a** (824 mg, 3.79 mmol) in dry THF (15 ml) at 0 °C was treated with sodium hydride (60 % suspension in paraffin oil, 354 mg, 8.85 mmol) and stirred at rt for 2 h under argon atmosphere. A solution of **19** (500 mg, 2.53 mmol) in dry THF (10 ml) was added dropwise at 0 °C and the mixture was heated under reflux for 5 h. The reaction was worked up according to general procedure **G.6** and purified by column chromatography (silica, petroleum ether/acetone 10/3) to give **24a** as white solid.

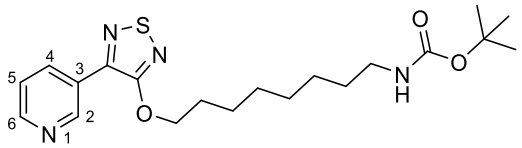
yield:	258 mg (28 %)
reaction control:	R_f = 0.47 (silica gel; petroleum ether/acetone: 10/3)

melting point [°C]:	55
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3346, 2931, 2859, 1694, 1512, 1413, 1257, 1168

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.39 (s, 1H, **H**-2), 8.65-8.62 (m, 1H, **H**-6), 8.51-8.44 (m, 1H, **H**-4), 7.48-7.41 (m, 1H, **H**-5), 4.60-4.48 (m, 3H, N**H**, OC**H$_2$**), 3.14-3.06 (m, 2H, NHC**H$_2$**), 1.92-1.84 (m, 2H, OCH_2C**H$_2$**), 1.55-1.45 (m, 4H, C**H$_2$**), 1.44-1.36 (m, 11H, C(C**H$_3$**)$_3$, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 163.0 (thiadiazolyl-N**C**O), 156.2 (**C**(O)NH), 149.2 (**C**-6), 147.7 (**C**-2), 139.5 (thiadiazolyl-**C$_q$**), 135.9 (**C**-4), 128.3 (**C**-3), 124.1 (**C**-5), 79.3 (**C**$(CH_3)_3$), 71.6 (O**C**H_2), 40.7 (NH**C**H_2), 30.2 (**C**H_2), 29.0 (**C**H_2), 28.7 (**C**H_3), 26.6 (**C**H_2), 26.0 (**C**H_2).

tert-Butyl (8-((4-(pyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)octyl)carbamate, ***24b*** *(FG_XA_13)*



$C_{20}H_{30}N_4O_3S$

M_r = 406.55 g/mol

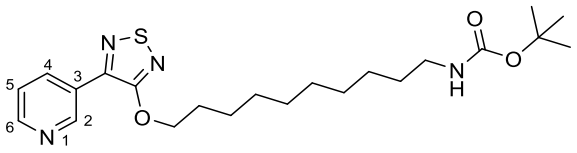
A solution of **9b** (695 mg, 2.83 mmol) in dry THF (15 ml) at 0 °C was treated with sodium hydride (60 % suspension in paraffin oil, 283 mg, 7.08 mmol) and stirred at rt for 2 h under argon atmosphere. A solution of **19** (400 mg, 2.02 mmol) in dry THF (10 ml) was added dropwise at 0 °C and the mixture was heated under reflux for 3 h. The reaction was worked up according to general procedure **G.6** and purified by column chromatography (silica, petroleum ether/acetone 10/3) to give **24b** as colorless oil.

yield:	286 mg (35 %)
reaction control:	R_f = 0.49 (silica gel; petroleum ether/acetone: 10/3)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3345, 2928, 2855, 1698, 1512, 1487, 1257, 1168

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.39 (d, ^4J = 2.0 Hz, 1H, **H**-2), 8.64 (dd, ^3J = 4.9 Hz, ^4J = 1.4 Hz, 1H, **H**-6), 8.49.-8.45 (m, 1H, **H**-4), 7.44 (dd, ^3J = 8.0 Hz, ^3J = 4.9 Hz, 1H, **H**-5), 4.61 (s, 1H, N**H**), 4.51 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 3.12-3.04 (m, 2H, NHC**H$_2$**), 1.91-1.82 (m, 2H, OCH_2C**H$_2$**), 1.51-1.28 (m, 19H, C(C**H$_3$**)$_3$, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 163.1 (thiadiazolyl-N**C**O), 156.2 (NH**C**(=O)O), 149.9 (**C**-6), 147.9 (**C**-2), 144.7 (thiadiazolyl-**C$_q$**), 135.8 (**C**-4), 128.3 (**C**-3), 124.0 (**C**-5), 79.2 (**C**$(CH_3)_3$), 71.7 (O**C**H_2), 40.8 (NH**C**H_2), 30.2 (**C**H_2), 29.3 (**C**H_2), 29.3 (**C**H_2), 29.0 (**C**H_2), 28.7 (**C**H_3), 26.9 (**C**H_2), 26.1 (**C**H_2).

tert-Butyl (10-((4-(pyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)decyl)carbamate, ***24c*** *(FG_XA_3)*



$C_{22}H_{34}N_4O_3S$

M_r = 434.60 g/mol

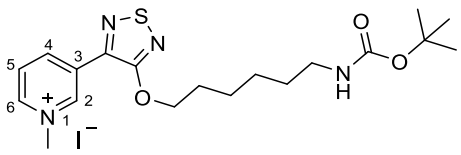
A solution of **9c** (1.04 g, 3.79 mmol) in dry THF (15 ml) at 0 °C was treated with sodium hydride (60 % suspension in paraffin oil, 354 mg, 8.85 mmol) and stirred at rt for 2 h under argon atmosphere. A solution of **19** (500 mg, 2.53 mmol) in dry THF (10 ml) was added dropwise at 0 °C and the mixture was heated under reflux for 3 h. The reaction was worked up according to general procedure **G.6** and purified by column chromatography (silica, petroleum ether/acetone 10/3) to give **24c** as white solid.

yield:	277 mg (25 %)
reaction control:	R_f = 0.52 (silica gel; petroleum ether/acetone: 10/3)
melting point [°C]:	49
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3370, 2918, 2853, 1681, 1510, 1413, 1250, 1167

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.37 (s, 1H, **H**-2), 8.64-8.61 (m, 1H, **H**-6), 8.44.-8.38 (m, 1H, **H**-4), 7.41-7.35 (m, 1H, **H**-5), 4.57- 4.45 (m, 3H, N**H**, OC**H$_2$**), 3.11-3.03 (m, 2H, NHC**H$_2$**), 1.92-1.81 (m, 2H, OCH_2C**H$_2$**), 1.49-1.21 (m, 23H, C(C**H$_3$**)$_3$, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 163.1 (thiadiazolyl-N**C**O), 156.2 (**C**(O)NH), 150.1 (**C**-6), 148.6 (**C**-2), 145.1 (thiadiazolyl-**C$_q$**), 135.1 (**C**-4), 128.0 (**C**-3), 123.7 (**C**-5), 79.2 (**C**$(CH_3)_3$), 71.7 (O**C**H_2), 40.8 (NH**C**H_2), 30.3 (**C**H_2), 29.6 (**C**H_2), 29.6 (**C**H_2), 29.4 (**C**H_2), 29.4 (**C**H_2), 29.1 (**C**H_2), 28.6 (**C**H_3), 27.0 (**C**H_2), 26.2 (**C**H_2).

3-(4-((6-((tert-Butoxycarbonyl)amino)hexyl)oxy)-1,2,5-thiadiazol-3-yl)-1-methylpyridin-1-iumiodide, ***25a*** *(FG_XA_4)*



$C_{19}H_{29}IN_4O_3S$

M_r = 520.43 g/mol

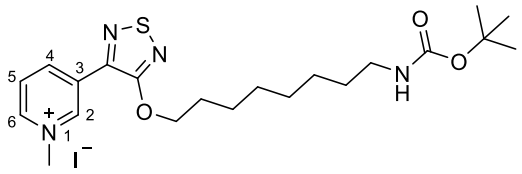
Methyl iodide (362 µl, 5.81 mmol) was added to a solution of compound **24a** (220 mg, 581 µmol) in dry acetone (10 ml) under argon atmosphere and stirred at rt for 24 h. The reaction was worked up according to general procedure **G.7** and purified by column chromatography (silica, DCM/CH_3OH 6/1) to give **25a** as dark yellow solid.

yield:	278 mg (92 %)
reaction control:	R_f = 0.40 (silica gel; DCM/CH_3OH: 6/1)
melting point [°C]:	118-119
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3326, 2923, 2853, 1684, 1516, 1364, 1273, 1169

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.55 (d, ^3J = 6.0 Hz, 1H, **H**-6), 9.43 (s, 1H, **H**-2), 9.12 (d, ^3J = 8.3 Hz, 1H, **H**-4), 8.26 (dd, ^3J = 8.2 Hz, ^3J = 6.2 Hz, 1H, **H**-5), 4.78 (s, 3H, $N^+C\mathbf{H_3}$), 4.61-4.50 (m, 3H, $OC\mathbf{H_2}$, N**H**), 3.14-3.05 (m, 2H, $NHC\mathbf{H_2}$), 1.97-1.88 (m, 2H, $OCH_2C\mathbf{H_2}$), 1.55-1.33 (m, 15H, $C(C\mathbf{H_3})_3$, $C\mathbf{H_2}$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 163.3 (thiadiazolyl-N**C**O), 156.3 (**C**(O)NH), 145.8 (**C**-6), 143.1 (**C**-2), 142.4 (**C**-4), 139.7 (thiadiazolyl-$\mathbf{C_q}$), 131.9 (**C**-3), 128.9 (**C**-5), 79.3 ($\mathbf{C}(CH_3)_3$), 72.6 ($O\mathbf{C}H_2$), 50.9 ($N^+\mathbf{C}H_3$), 40.5 ($NH\mathbf{C}H_2$), 30.2 ($\mathbf{C}H_2$), 28.9 ($\mathbf{C}H_2$), 28.6 ($\mathbf{C}H_3$), 26.4 ($\mathbf{C}H_2$), 25.7 ($\mathbf{C}H_2$).

3-(4-((8-((tert-Butoxycarbonyl)amino)octyl)oxy)-1,2,5-thiadiazol-3-yl)-1-methylpyridin-1-ium iodide, ***25b*** *(FG_XA_14)*



$C_{21}H_{33}IN_4O_3S$

M_r = 548.48 g/mol

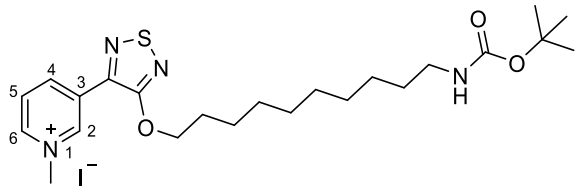
Methyl iodide (270 µl, 4.33 mmol) was added to a solution of compound **24b** (176 mg, 433 µmol) in dry acetone (10 ml) under argon atmosphere and stirred at rt for 24 h. The reaction was worked up according to general procedure **G.7** and purified by column chromatography (silica, DCM/CH_3OH 6/1) to give **25b** as brownish solid.

yield:	146 mg (61 %)
reaction control:	R_f = 0.42 (silica gel; DCM/CH_3OH: 6/1)
melting point [°C]:	73
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3472, 3374, 2926, 2857, 1681, 1519, 1365, 1168

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.58 (d, ^3J = 5.0 Hz, 1H, **H**-6), 9.42 (s, 1H, **H**-2), 9.11 (d, ^3J = 7.9 Hz, 1H, **H**-4), 8.29-8.22 (m, 1H, **H**-5), 4.77 (s, 3H, $N^+C\mathbf{H_3}$), 4.62-4.46 (m, 3H, $OC\mathbf{H_2}$, N**H**), 3.16-2.99 (m, 2H, $NHC\mathbf{H_2}$), 1.98-1.85 (m, 2H, $OCH_2C\mathbf{H_2}$), 1.54-1.21 (m, 19H, $C(C\mathbf{H_3})_3$, $C\mathbf{H_2}$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 163.4 (thiadiazolyl-N**C**O), 156.2 (**C**(O)NH), 146.0 (**C**-6), 143.1 (**C**-2), 142.2 (**C**-4), 139.7 (thiadiazolyl-**C_q**), 131.8 (**C**-3), 128.9 (**C**-5), 79.2 (**C**$(CH_3)_3$), 72.7 (O**C**H_2), 50.9 (N^+**C**H_3), 40.7 (NH**C**H_2), 30.2 (**C**H_2), 29.3 (**C**H_2), 29.3 (**C**H_2), 28.9 (**C**H_2), 28.6 (**C**H_3), 26.8 (**C**H_2), 26.0 (**C**H_2).

3-(4-((10-((tert-Butoxycarbonyl)amino)decyl)oxy)-1,2,5-thiadiazol-3-yl)-1-methylpyridin-1-ium iodide, ***25c*** *(FG_XA_5)*



$C_{23}H_{37}IN_4O_3S$

M_r = 576.54 g/mol

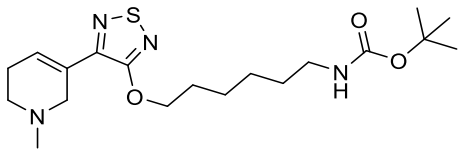
Methyl iodide (315 µl, 5.06 mmol) was added to a solution of compound **24c** (220 mg, 506 µmol) in dry acetone (10 ml) under argon atmosphere and stirred at rt for 24 h. The reaction was worked up according to general procedure **G.7** and purified by column chromatography (silica, DCM/CH_3OH 6/1) to give **25c** as yellowish oil.

yield:	209 mg (72 %)
reaction control:	R_f = 0.45 (silica; DCM/CH_3OH: 6/1)
melting point [°C]:	87-88
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3373, 2925, 2855, 1682, 1519, 1365, 1250, 1168

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 9.66 (d, ^3J = 5.8 Hz, 1H, **H**-6), 9.40 (s, 1H, **H**-2), 9.11 (d, ^3J = 8.2 Hz, 1H, **H**-4), 8.24 (m, 1H, **H**-5), 4.79 (s, 3H, N^+C**H$_3$**), 4.58 (t, ^3J = 5.8 Hz, 2H, OC**H$_2$**), 4.50 (s, 1H, N**H**), 3.10-3.03 (m, 2H, NHC**H$_2$**), 1.96-1.87 (m, 2H, OCH_2C**H$_2$**), 1.47-1.25 (m, 23H, C(C**H$_3$**)$_3$, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 163.4 (thiadiazolyl-N**C**O), 156.3 (**C**(O)NH), 146.1 (**C**-6), 143.0 (**C**-2), 142.1 (**C**-4), 139.6 (thiadiazolyl-**C_q**), 131.8 (**C**-3), 128.9 (**C**-5), 79.2 (**C**$(CH_3)_3$), 72.7 (O**C**H_2), 50.8 (N^+**C**H_3), 40.8 (NH**C**H_2), 30.3 (**C**H_2), 29.6 (**C**H_2), 29.6 (**C**H_2), 29.4 (**C**H_2), 29.4 (**C**H_2), 29.0 (**C**H_2), 28.6 (**C**H_3), 27.0 (**C**H_2), 26.1 (**C**H_2).

*tert-Butyl (6-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)hexyl)-carb-amate, **26a** (FG_XA_6)*



$C_{19}H_{32}N_4O_3S$

M_r = 396.55 g/mol

A solution of compound **25a** (200.0 mg, 384 µmol) in dry ethanol (15 ml) at 0 °C was treated with sodium borohydride (72.7 mg, 1.92 mmol) under argon atmosphere. The reaction mixture was stirred for 20 h at rt, heated under reflux for 2 h, and worked up according to **G.8**. Purification of the crude product was achieved by column chromatography (silica, EtOAc/CH_3OH 3/1) to give **26a** as brown solid.

yield:	77 mg (51 %)
reaction control:	R_f = 0.61 (silica; EtOAc/CH_3OH: 3/1)
melting point [°C]:	73
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3355, 2928, 2856, 1691, 1509, 1447, 1247, 1169

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 7.13-7.09 (m, 1H, tetrahydropyridinyl-C**H**), 4.48 (t, ^3J = 6.5 Hz, 2H, OC**H₂**), 3.54-3.50 (m, 2H, tetrahydropyridinyl-NC**H₂**C(C)=C), 3.04 (t, ^3J = 6.9 Hz, 2H, NHC**H₂**), 2.69 (t, ^3J = 5.9 Hz, 2H, tetrahydropyridinyl-NC**H₂**CH_2), 2.52-2.46 (m, 5H, tetrahydropyridinyl-NCH_2C**H₂**, NC**H₃**), 1.90-1.83 (m, 2H, OCH_2C**H₂**), 1.55-1.36 (m, 15H, C(C**H₃**)$_3$, C**H₂**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 164.0 (thiadiazolyl-N**C**O), 158.7 (**C**(O)NH), 147.8 (thiadiazolyl-**C$_q$**), 129.8 (tetrahydropyridinyl-**C$_q$**), 129.5 (tetrahydropyridinyl-**C**H), 79.9 (**C**$(CH_3)_3$), 72.3 (O**C**H_2), 55.6 (tetrahydropyridinyl-N**C**H_2C(C)=C), 52.1 (tetrahydropyridinyl-N**C**H_2CH_2), 45.8 (N**C**H_3), 41.4 (NH**C**H_2), 31.0 (**C**H_2), 30.0 (**C**H_2), 28.9 (**C**H_3), 27.6 (**C**H_2), 27.0 (**C**H_2), 26.8 (tetrahydropyridinyl-NCH_2**C**H_2).

*tert-Butyl (8-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)octyl)carbamate, **26b** (FG_XA_15)*

$C_{21}H_{36}N_4O_3S$

M_r = 424.60 g/mol

A solution of compound **25b** (130.0 mg, 237 µmol) in dry ethanol (15 ml) at 0 °C was treated with sodium borohydride (44.8 mg, 1.19 mmol) under argon atmosphere. The reaction mixture

was stirred for 20 h at rt, heated under reflux for 2 h, and worked up according to **G.8**. Purification of the crude product was achieved by column chromatography (silica, EtOAc/CH_3OH 3/1) to give **26b** as brownish solid.

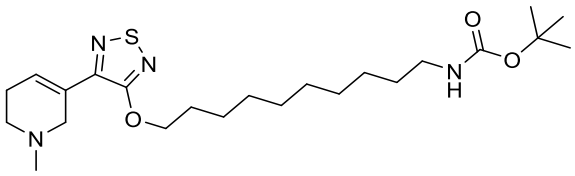
yield:	60 mg (60 %)
reaction control:	R_f = 0.58 (silica; EtOAc/CH_3OH: 4/1)
melting point [°C]:	69
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3343, 2926, 2854, 1692, 1509, 1447, 1247, 1169

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.04-7.00 (m, 1H, tetrahydropyridinyl-C**H**), 4.39 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 3.45-3.43 (m, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 3.09-3.01 (m, 2H, NHC**H$_2$**), 2.57 (t, ^3J = 5.7 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.45-2.38 (m, 5H, tetrahydropyridinyl-NCH_2C**H$_2$**, NC**H$_3$**), 1.84-1.75 (m, 2H, OCH_2C**H$_2$**), 1.47-1.36 (m, 19H, C(C**H$_3$**)$_3$, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.7 (thiadiazolyl-N**C**O), 156.2 (**C**(O)NH), 146.9 (thiadiazolyl-**C$_q$**), 129.3 (tetrahydropyridinyl-**C$_q$**), 128.4 (tetrahydropyridinyl-**C**H), 79.2 (**C**-$(CH_3)_3$), 71.1 (O**C**H_2), 55.0 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.3 (tetrahydropyridinyl-N**C**H_2CH_2), 45.9 (N^+**C**H_3), 40.8 (NH**C**H_2), 30.2 (**C**H_2), 29.3 (**C**H_2), 29.3 (**C**H_2), 29.0 (**C**H_2), 28.6 (**C**H_3), 26.9 (**C**H_2), 26.6 (**C**H_2), 26.1 (tetrahydropyridinyl-NCH_2**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{21}H_{37}N_4O_3S^+$: 425.3. Found: 397.1.

*tert-Butyl (10-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)decyl)-carbamate, **26c** (FG_XA_7)*



$C_{23}H_{40}N_4O_3S$

M_r = 452.66 g/mol

A solution of compound **25c** (158.0 mg, 274 µmol) in dry ethanol (15 ml) at 0 °C was treated with sodium borohydride (51.8 mg, 1.37 mmol) under argon atmosphere. The reaction mixture was stirred for 20 h at rt, heated under reflux for 2 h, and worked up according to **G.8**. Purification of the crude product was achieved by column chromatography (EtOAc/CH_3OH) to give **26c** as brown oil.

yield:	63 mg (51 %)
reaction control:	R_f = 0.63 (silica; EtOAc/CH_3OH: 3/1)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3353, 2925, 2853, 1692, 1511, 1449, 1247, 1169

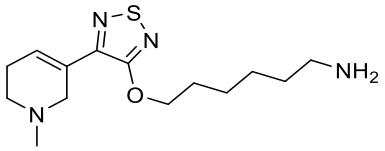
^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 7.12-7.08 (m, 1H, tetrahydropyridinyl-C**H**), 4.47 (t, ^{3}J = 6.5 Hz, 2H, OC**H$_2$**), 3.47-3.44 (m, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 3.01 (t, ^{3}J = 7.0 Hz, 2H, NHC**H$_2$**), 2.63 (t, ^{3}J = 5.8 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.49-2.43 (m, 5H, tetrahydropyridinyl-NCH_2C**H$_2$**, NC**H$_3$**), 1.87-1.80 (m, 2H, OCH_2C**H$_2$**), 1.52-1.24 (m, 23H, C(C**H$_3$**)$_3$, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 164.0 (thiadiazolyl-N**C**O), 158.7 (**C**(O)NH), 147.9 (thiadiazolyl-**C$_q$**), 130.1 (tetrahydropyridinyl-**C$_q$**), 128.0 (tetrahydropyridinyl-**C**H), 79.9 (**C**$(CH_3)_3$), 72.3 (O**C**H_2), 55.8 (tetrahydropyridinyl-N**C**H_2C(C)=C), 52.2 (tetrahydropyridinyl-N**C**H_2CH_2), 46.0 (N^+**C**H_3), 41.5 (NH**C**H_2), 31.1 (**C**H_2), 30.7 (**C**H_2), 30.7 (**C**H_2), 30.5 (**C**H_2), 30.4 (**C**H_2), 30.1 (**C**H_2), 29.0 (**C**H_3), 28.0 (**C**H_2), 27.3 (**C**H_2), 27.1 (tetrahydropyridinyl-NCH_2**C**H_2).

*General procedure **G.9** for preparation of compounds **4-Ln***

The Boc deprotection was performed according to a procedure from She *et al*[113]. The appropriate Boc protected linker **26a-c** (1 equiv) was dissolved in dry DCM (2 ml) under argon atmosphere and cooled to -20 °C in a sodium chloride ice bath. After the addition of TFA (100 equiv), the reaction mixture was stirred for 30 min at -20 °C and for 16 h at rt. The mixture was concentrated, followed by the addition of water (10 ml) and adjustment of pH 9 with 25 % aqueous ammonia solution. The mixture was extracted with DCM (5x10 ml). After phase separation, the combined organic layers were dried over sodium sulfate and concentrated *in vacuo* to give **4-Ln**.

*6-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)hexan-1-amine, **4-L6** (GB_hxn_fx)*



$C_{14}H_{24}N_4OS$

M_r = 296.43 g/mol

TFA (3.47 ml, 45.4 mmol) was added to a solution of compound **26c** (180.0 mg, 454 µmol) in dry DCM (5 ml) at -20 °C under argon atmosphere, stirred for 30 min at -20 °C and for 16 h at rt. The reaction mixture was worked up according to **G.9** to give **4-L6** as brown oil.

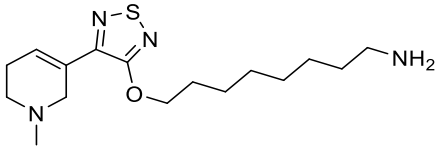
yield: 78 mg (58 %)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 2934, 2855, 2785, 1674, 1507, 1444, 1126

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.01-6.97 (m, 1H, tetrahydropyridinyl-C**H**), 4.38 (t, ^{3}J = 6.6 Hz, 2H, OC**H$_2$**), 3.40-3.36 (m, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 2.65 (t, ^{3}J = 6.9 Hz, 2H, NH_2C**H$_2$**), 2.51 (t, ^{3}J = 5.7 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.42-2.35 (m, 5H, tetrahydropyridinyl-NCH_2C**H$_2$**, NC**H$_3$**), 1.83-1.74 (m, 2H, OCH_2C**H$_2$**), 1,48-1.30 (m, 6H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.6 (thiadiazolyl-N**C**O), 146.9 (thiadiazolyl-**C$_q$**), 129.5 (tetrahydropyridinyl-**C$_q$**), 128.5 (tetrahydropyridinyl-**C**H), 70.9 (O**C**H_2), 55.1 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.3 (tetrahydropyridinyl-N**C**H_2CH_2), 46.0 (N**C**H_3), 41.9 (NH_2**C**H_2), 33.0 (**C**H_2), 28.9 (**C**H_2), 26.7 (**C**H_2), 26.6 (**C**H_2) 26.0 (**C**H_2).

8-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)octan-1-amine, ***4-L8*** *(GB_oct_fx)*



$C_{16}H_{28}N_4OS$

M_r = 324.49 g/mol

TFA (7.21 ml, 94.2 mmol) was added to a solution of compound **26b** (400.0 mg, 942 µmol) in dry DCM (7 ml) at -20 °C under argon atmosphere, stirred for 30 min at -20 °C and for 16 h at rt. The reaction mixture was worked up according to **G.9** to give **4-L8** as brown oil.

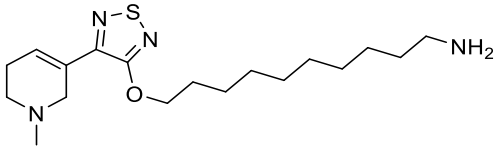
yield: 199 mg (65 %)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 2963, 2857, 1623, 1506, 1446, 1084

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.04-7.01 (m, 1H, tetrahydropyridinyl-C**H**), 4.41 (t, ^{3}J = 6.6 Hz, 2H, OC**H$_2$**), 3.43-3.40 (m, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 2.67 (t, ^{3}J = 7.0 Hz, 2H, NH_2C**H$_2$**), 2.54 (t, ^{3}J = 5.7 Hz, 2H tetrahydropyridinyl-NC**H$_2$**CH_2), 2.45-2.38 (m, 5H, tetrahydropyridinyl-NCH_2C**H$_2$**, NC**H$_3$**), 1.85-1.76 (m, 2H, OCH_2C**H$_2$**), 1.60-1.30 (m, 10H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.7 (thiadiazolyl-N**C**O), 147.0 (thiadiazolyl-**C$_q$**), 129.6 (tetrahydropyridinyl-**C$_q$**), 128.5 (tetrahydropyridinyl-**C**H), 71.1 (O**C**H_2), 55.2 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.4 (tetrahydropyridinyl-N**C**H_2CH_2), 46.1 (N**C**H_3), 42.1 (NH_2**C**H_2), 33.1 (**C**H_2), 29.5 (**C**H_2), 29.4 (**C**H_2), 29.0 (**C**H_2), 27.0 (**C**H_2), 26.8 (**C**H_2), 26.1 (**C**H_2).

10-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)decan-1-amine, ***4-L10*** *(GB_dec_fx)*



$C_{18}H_{32}N_4OS$

M_r = 352.54 g/mol

TFA (6.76 ml, 88.4 mmol) was added to a solution of compound **26c** (400.0 mg, 884 µmol) in dry DCM (7 ml) at -20 °C under argon atmosphere, stirred for 30 min at -20 °C, and for 16 h at rt. The reaction mixture was worked up according to **G.9** to give **4-L10** as brown oil.

yield:	208 mg (67 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2928, 2850, 1673, 1592, 1424, 1027

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.04-7.02 (m, 1H, tetrahydropyridinyl-C**H**), 4.40 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 3.41 (dd, ^3J = 4.3 Hz, ^4J = 2.4 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 2.66 (t, ^3J = 7.1 Hz, 2H, NH_2C**H$_2$**), 2.53 (t, ^3J = 5.7 Hz, 2H tetrahydropyridinyl-NC**H$_2$**CH_2), 2.43-2.38 (m, 5H, tetrahydropyridinyl-NCH_2C**H$_2$**, NC**H$_3$**), 1.84-1.76 (m, 2H, OCH_2C**H$_2$**), 1.45-1.23 (m, 14H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 162.7 (thiadiazolyl-N**C**O), 147.0 (thiadiazolyl-**C$_q$**), 129.5 (tetrahydropyridinyl-**C$_q$**), 128.5 (tetrahydropyridinyl-**C**H), 71.1 (O**C**H_2), 55.2 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.4 (tetrahydropyridinyl-N**C**H_2CH_2), 46.1 (N**C**H_3), 42.1 (NH_2**C**H_2), 33.2 (**C**H_2), 29.6 (**C**H_2), 29.6 (**C**H_2), 29.6 (**C**H_2), 29.4 (**C**H_2), 29.0 (**C**H_2), 27.0 (**C**H_2), 26.8 (**C**H_2), 26.1 (**C**H_2).

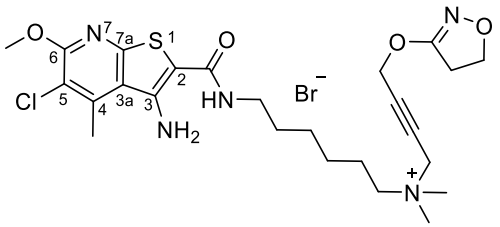
7.2.6 Preparation of LY20-An hybrids through amide coupling

General procedure ***G.10*** *for PyBOP mediated amide coupling*

Using a modified procedure from Szabo *et al.*[109], the appropriate carboxylic acid (1 equiv) was dissolved in dry DMF under argon atmosphere and treated subsequently with DIPEA (1.05 equiv) and PyBOP reagent (1 equiv). The mixture was stirred at rt for 15 min. Simultaneously, the required amine (1 equiv) was dissolved in dry DMF (3 ml) under argon atmosphere in a second vessel, followed by the addition of DIPEA (1.05 equiv) and stirring at rt for 10 min. The amine solution was then added dropwise to the reaction mixture and stirred at rt for 3-4 h before the solvent was removed *in vacuo*. The resulting residue was diluted with DCM (20 ml) and washed with saturated sodium bicarbonate solution (40 ml). The phases were separated and the aqueous layer was further extracted with DCM (3x20 ml). After phase separation, the combined organic layers were washed with water (20 ml) and brine (20 ml),

respectively. The organic fraction was then dried over sodium sulfate and the solvent was removed *in vacuo* to give the crude product. Further purification of hybrid compounds was achieved by column chromatography as indicated.

6-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethylhexan-1-aminium bromide, ***LY20-A6-iper****, (FG_B_39)*



$C_{25}H_{35}BrClN_5O_4S$

M_r = 617.00 g/mol

Carboxylic acid **1** (70.0 mg, 257 µmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (47 µl, 269 µmol) and PyBOP (133.6 mg, 257 µmol) were successively added. Compound **1-L6** (122.3 mg, 257 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (47 µl, 269 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/1 → 10/1) to give **LY20-A6-iper** as yellowish crystals.

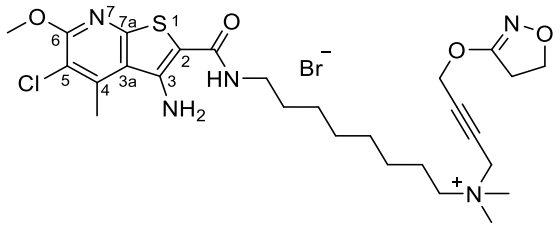
yield:	40 mg (25 %)
HPLC purity:	98 % (HPLC Method **I**, B: 65 %)
reaction control:	R_f = 0.15 (alox basic; $CHCl_3$/CH_3OH: 20/1; Dragendorff-reagent)
melting point [°C]:	80
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3420, 3320, 2930, 2860, 1622, 1593, 1520, 1359, 1280

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.40 (s, 2H, N**H$_2$**), 6.28 (t, 1H, ^3J = 5.6 Hz, N**H**), 4.84 (t, ^5J = 1.6 Hz, 2H, OC**H$_2$**C≡C), 4.34 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 4.18-4.15 (m, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 3.99 (s, 3H, OC**H$_3$**), 3.35–3.27 (m, 4H, NHC**H$_2$**$(CH_2)_4$C**H$_2$**$N^+(CH_3)_2$), 3.05 (s, 6H, N^+(C**H$_3$**)$_2$), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.76 (s, 3H, C**H$_3$**), 1.78-1.68 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.62-1.52 (m, 2H, NHCH_2C**H$_2$**), 1.45-1.34 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 168.4 (isoxazolinyl-**C$_q$**), 166.8 (**C**(O)NH), 160.3 (**C**-6), 155.7 (**C$_q$**), 148.9 (**C$_q$**), 145.3 (**C**-4), 122.5 (**C$_q$**), 117.2 (**C$_q$**), 99.3 (**C$_q$**), 88.0 (C≡**C**CH_2O), 76.2 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 65.6 ($N^+(CH_3)_2$**C**H_2), 58.5 (O**C**H_2C≡C), 55.8 (O**C**H_3), 55.5 ($N^+(CH_3)_2$**C**H_2C≡C), 51.8 (N^+(**C**$H_3)_2$), 40.1 (**C**H_2NH), 33.8 (isoxazolinyl-OCH_2**C**H_2), 30.5 (NHCH_2**C**H_2), 27.3 (**C**H_2), 26.8 (**C**H_2), 23.5 (**C**$H_2CH_2N^+(CH_3)_2$), 17.0 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{25}H_{35}ClN_5O_4S^+$: 536.2. Found: 536.3.

8-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyloctan-1-aminium bromide, ***LY20-A8-iper*** *(FG_B_45)*



$C_{27}H_{39}BrClN_5O_4S$

M_r = 645.05 g/mol

Carboxylic acid **1** (125.0 mg, 458 µmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (84 µl, 482 µmol) and PyBOP (238.5 mg, 458 µmol) were successively added. Compound **1-L8** (231.2 mg, 458 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (84 µl, 482 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/1 → 10/1) to give **LY20-A8-iper** as yellowish crystals.

yield:	40 mg (14 %)
HPLC purity:	99 % (HPLC Method **I**, B: 65 %)
reaction control:	R_f = 0.16 (alox basic; $CHCl_3$/CH_3OH: 20/1; Dragendorff-reagent)
melting point:	78
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3420, 3317, 2930, 2860, 1626, 1594, 1522, 1360, 1280

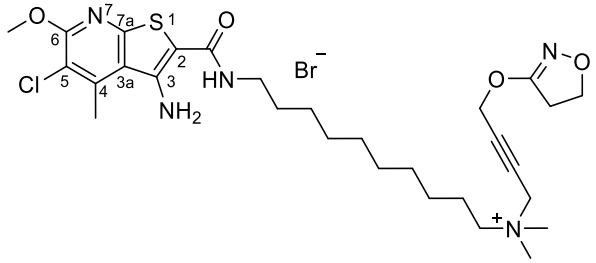
^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.39 (s, 2H, N**H$_2$**), 6.19 (t, ^3J = 5.6 Hz, 1H, N**H**), 4.84 (t, ^5J = 1.8 Hz, 2H, OC**H$_2$**C≡C), 4.34 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 4.12 (t, ^5J = 1.7 Hz, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 4.00 (s, 3H, OC**H$_3$**), 3.32–3.25 (m, 4H, NHC**H$_2$**$(CH_2)_6$C**H$_2$**-

$N^+(CH_3)_2$), 3.04 (s, 6H, $N^+(C\mathbf{H_3})_2$), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OCH_2C\mathbf{H_2}$), 2.76 (s, 3H, $C\mathbf{H_3}$), 1.78-1.68 (m, 2H, $C\mathbf{H_2}CH_2N^+(CH_3)_2$), 1.58-1.53 (m, 2H, $NHCH_2C\mathbf{H_2}$), 1.38-1.27 (m, 8H, $C\mathbf{H_2}$).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 168.4 (isoxazolinyl-$\mathbf{C_q}$), 166.7 (**C**(O)NH), 160.3 (**C**-6), 155.7 ($\mathbf{C_q}$), 148.8 ($\mathbf{C_q}$), 145.3 (**C**-4), 122.5 ($\mathbf{C_q}$), 117.2 ($\mathbf{C_q}$), 99.3 ($\mathbf{C_q}$), 88.1 (C≡**C**CH_2O), 76.1 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 65.6 ($N^+(CH_3)_2\mathbf{C}H_2$), 58.5 (O**C**H_2C≡C), 55.9 (O**C**H_3), 55.5 ($N^+(CH_3)_2\mathbf{C}H_2$C≡C), 51.8 ($N^+(\mathbf{C}H_3)_2$), 40.4 (**C**H_2NH), 33.8 (isoxazolinyl-$OCH_2\mathbf{C}H_2$), 30.8 ($NHCH_2\mathbf{C}H_2$), 30.0 (**C**H_2), 29.9 (**C**H_2) 27.8 (**C**H_2) 27.0 (**C**H_2), 23.5 (**C**$H_2CH_2N^+(CH_3)_2$), 17.0 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{27}H_{39}ClN_5O_4S^+$: 564.1. Found: 564.2.

10-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyldecan-1-aminium bromide, ***LY20-A10-iper*** *(FG_B_57)*



$C_{29}H_{43}BrClN_5O_4S$

M_r = 673.11 g/mol

Carboxylic acid **1** (150.0 mg, 550 µmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (101 µl, 580 µmol) and PyBOP (286.3mg, 550 µmol) were successively added. Compound **1-L10** (292.9 mg, 550 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (101 µl, 580 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/1 → 10/1) to give **LY20-A10-iper** as yellowish crystals.

yield:	72 mg (20 %)
HPLC purity:	95 % (HPLC Method **I**, B: 70 %)
reaction control:	R_f = 0.16 (alox basic; $CHCl_3$/CH_3OH: 20/1; Dragendorff-reagent)
melting point [°C]:	72

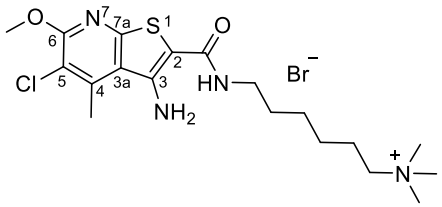
IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3419, 3326, 2926, 2855, 1626, 1594, 1523, 1360, 1279

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.42 (s, 2H, N**H$_2$**), 6.21 (t, ^3J = 5.7 Hz, 1H, N**H**), 4.85 (t, ^5J = 1.6 Hz, 2H, OC**H$_2$**C≡C), 4.35 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 4.11 (t, ^5J = 1.6 Hz, 2H, $N^+(CH_3)_2C$**H$_2$**C≡C), 4.01 (s, 3H, OC**H$_3$**), 3.32–3.25 (m, 4H, NHC**H$_2$**$(CH_2)_8$C**H$_2$**-$N^+(CH_3)_2$), 3.03 (s, 6H, $N^+(C$**H$_3$**$)_2$), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.79 (s, 3H, C**H$_3$**), 1.73-1.64 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_2$), 1.59-1.51 (m, 2H, $NHCH_2C$**H$_2$**), 1.37-1.28 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 168.4 (isoxazolinyl-**C$_q$**), 166.7 (**C**(O)NH), 160.4 (**C**-6), 155.8 (**C$_q$**), 148.9 (**C$_q$**), 145.4 (**C**-4), 122.6 (**C$_q$**), 117.2 (**C$_q$**), 99.3 (**C$_q$**), 88.1 (C≡**C**CH_2O), 76.1 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 65.7 ($N^+(CH_3)_2$**C**H_2), 58.5 (O**C**H_2C≡C), 55.9 (O**C**H_3), 55.6 ($N^+(CH_3)_2$**C**H_2C≡C), 51.8 (N^+(**C**$H_3)_2$), 40.4 (**C**H_2NH), 33.9 (isoxazolinyl-OCH_2**C**H_2), 30.9 ($NHCH_2$**C**H_2), 30.4 (**C**H_2), 30.3 (**C**H_2), 30.2 (**C**H_2), 29.9 (**C**H_2), 28.0 (**C**H_2), 27.0 (**C**H_2), 23.6 (**C**$H_2CH_2N^+(CH_3)_2$), 17.1 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{29}H_{43}ClN_5O_4S^+$: 592.2. Found: 592.3.

6-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethylhexan-1-aminium bromide, ***LY20-A6-TMA*** *(FG_B_110)*



$C_{19}H_{30}BrClN_4O_2S$

M_r = 493.89 g/mol

Carboxylic acid **1** (103.0 mg, 378 µmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (69 µl, 397 µmol) and PyBOP (196.6 mg, 378 µmol) were successively added. Compound **2-L6** (133.4 mg, 378 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (69 µl, 397 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/3 → 10/1) to give **LY20-A6-TMA** as yellowish crystals.

yield: 64 mg (34 %)

HPLC purity: 99 % (HPLC Method **I**, B: 65 %)

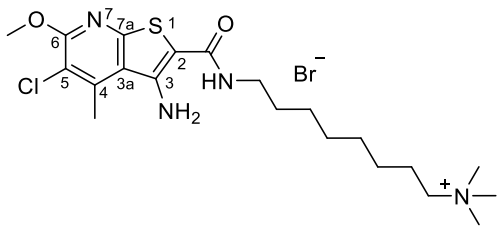
reaction control:	R_f = 0.30 (alox basic; $CHCl_3/CH_3OH$: 10/1; Dragendorff-reagent)
melting point [°C]:	111-118
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3317, 2935, 2861, 1595, 1521, 1469, 1360

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.41 (s, 2H, N**H$_2$**), 6.23 (t, ^3J = 5.2 Hz, 1H, N**H**), 4.01 (s, 3H, OC**H$_3$**), 3.34–3.27 (m, 2H, NHC**H$_2$**), 3.23-3.17 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.00 (s, 9H, $N(CH_3)_3$), 2.78 (s, 3H, C**H$_3$**), 1.77-1.67 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.62-1.52 (m, 2H, $NHCH_2$C**H$_2$**), 1.45-1.32 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.8 (**C**(O)NH), 160.4 (**C**-6), 155.8 (**C$_q$**), 149.0 (**C$_q$**), 145.4 (**C**-4), 122.5 (**C$_q$**), 117.2 (**C$_q$**), 99.2 (**C$_q$**), 68.0 (**C**$H_2N^+(CH_3)_3$), 55.9 (O**C**H_3), 54.2 (N^+(**C**H_3)$_3$), 40.2 (**C**H_2NH), 30.6 ($NHCH_2$**C**H_2), 27.3 (**C**H_2), 26.8 (**C**H_2), 23.8 (**C**$H_2CH_2N^+(CH_3)_3$), 17.1 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{19}H_{30}ClN_4O_2S^+$: 413.2. Found: 413.1.

8-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyloctan-1-aminium bromide, ***LY20-A8-TMA*** *(FG_B_115)*



$C_{21}H_{34}BrClN_4O_2S$

M_r = 521.94 g/mol

Carboxylic acid **1** (103.2 mg, 378 µmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (69 µl, 397 µmol) and PyBOP (196.9 mg, 378 µmol) were successively added. Compound **2-L8** (144.3 mg, 378 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (69 µl, 397 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3/CH_3OH$ 100/3 → 10/1) to give **LY20-A8-TMA** as white crystals.

yield:	29 mg (15 %)
HPLC purity:	99 % (HPLC Method **I**, B: 65 %)

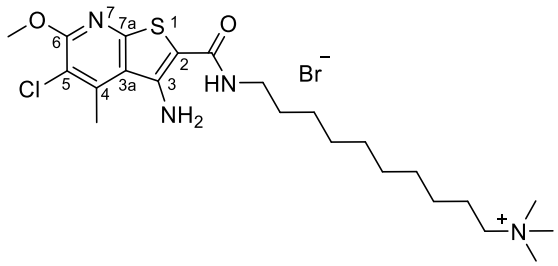
reaction control:	R_f = 0.32 (alox basic; $CHCl_3/CH_3OH$: 20/1; Dragendorff-reagent)
melting point [°C]:	187
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3311, 2928, 2851, 1600, 1513, 1469, 1358

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.39 (s, 2H, N**H$_2$**), 6.19 (t, ^3J = 5.3 Hz, 1H, N**H**), 4.00 (s, 3H, OC**H$_3$**), 3.29 (dd, ^3J = 6.6 Hz, ^3J = 13.4 Hz, 2H, NHC**H$_2$**), 3.21-3.15 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.99 (s, 9H, $N^+(CH_3)_3$), 2.76 (s, 3H, C**H$_3$**), 1.75-1.65 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.58-1.52 (m, 2H, $NHCH_2$C**H$_2$**), 1.37-1.25 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.7 (**C**(O)NH), 160.3 (**C**-6), 155.7 (**C$_q$**), 148.9 (**C$_q$**), 145.3 (**C**-4), 122.5 (**C$_q$**), 117.2 (**C$_q$**), 99.3 (**C$_q$**), 68.0 (**C**$H_2N^+(CH_3)_3$), 55.8 (O**C**H_3), 54.2 (N^+(**C**H_3)$_3$), 40.4 (**C**H_2NH), 30.8 ($NHCH_2$**C**H_2), 30.0 (**C**H_2), 29.9 (**C**H_2), 27.6 (**C**H_2), 27.0 (**C**H_2), 23.8 (**C**$H_2CH_2N^+(CH_3)_3$), 17.0 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{21}H_{34}ClN_4O_2S^+$: 441.2. Found: 441.2.

10-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyldecan-1-aminium bromide, ***LY20-A10-TMA*** *(FG_B_120)*



$C_{23}H_{38}BrClN_4O_2S$

M_r = 555.00 g/mol

Carboxylic acid **1** (72.0 mg, 264 µmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (48 µl, 277 µmol) and PyBOP (137.4 mg, 264 µmol) were successively added. Compound **2-L10** (108.0 mg, 264 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (48 µl, 277 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3/CH_3OH$ 100/3 → 10/1) to give **LY20-A10-TMA** as white crystals.

yield:	42 mg (15 %)
HPLC purity:	99 % (HPLC Method **I**, B: 70 %)

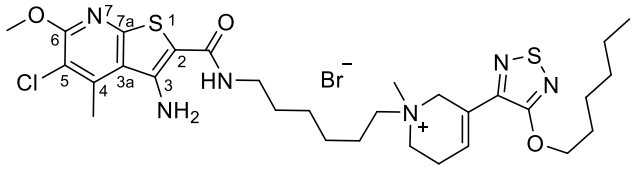
reaction control:	R_f = 0.32 (alox basic; $CHCl_3/CH_3OH$: 10/1; Dragendorff-reagent)
melting point [°C]:	166
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3314, 2925, 2852, 1591, 1530, 1469, 1360

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.37 (s, 2H, N**H$_2$**), 6.26 (t, ^3J = 5.6 Hz, 1H, N**H**), 3.98 (s, 3H, OC**H$_3$**), 3.27 (q, ^3J = 7.7 Hz, 2H, NHC**H$_2$**), 3.21-3.16 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.00 (s, 9H, $N^+(CH_3)_3$), 2.73 (s, 3H, C**H$_3$**), 1.74-1.65 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.58-1.50 (m, 2H, $NHCH_2$C**H$_2$**), 1.40-1.22 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.7 (**C**(O)NH), 160.2 (**C**-6), 155.6 (**C$_q$**), 148.7 (**C$_q$**), 145.2 (**C**-4), 122.4 (**C$_q$**), 117.1 (**C$_q$**), 99.4 (**C$_q$**), 68.0 (**C**$H_2N^+(CH_3)_3$), 55.8 (O**C**H_3), 54.2 (N^+(**C**H_3)$_3$), 40.5 (**C**H_2NH), 30.9 ($NHCH_2$**C**H_2), 30.5 (**C**H_2), 30.4 (**C**H_2), 30.3 (**C**H_2), 30.0 (**C**H_2), 28.0 (**C**H_2), 27.1 (**C**H_2), 23.8 (**C**$H_2CH_2N^+(CH_3)_3$), 17.0 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{23}H_{38}ClN_4O_2S^+$: 469.2. Found: 469.2.

1-(6-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)hexyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium bromide, ***LY20-A6-XanA*** *(FG_XA_19)*



$C_{30}H_{44}BrClN_6O_3S_2$

M_r = 716.20 g/mol

Carboxylic acid **1** (25.0 mg, 92 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (18 µl, 97 µmol) and PyBOP (47.7 mg, 92 µmol) were successively added. Compound **3-L6** (52.8 mg, 92 µmol) was dissolved in dry DMF (2 ml), treated with DIPEA (18 µl, 97 µmol) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/MeOH 100/1 → 10/1) to give **LY20-A6-XanA** as colorless oil.

yield:	22 mg (34 %)
HPLC purity:	98 % (HPLC Method **I**, B: 85 %)

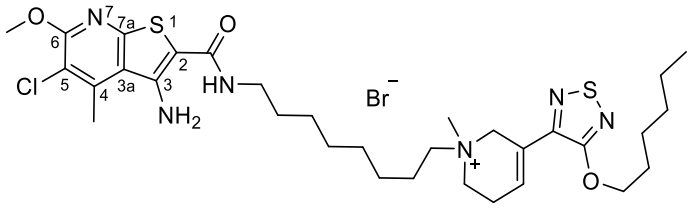
reaction control:	R_f = 0.31 (alox basic; $CHCl_3$/MeOH: 30/1, Dragendorff reagent)
melting point [°C]:	107
IR (ATR, [cm^{-1}]):	3504, 2924, 2856, 1593, 1512, 1451, 1359, 1075

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.19-7.15 (m, 1H, tetrahydropyridinyl-C**H**), 6.21 (s, 2H, N**H$_2$**), 5.69 (t, ^3J = 5.8 Hz, 1H, N**H**), 4.41 (t, ^3J = 6.8 Hz, 2H, OC**H$_2$**), 4.31-4.18 (m, 2H, tetrahydropyridinyl-N^+C**H$_2$**C(C)=C), 4.00 (s, 3H, OC**H$_3$**), 3.68-3.50 (m, 2H, tetrahydropyridinyl-N^+C**H$_2$**CH_2), 3.45-3.37 (m, 2H, $N^+(CH_3)$C**H$_2$**), 3.37-3.30 (m, 2H, NHC**H$_2$**), 3.14 (s, 3H, NC**H$_3$**), 2.80-2.71 (m, 5H, tetrahydropyridinyl-NCH_2C**H$_2$**, C**H$_3$**), 1.90-1.77 (m, 4H, $N^+(CH_3)CH_2$C**H$_2$**, OCH_2C**H$_2$**), 1.66-1.53 (m, 4H, C**H$_2$**), 1.47-1.23 (m, 12H, C**H$_2$**), 0.90-0.85 (m, 3H, CH_2C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.9 (**C**(O)NH), 162.3 (thiadiazolyl-N**C**O), 159.2 (**C**-6), 154.6 (**C$_q$**), 147.4 (**C$_q$**), 143.6 (thiadiazolyl-**C$_q$**), 143.2 (**C**-4), 126.1 (tetrahydropyridinyl-**C**H), 122.9 (tetrahydropyridinyl-**C$_q$**), 121.2 (**C$_q$**), 116.5 (**C$_q$**), 98.6 (**C$_q$**), 71.9 (O**C**H_2), 64.5 ($N^+(CH_3)$**C**H_2), 59.4 (tetrahydropyridinyl-N^+**C**H_2C(C)=C), 56.9 (tetrahydropyridinyl-N^+**C**H_2CH_2), 55.2 (O**C**H_3), 48.1 (N^+**C**H_3), 39.5 (NH**C**H_2), 31.6 (**C**H_2), 29.9 (**C**H_2), 29.4 (**C**H_2), 28.9 (**C**H_2), 26.1 (**C**H_2), 25.8 (**C**H_2), 25.7 (**C**H_2), 22.7 (**C**H_2), 21.8 (tetrahydropyridinyl-N^+CH_2**C**H_2), 21.8 (**C**H_2), 22.1 (**C**H_2), 16.3 (**C**H_3), 14.2 (CH_2**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{30}H_{44}ClN_6O_3S_2^+$: 635.3. Found: 635.2.

1-(8-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)octyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium bromide, ***LY20-A8-XanA*** *(FG_XA_23)*



$C_{32}H_{48}BrClN_6O_3S_2$

M_r = 744.25 g/mol

Carboxylic acid **1** (35.0 mg, 128 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (24 µl, 135 µmol) and PyBOP (66.8 mg, 128 µmol) were successively added. Compound **3-L8** (77.5 mg, 128 µmol) was dissolved in dry DMF (2 ml), treated with DIPEA (24 µl, 135 µmol) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column

chromatography (alox basic, $CHCl_3/CH_3OH$ 100/1 → 10/1) to give **LY20-A8-XanA** as colorless oil.

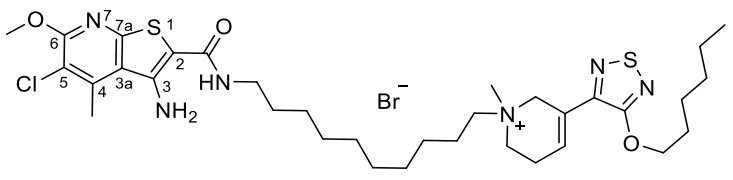
yield:	44 mg (46 %)
HPLC purity:	93 % (HPLC Method **I**, B: 85 %)
reaction control:	R_f = 0.36 (alox basic; $CHCl_3/CH_3OH$: 30/1, Dragendorff reagent)
melting point [°C]:	117-118
IR (ATR, [cm^{-1}]):	3420, 2928, 2857, 1594, 1513, 1450, 1361, 1281, 1075

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.21-7.18 (m, 1H, tetrahydropyridinyl-C**H**), 6.23 (s, 2H, N**H$_2$**), 5.52 (t, ^3J = 5.7 Hz, 1H, N**H**), 4.43 (t, ^3J = 6.8 Hz, 2H, OC**H$_2$**), 4.32-4.19 (t, 2H, tetrahydropyridinyl-N^+C**H$_2$**C(C)=C), 4.02 (s, 3H, OC**H$_3$**), 3.71-3.50 (m, 2H, tetrahydropyridinyl-N^+C**H$_2$**CH_2), 3.44-3.31 (m, 4H, $N^+(CH_3)$C**H$_2$**, NHC**H$_2$**), 3.15 (s, 3H, N^+C**H$_3$**), 2.87-2.66 (m, 5H, tetrahydropyridinyl-N^+CH_2C**H$_2$**, C**H$_3$**), 1.87-1.77 (m, 4H, $N^+(CH_3)CH_2$C**H$_2$**, OCH_2C**H$_2$**), 1.66-1.50 (m, 4H, C**H$_2$**), 1.46-1.22 (m, 16H, C**H$_2$**), 0.88 (t, ^3J = 6.9 Hz, 3H, CH_2C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.9 (**C**(O)NH), 162.8 (thiadiazolyl-N**C**O), 159.2 (**C**-6), 154.5 (**C$_q$**), 147.3 (**C$_q$**), 143.6 (thiadiazolyl-**C$_q$**), 143.2 (**C**-4), 126.1 (tetrahydropyridinyl-**C**H), 122.9 (tetrahydropyridinyl-**C$_q$**), 121.3 (**C$_q$**), 116.6 (**C$_q$**), 98.6 (**C$_q$**), 71.9 (O**C**H_2), 64.8 ($N^+(CH_3)$**C**H_2), 59.3 (tetrahydropyridinyl-N^+**C**H_2C(C)=C), 57.0 (tetrahydropyridinyl-N^+**C**H_2CH_2), 55.2 (O**C**H_3), 48.2 (N^+**C**H_3), 39.7 (NH**C**H_2), 31.6 (**C**H_2), 29.8 (**C**H_2), 28.9 (**C**H_2), 28.8 (**C**H_2), 28.8 (**C**H_2), 26.7 (**C**H_2), 26.1 (**C**H_2), 25.8 (**C**H_2), 22.7 (**C**H_2), 22.2 (**C**H_2), 21.8 (tetrahydropyridinyl-N^+CH_2**C**H_2), 16.4 (**C**H_3), 14.2 (CH_2**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{32}H_{48}ClN_6O_3S_2^+$: 663.2. Found: 663.2.

1-(10-(3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamido)decyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium, ***LY20-A10-XanA*** *(FG_XA_24)*



$C_{34}H_{52}BrClN_6O_3S_2$

M_r = 772.30 g/mol

Carboxylic acid **1** (30.0 mg, 110 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (20 µl, 116 µmol) and PyBOP (57.3 mg, 121 µmol) were successively added. Compound **3-L10** (69.5 mg, 110 µmol) was dissolved in dry DMF (2 ml), treated with DIPEA (20 µl, 116 µmol) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3/CH_3OH$ 100/1 → 10/1) to give **LY20-A10-XanA** as yellowish solid.

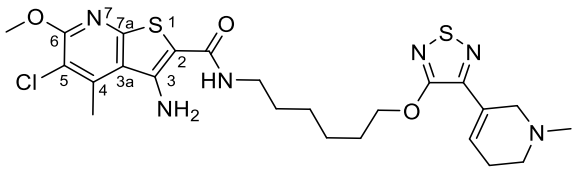
yield:	49 mg (58 %)
HPLC purity:	97 % (HPLC Method **I**, B: 85 %)
reaction control:	R_f = 0.39 (alox basic; $CHCl_3/CH_3OH$: 30/1, Dragendorff reagent)
melting point [°C]:	111-114
IR (ATR, [cm^{-1}]):	3420, 2921, 2851, 1594, 1509, 1451, 1360, 1075

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.22-7.19 (m, 1H, tetrahydropyridinyl-C**H**), 6.24 (s, 2H, N**H$_2$**), 5.45 (t, ^3J = 5.6 Hz, 1H, N**H**), 4.43 (t, ^3J = 6.8 Hz, 2H, OC**H$_2$**), 4.33-4.19 (m, 2H, tetrahydropyridinyl-N^+C**H$_2$**C(C)=C), 4.02 (s, 3H, OC**H$_3$**), 3.70-3.51 (m, 2H, tetrahydropyridinyl-N^+C**H$_2$**CH_2), 3.43-3.32 (m, 4H, $N^+(CH_3)$C**H$_2$**, NHC**H$_2$**), 3.14 (s, 3H, N^+C**H$_3$**), 2.87-2.67 (m, 5H, tetrahydropyridinyl-N^+CH_2C**H$_2$**, C**H$_3$**), 1.87-1.76 (m, 4H, $N^+(CH_3)CH_2$C**H$_2$**, OCH_2C**H$_2$**), 1.63-1.52 (m, 4H, C**H$_2$**), 1.45-1.20 (m, 20H, C**H$_2$**), 0.88 (t, ^3J = 7.0 Hz, 3H, CH_2C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.8 (**C**ONH), 162.8 (thiadiazolyl-N**C**O), 159.2 (**C**-6), 154.5 (**C$_q$**), 147.3 (**C$_q$**), 143.6 (thiadiazolyl-**C$_q$**), 143.2 (**C**-4), 126.2 (tetrahydropyridinyl-**C**H), 122.9 (tetrahydropyridinyl-**C$_q$**), 121.3 (**C$_q$**), 116.6 (**C$_q$**), 98.5 (**C$_q$**), 71.9 (O**C**H_2), 64.7 ($N^+(CH_3)$**C**H_2), 59.2 (tetrahydropyridinyl-N^+**C**H_2C(C)=C), 57.1 (tetrahydropyridinyl-N^+**C**H_2CH_2), 55.2 (O**C**H_3), 48.2 (N^+**C**H_3), 39.9 (NH**C**H_2), 31.6 (**C**H_2), 30.0 (**C**H_2), 29.3 (**C**H_2), 29.2 (**C**H_2), 29.1 (**C**H_2), 28.9 (**C**H_2), 27.0 (**C**H_2), 26.7 (**C**H_2), 26.2 (**C**H_2), 25.8 (**C**H_2), 22.7 (**C**H_2), 22.4 (**C**H_2), 21.8 (tetrahydropyridinyl-N^+CH_2**C**H_2), 16.4 (**C**H_3), 14.2 (CH_2**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{34}H_{52}ClN_6O_3S_2^+$: 691.3. Found: 691.2

3-Amino-5-chloro-6-methoxy-4-methyl-N-(6-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)hexyl)thieno[2,3-b]pyridine-2-carboxamide, ***LY20-A6-XanB*** *(FG_XA_8)*



$C_{24}H_{31}ClN_6O_3S_2$

M_r = 551.12 g/mol

Carboxylic acid **1** (92.0 mg, 337 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (63 µl, 371 µmol) and PyBOP (175.6 mg, 337 µmol) were successively added and the mixture was stirred for 15 min at rt. Compound **4-L6** (138.5 mg, 337 µmol) was dissolved in dry DMF (3 ml) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 400/1) to give **LY20-A6-XanB** as yellowish crystals.

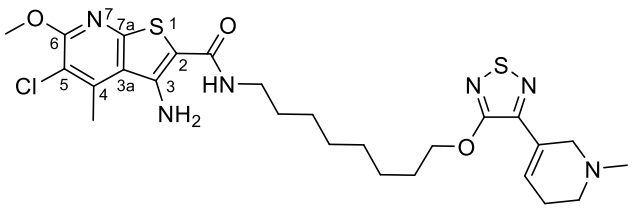
yield:	41 mg (22 %)
HPLC purity:	96 % (HPLC Method **IIb**)
reaction control:	R_f = 0.12 (alox basic; $CHCl_3$/CH_3OH: 400/1)
melting point [°C]:	117
IR (ATR, [cm^{-1}]):	3305, 2927, 2853, 2790, 1591, 1508, 1447, 1358

^{1}H NMR (400 MHz, $CDCl_3$, [ppm]): 7.05–7.01 (m, 1H, tetrahydropyridinyl-C**H**), 6.26 (s, 2H, N**H$_2$**), 5.40 (t, ^3J = 5.5 Hz, 1H, N**H**), 4.43 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 4.04 (s, 3H, OC**H$_3$**), 3.46 (d, ^4J = 1.4 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 3.39 (dd, ^3J = 13.3 Hz, ^3J = 6.8 Hz, 2H, NHC**H$_2$**), 2.80 (s, 3H, C**H$_3$**), 2.59 (t, ^3J = 5.7 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.48–2.42 (m, 5H, tetrahydropyridinyl-N(C**H$_3$**)CH_2C**H$_2$**), 1.88-1.80 (m, 2H, OCH_2C**H$_2$**), 1.62–1.57 (m, 2H, $NHCH_2$C**H$_2$**), 1.54–1.41 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, [ppm]): 165.9 (**C**(O)NH), 162.7 (thiadiazolyl-N**C**O), 159.4 (**C**-6), 154.5 (**C$_q$**), 147.5 (**C$_q$**), 146.8 (thiadiazolyl-**C$_q$**), 143.1 (**C**-4), 129.1 (tetrahydropyridinyl-**C$_q$**), 128.5 (tetrahydropyridinyl-**C**H), 121.3 (**C$_q$**), 116.7 (**C$_q$**), 98.3 (**C$_q$**), 71.0 (O**C**H_2), 55.2 (O**C**H_3), 55.0 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.4 (tetrahydropyridinyl-N**C**H_2CH_2), 45.9 (N**C**H_3), 39.8 (NH**C**H_2), 30.0 ($NHCH_2$**C**H_2), 29.0 (**C**H_2), 26.8 (**C**H_2), 26.5 (tetrahydropyridinyl-NCH_2**C**H_2), 25.9 (**C**H_2), 16.4 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{24}H_{32}ClN_6O_3S_2^+$: 551.2. Found: 551.1.

3-Amino-5-chloro-6-methoxy-4-methyl-N-(8-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)octyl)thieno[2,3-b]pyridine-2-carboxamide, ***LY20-A8-XanB*** *(FG_XA_1)*



$C_{26}H_{35}ClN_6O_3S_2$

M_r = 579.18 g/mol

Carboxylic acid **1** (68.0 mg, 249 µmol) was dissolved in dry DMF (3 ml) under argon atmosphere. DIPEA (47 µl, 274 µmol) and PyBOP (129.8 mg, 249 µmol) were successively added and the mixture was stirred for 15 min at rt. Compound **4-L8** (109.3 mg, 249 µmol) was dissolved in dry DMF (3 ml) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 400/1) to give **LY20-A8-XanB** as yellowish crystals.

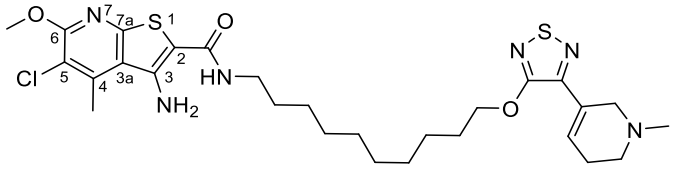
yield:	30 mg (22 %)
HPLC purity:	95 % (HPLC Method **IIa**)
reaction control:	R_f = 0.15 (alox basic; $CHCl_3$/CH_3OH: 400/1)
melting point [°C]:	111
IR (ATR, [cm^{-1}]):	3453, 3418, 3287, 2925, 2855, 1640, 1593, 1375

^{1}H NMR (400 MHz, $CDCl_3$, [ppm]): 7.05–7.01 (m, 1H, tetrahydropyridinyl-C**H**), 6.26 (s, 2H, N**H$_2$**), 5.39 (t, ^3J = 5.4 Hz, 1H, N**H**), 4.41 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 4.04 (s, 3H, OC**H$_3$**), 3.45 (d, ^4J = 1.8 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 3.40–3.30 (m, 2H, NHC**H$_2$**), 2.79 (s, 3H, C**H$_3$**), 2.58 (t, ^3J = 5.7 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.46–2.42 (m, 5H, tetrahydropyridinyl-N(C**H$_3$**)CH_2C**H$_2$**), 1.81 (m, 2H, OCH_2C**H$_2$**), 1.62–1.53 (m, 2H, $NHCH_2$C**H$_2$**), 1.46–1.32 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, [ppm]): 165.8 (**C**(O)NH), 162.8 (thiadiazolyl-N**C**O), 159.3 (**C**-6), 154.5 (**C$_q$**), 147.4 (**C$_q$**), 146.9 (thiadiazolyl-**C$_q$**), 143.1 (**C**-4), 129.2 (tetrahydropyridinyl-**C$_q$**), 128.4 (tetrahydropyridinyl-**C**H), 121.3 (**C$_q$**), 116.7 (**C$_q$**), 98.4 (**C$_q$**), 71.1 (O**C**H_2), 55.2 (O**C**H_3), 55.0 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.4 (tetrahydropyridinyl-N**C**H_2CH_2), 45.9 (N**C**H_3), 39.8 (NH**C**H_2), 30.0 ($NHCH_2$**C**H_2), 29.4 (**C**H_2), 29.3 (**C**H_2), 29.0 (**C**H_2), 27.0 (**C**H_2), 26.6 (tetrahydropyridinyl-NCH_2**C**H_2), 26.1 (**C**H_2), 16.4 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{26}H_{36}ClN_6O_3S_2^+$: 579.2. Found: 579.1.

3-Amino-5-chloro-6-methoxy-4-methyl-N-(10-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)decyl)thieno[2,3-b]pyridine-2-carboxamide, ***LY20-A10-XanB*** *(FG_XA_9)*



$C_{28}H_{39}ClN_6O_3S_2$

M_r = 607.23 g/mol

Carboxylic acid **1** (98.0 mg, 359 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (67 µl, 395 µmol) and PyBOP (187.0 mg, 359 µmol) were successively added and the mixture was stirred for 15 min at rt. Compound **4-L10** (167.7 mg, 359 µmol) was dissolved in dry DMF (2 ml) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 400/1) to give **LY20-A10-XanB** as light brown solid.

yield:	119 mg (55 %)
HPLC purity:	99 % (HPLC Method **IIa**)
reaction control:	R_f = 0.15 (alox basic; $CHCl_3$/CH_3OH: 400/1)
melting point [°C]:	119
IR (ATR, [cm^{-1}]):	3457, 3419, 3285, 2924, 2854, 1640, 1593, 1376

^{1}H NMR (400 MHz, $CDCl_3$, [ppm]): 7.07–7.03 (m, 1H, tetrahydropyridinyl-C**H**), 6.27 (s, 1H, N**H$_2$**), 5.38 (t, ^3J = 5.4 Hz, 1H, N**H**), 4.41 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 4.04 (s, 3H, OC**H$_3$**), 3.50 (s, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 3.37 (dd, ^3J = 13.2 Hz, ^3J = 6.9 Hz, 2H, NHC**H$_2$**), 2.81 (s, 3H, C**H$_3$**), 2.63 (t, ^3J = 5.7 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.50–2.43 (m, 5H, tetrahydropyridinyl-N(C**H$_3$**)CH_2C**H$_2$**), 1.85-1.76 (m, 2H, OCH_2C**H$_2$**), 1.62–1.53 (m, 2H, $NHCH_2$C**H$_2$**), 1.46–1.25 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, [ppm]): 165.8 (**C**(O)NH), 162.8 (thiadiazolyl-N**C**O), 159.3 (**C**-6), 154.5 (**C$_q$**), 147.4 (**C$_q$**), 146.7 (thiadiazolyl-**C$_q$**), 143.1 (**C**-4), 128.9 (tetrahydropyridinyl-**C$_q$**), 128.4 (tetrahydropyridinyl-**C**H), 121.4 (**C$_q$**), 116.7 (**C$_q$**), 98.4 (**C$_q$**), 71.2 (O**C**H_2), 55.2 (O**C**H_3), 54.9 (tetrahydropyridinyl-N**C**H_2C(C)=C), 51.3 (tetrahydropyridinyl-N**C**H_2CH_2), 45.8

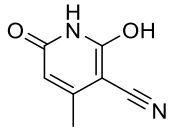
(N**C**H$_3$), 39.9 (NH**C**H$_2$), 30.1 (NHCH$_2$**C**H$_2$), 29.6 (**C**H$_2$), 29.6 (**C**H$_2$), 29.5 (**C**H$_2$), 29.4 (**C**H$_2$), 29.1 (**C**H$_2$), 27.1 (**C**H$_2$), 26.4 (tetrahydropyridinyl-NCH$_2$**C**H$_2$), 26.2 (**C**H$_2$), 16.4 (**C**H$_3$).

MS (ESI) m/z [M$^+$] Calcd for $C_{28}H_{40}ClN_6O_3S_2^+$: 607.2. Found: 607.1

7.3 Synthesis of LY21-An hybrids

7.3.1 Preparation of thienopyridine carboxylic acid scaffold 27

2-Hydroxy-4-methyl-6-oxo-1,6-dihydropyridine-3-carbonitrile, ***28*** *(FG_B_3)*



$C_7H_6N_2O_2$

M_r = 150.14 g/mol

According to Renck *et al.*[117], ethyl 3-oxobutanoate (19.7 ml, 154.6 mmol) and cyanoacetamide (13.0 g, 154.6 mmol) were dissolved in methanol (125 ml), followed by the addition of a solution of potassium hydroxide (9.1 g, 162.4 mmol) in methanol (75 ml). The reaction mixture was heated at 65 °C for 16 h. Thereby, a white solid precipitated. After cooling to rt, the amorphous solid was filtered, air dried, and dissolved in warm water (100 ml). The solution was acidified with 6 M hydrochloric acid, whereupon **28** precipitated as a white solid. The product was filtered, washed with cold water (30 ml) and cold methanol (30 ml), and dried *in vacuo*.

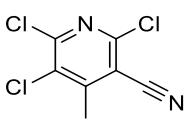
yield:	17.0 g (73 %, Lit.:[117] 52 %)
melting point [°C]:	316-317 (Lit.:[117] 302-306)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3031, 2887, 2224, 1603, 1503, 1307, 1174

^{1}H NMR (400 MHz, DMSO-d$_6$, δ [ppm]): 5.59 (s, 1H, C**H**), 2.23 (s, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, DMSO-d$_6$, δ [ppm]): 162.2 (**C$_q$**), 161.5 (**C$_q$**), 160.0 (**C$_q$**), 117.5 (**C$_q$**), 93.2 (**C**H), 88.2 (**C$_q$**), 20.7 (**C**H$_3$).

The obtained spectroscopic data are consistent with that found in literature.[117]

2,5,6-Trichloro-4-methylnicotinonitrile, ***29*** *(FG_B_5)*



$C_7H_3Cl_3N_2$

M_r = 221.47 g/mol

Following a modified procedure from Lounasmaa *et al.*[118], **28** (10.0 g, 66.6 mmol) and phosphorus pentachloride (41.6 g, 199.8 mmol) were grinded and placed in a reaction vessel. The mixture was heated at 160 °C for 20 h and the formed $POCl_3$ was passed through a water-filled gas washing bottle. After cooling to rt, the dark brown solution was transferred carefully into ice cooled water (100 ml) and the mixture was extracted with DCM (6x100 ml). After phase separation, the combined organics were washed with ice water. Phases were again separated and the organic layer was dried over sodium sulfate, followed by solvent removal *in vacuo*. The residue was purified by column chromatography (silica, toluene/cyclohexane 11/9 → 2/1) to give a yellow oil. The addition of ice cooled methanol (5 ml) led to precipitation of a white solid which was filtered, washed with ice cooled methanol (3x5 ml), and air-dried to give **29**.

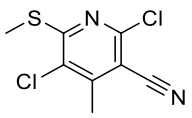
yield:	3.0 g (20 %, Lit.:[118] 61 %)
reaction control:	R_f = 0.54 (silica; toluene/Cy: 2/1)
melting point [°C]:	124-127 (Lit.:[118] 120-122)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2928, 2870, 2230, 1526, 1335, 1224, 1163

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 2.63 (s, 3H, C**H**$_3$).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 155.4 (**C$_q$**), 150.5 (**C$_q$**), 148.1 (**C$_q$**), 129.8 (**C$_q$**), 113.5 (**C$_q$**), 111.4 (**C$_q$**), 20.6 (**C**H_3).

The obtained physical and spectroscopic data are consistent with that found in literature. Lounasmaa *et al.* do not provide ^{13}C NMR or IR-data.[118]

*2,5-Dichloro-4-methyl-6-(methylthio)nicotinonitrile, **30** (FG_B_6)*



$C_8H_6Cl_2N_2S$

M_r = 233.11 g/mol

According to Rubio *et al.*[67], **29** (3.4 g, 15.2 mmol) was suspended in dry methanol (50 ml) under argon atmosphere and cooled to 0 °C in an ice bath. After addition of sodium methanethiolate (1.1 g, 15.2 mmol), the mixture was stirred for 1 h at 0 °C before the ice bath was removed and stirring continued at rt for 1 h. The suspension was quenched with water (30 ml) and extracted with EtOAc (2x20 ml). After phase separation, the combined organic

layers were dried over magnesium sulfate and the solvent was removed to give product **30** as yellowish solid.

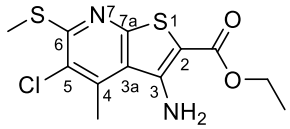
yield:	3.1 g (88 %, Lit.:[67] 99 %)
reaction control:	R_f = 0.58 (silica; EtOAc/Cy: 3/100)
melting point [°C]:	88-92
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3009, 2926, 2230, 1549, 1328

^{1}H NMR (400 MHz, DMSO-d$_6$, δ [ppm]): 2.55 (s, 3H, SC**H$_3$**), 2.51 (s, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, DMSO-d$_6$, δ [ppm]): 163.2 (**C$_q$**), 150.2 (**C$_q$**), 149.0 (**C$_q$**), 126.9 (**C$_q$**), 114.3 (**C$_q$**), 106.3 (**C$_q$**), 19.1 (**C**H$_3$), 14.0 (S**C**H$_3$).

Rubio *et al.* did not provide a melting point.

Ethyl 3-amino-5-chloro-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxylate, ***31*** *(FG_B_56)*



$C_{12}H_{13}ClN_2O_2S_2$

M_r = 316.82 g/mol

Compound **30** (1.6 g, 6.9 mmol) was suspended in dry ethanol (50 ml) under argon atmosphere and treated with sodium carbonate (1.5 g, 13.8 mmol), followed by the addition of ethyl 2-mercaptoacetate (954 µl, 8.7 mmol). The reaction mixture was heated under reflux for 12 h. After cooling to rt, the reaction mixture was treated with water (25 ml) and stirred at 0 °C for 30 min. The formed yellowish precipitate was filtered, dried *in vacuo*, and purified by column chromatography (silica, $CHCl_3$) to give compound **31** as yellow solid.

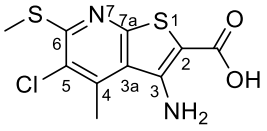
yield:	1.5 g (69 %)
reaction control:	R_f = 0.57 (silica gel; $CHCl_3$)
melting point [°C]:	210-214
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3479, 3358, 2981, 2925, 1660, 1603, 1534, 1263

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.10 (s, 2H, N**H$_2$**), 4.32 (q, ^3J = 7.1 Hz, 2H, C**H$_2$**CH$_3$), 2.79 (s, 3H, C**H$_3$**), 2.57 (s, 3H, SC**H$_3$**), 1.36 (t, ^3J = 7.1 Hz, 3H, CH$_2$C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.8 (**C**(O)O), 160.2 (**C**-6), 159.1 (**C_q**), 148.9 (**C_q**), 140.0 (**C**-4), 125.9 (**C_q**), 121.7 (**C_q**), 98.0 (**C**-3), 60.8 (**C**$H_2$$CH_3$), 16.4. (**C**$H_3$), 14.7 ($CH_2$**C**$H_3$), 14.6 (S**C**$H_3$).

MS (ESI) m/z [M^+] Calcd for $C_{12}H_{14}ClN_2O_2S_2^+$: 317.0. Found: 316.9

3-Amino-5-chloro-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxylic acid, ***27*** *(FG_B_67)*



$C_{10}H_9ClN_2O_2S_2$

M_r = 288.76 g/mol

Compound **31** (234.6 mg, 740.5 µmol) was suspended in a 1/1 mixture of ethanol and 2 M aqueous sodium hydroxide solution (50 ml) and heated under reflux for 2 h. After cooling to rt, an excess of 6 M hydrochloric acid was added to cause precipitation of compound **27** as beige solid. For complete precipitation, the mixture was cooled in an ice bath. The precipitate was filtered, washed with cold water, and dried *in vacuo***.**

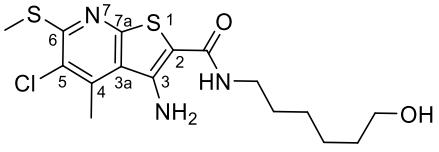
yield:	175 mg (82 %)
reaction control:	R_f = 0.08 (silica gel; EtOAc/Cy: 2/3)
melting point [°C]:	147-149
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3504, 3443, 3331, 2925, 2834, 1653, 1530, 1266

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 12.66 (br s, 1H, C(O)O**H**), 6.77 (s, 2H, N**H_2**), 2.79 (s, 3H, C**H_3**), 2.53 (s, 3H, SC**H_3**).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 166.5 (**C**(O)O), 159.0 (**C**-6), 158.1 (**C_q**), 149.5 (**C_q**), 141.9 (**C**-4), 124.8 (**C_q**), 122.1 (**C_q**), 96.4 (**C_q**), 16.4. (**C**H_3), 14.0 (S**C**H_3).

7.3.2 Preparation of LY21-An-iper hybrids

3-Amino-5-chloro-N-(6-hydroxyhexyl)-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxamide, ***32a*** *(FG_B_76)*



$C_{16}H_{22}ClN_3O_2S_2$

M_r = 387.94 g/mol

Carboxylic acid **27** (300.0 mg, 1.04 mmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (380 µl, 2.18 mmol) and PyBOP (567.7 mg, 1.09 mmol) were successively added and the mixture was stirred for 15 min at rt. After the addition of 6-amino-1-hexanol (121.8 mg, 1.04 mmol), the mixture was stirred at rt for 3 h and worked up as described in **G.10**. Purification was achieved by column chromatography (silica; EtOAc/Cy 1/2 to 2/1) to yield **32a** as yellowish solid.

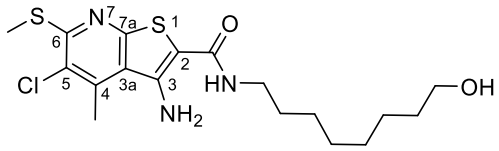
yield:	357 mg (89 %)
reaction control:	R_f = 0.25 (silica; EtOAc/Cy 2/1)
melting point [°C]:	133-135
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3363, 3308, 2939, 2855, 1594, 1519, 1264

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 3.46 (t, ^3J = 6.6 Hz, 2H, C**H$_2$**OH), 3.37–3.32 (m, 2H, NHC**H$_2$**), 2.86 (s, 3H, C**H$_3$**), 2.58 (s, 3H, SC**H$_3$**), 1.67–1.56 (m, 4H, C**H$_2$**CH$_2$NH, C**H$_2$**CH$_2$OH), 1.47-1.40 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 167.8 (**C**(O)NH), 160.5 (**C**-6), 158.7 (**C$_q$**), 149.0 (**C$_q$**), 142.4 (**C**-4), 126.8 (**C$_q$**), 124.2 (**C$_q$**), 101.1 (**C$_q$**), 63.1 (**C**H$_2$OH), 40.7 (**C**H$_2$NH), 33.7 (**C**H$_2$CH$_2$-OH), 31.0 (**C**H$_2$CH$_2$NH), 28.0 (**C**H$_2$), 26.8 (**C**H$_2$), 16.5 (**C**H$_3$), 14.4 (S**C**H$_3$).

MS (ESI) m/z [M^+] Calcd for $C_{16}H_{23}ClN_3O_2S_2^+$: 388.1. Found: 388.0.

3-Amino-5-chloro-N-(8-hydroxyoctyl)-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxamide, ***32b*** *(FG_B_94)*



$C_{18}H_{26}ClN_3O_2S_2$

M_r = 416.00 g/mol

Carboxylic acid **27** (350.0 mg, 1.21 mmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (443 µl, 2.55 mmol) and PyBOP (662.3 mg, 1.27 mmol) were successively added and the mixture was stirred for 15 min at rt. After the addition of 8-amino-1-octanol (176.1 mg, 1.21 mmol), the mixture was stirred at rt for 3 h and worked up as described in **G.10**. Purification was achieved by column chromatography (silica; EtOAc/Cy 1/2 to 2/1) to yield **32b** as yellowish solid.

yield:	352 mg (70 %)

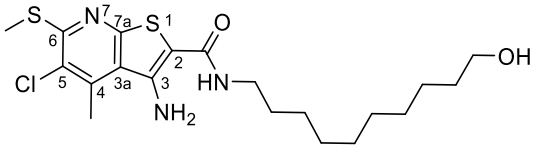
reaction control:	R_f = 0.27 (silica; EtOAc/Cy 2/1)
melting point [°C]:	131-134
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3428, 3348, 2927, 2850, 1592, 1525, 1267

^{1}H NMR (400 MHz, DMSO-d$_6$, δ [ppm]): 7.73 (t, ^3J = 5.7 Hz, 1H, N**H**), 6.82 (s, 2H, N**H$_2$**), 4.30 (t, ^3J = 5.2 Hz, 1H, O**H**), 3.37 (m, 2H, C**H$_2$**OH), 3.19 (m, 2H, NHC**H$_2$**), 2.80 (s, 3H, C**H$_3$**), 2.55 (s, 3H, SC**H$_3$**), 1.54–1.36 (m, 4H, C**H$_2$**CH$_2$NH, C**H$_2$**CH$_2$OH), 1.33-1.24 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, DMSO-d$_6$, δ [ppm]): 164.8 (**C**(O)NH), 157.7 (**C**-6), 156.5 (**C$_q$**), 147.2 (**C$_q$**), 141.2 (**C**-4), 124.7 (**C$_q$**), 122.7 (**C$_q$**), 99.2 (**C$_q$**), 60.7 (**C**H$_2$OH), 38.9 (**C**H$_2$NH), 32.5 (**C**H$_2$CH$_2$OH), 29.3 (**C**H$_2$CH$_2$NH), 28.9 (**C**H$_2$), 28.8 (**C**H$_2$), 26.5 (**C**H$_2$), 25.5 (**C**H$_2$), 16.1 (**C**H$_3$), 13.7 (S**C**H$_3$).

MS (ESI) m/z [M$^+$] Calcd for $C_{18}H_{27}ClN_3O_2S_2^+$: 416.1. Found: 416.1.

*3-Amino-5-chloro-N-(10-hydroxydecyl)-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxamide, **32c** (FG_B_100)*



$C_{20}H_{30}ClN_3O_2S_2$

M_r = 444.05 g/mol

Carboxylic acid **27** (606.0 mg, 2.10 mmol) was dissolved in dry DMF (15 ml) under argon atmosphere. DIPEA (443 µl, 2.55 mmol) and PyBOP (1.05 g, 2.20 mmol) were successively added and the mixture was stirred for 15 min at rt. After the addition of 10-amino-1-decanol (363.7 mg, 2.10 mmol), the mixture was stirred at rt for 3 h and worked up as described in **G.10**. Purification was achieved by column chromatography (silica; EtOAc/Cy 1/2 → 2/1) to yield **32c** as yellowish solid.

yield:	699 mg (75 %)
reaction control:	R_f = 0.27 (silica; EtOAc/Cy 2/1)
melting point [°C]:	117-120
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3470, 3421, 3340, 2920, 2851, 1590, 1265

^{1}H NMR (400 MHz, DMSO-d$_6$, δ [ppm]): 7.75-7.69 (m, 1H, N**H**), 6.82 (bs, 2H, N**H$_2$**), 4.29 (t, ^3J = 5.1 Hz, 1H, O**H**), 3.40-3.32 (m, 2H, C**H$_2$**OH), 3.22–3.15 (m, 2H, NHC**H$_2$**), 2.80 (s, 3H, C**H$_3$**), 2.55 (s, 3H, SC**H$_3$**), 1.55–1.45 (m, 2H, C**H$_2$**CH$_2$OH), 1.45–1.35 (m, 2H, C**H$_2$**CH$_2$NH), 1.34-1.18 (m, 12H, C**H$_2$**).

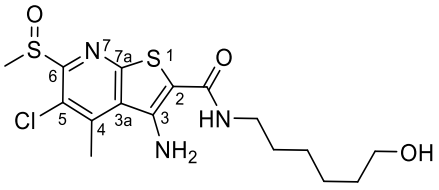
^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 164.8 (**C**(O)NH), 157.7 (**C**-6), 156.5 (**C$_q$**), 147.2 (**C$_q$**), 141.4 (**C**-4), 124.7 (**C$_q$**), 122.7 (**C$_q$**), 99.2 (**C$_q$**), 60.7 (**C**H_2OH), 38.9 (**C**H_2NH), 32.5 (**C**$H_2$$CH_2$OH), 29.3 (**C**$H_2$$CH_2$NH), 29.0 (**C**$H_2$), 29.0 (**C**$H_2$), 29.0 (**C**$H_2$), 28.8 (**C**$H_2$), 26.4 (**C**$H_2$), 25.5 (**C**$H_2$), 16.1 (**C**$H_3$), 13.7 (S**C**$H_3$).

MS (ESI) m/z [M^+] Calcd for $C_{20}H_{31}ClN_3O_2S_2^+$: 444.2. Found: 444.1.

*General procedure **G.11** for oxidation of organic sulfides to sulfoxides with hydrogen peroxide*

According to a procedure adapted from Rubio *et al.*[67], the appropriate sulfide (1.00 equiv) was dissolved in glacial acetic acid and treated with an aqueous solution of hydrogen peroxide (35 % wt.,1.15 equiv). The solution was stirred at 35 °C for 24-48 h. The reaction mixture was poured into water and extracted with DCM (3x10 ml). After phase separation, the combined organic layers were dried over magnesium sulfate and concentrated *in* vacuo. The residue was purified by column chromatography (silica, $CHCl_3$/CH_3OH 100/1 → 100/5).

*3-Amino-5-chloro-N-(6-hydroxyhexyl)-4-methyl-6-(methylsulfinyl)thieno[2,3-b]pyridine-2-carboxamide, **33a** (FG_B_77)*



$C_{16}H_{22}ClN_3O_3S_2$

M_r = 403.94 g/mol

A solution of compound **32a** (300.0 mg, 773 µmol) in glacial acetic acid (5 ml) was treated with an aqueous solution of hydrogen peroxide (35 % wt., 78 µl, 889 µmol) and stirred at 35 °C for 24 h. The reaction mixture was worked up according to **G.11** to obtain **33a** as yellow solid.

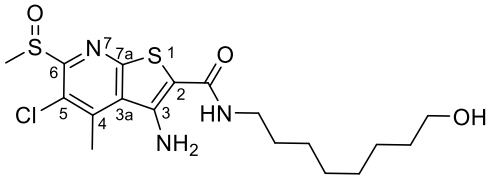
yield:	200 mg (64 %)
reaction control:	R_f = 0.35 (silica, $CHCl_3$/CH_3OH 100/5)
melting point [°C]:	184-187
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3300, 2928, 2854, 1594, 1530, 1275, 1042

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 8.00 (t, ^3J = 5.5 Hz, 1H, N**H**), 6.91 (s, 2H, N**H$_2$**), 4.33 (t, ^3J = 5.1, 1H, O**H**), 3.41-3.35 (m, 2H, C**H$_2$**OH), 3.25–3.18 (m, 2H, NHC**H$_2$**), 2.87 (bs, 6H, S(O)C**H$_3$**, C**H$_3$**), 1.56–1.47 (m, 2H, NHCH_2C**H$_2$**), 1.46–1.37 (m, 2H, CH_2C**H$_2$**OH) 1.35-1.25 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 164.4 (**C**(O)NH), 159.2 (**C**-6), 156.6 (**C_q**), 146.6 (**C_q**), 144.1 (**C**-4), 127.9 (**C_q**), 124.9 (**C_q**), 102.8 (**C_q**), 60.6 (**C**H_2OH), 39.0 (**C**H_2NH), 38.8 (S(O)**C**H_3), 32.5 (**C**H_2CH_2OH), 29.3 (**C**H_2CH_2NH), 26.4 (**C**H_2), 25.3 (**C**H_2), 15.8 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{16}H_{23}ClN_3O_3S_2^+$: 404.1. Found: 404.0.

3-Amino-5-chloro-N-(8-hydroxyoctyl)-4-methyl-6-(methylsulfinyl)thieno[2,3-b]pyridine-2-carboxamide, ***33b*** *(FG_B_95)*



$C_{18}H_{26}ClN_3O_3S_2$

M_r = 431.99 g/mol

A solution of compound **32b** (312.0 mg, 750 µmol) in glacial acetic acid (5 ml) was treated with an aqueous solution of hydrogen peroxide (35 % wt., 72 µl, 825 µmol) and stirred at 35 °C for 48 h. The reaction mixture was worked up according to **G.11** to obtain **33b** as yellow solid.

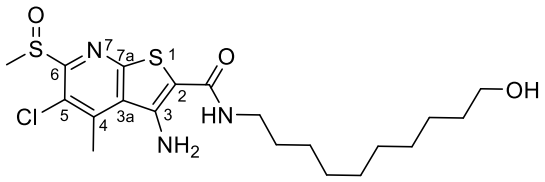
yield:	183 mg (57 %)
reaction control:	R_f = 0.30 (silica; EtOAc/Cy 2/1)
melting point [°C]:	105-108
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3319, 2925, 2852, 1596, 1530, 1274, 1028

^{1}H NMR (400 MHz, CD_3OD δ [ppm]): 3.54 (t, ^3J = 6.6 Hz, 2H, C**H_2**OH), 3.33 (t, ^3J = 7.0 Hz, 2H, C**H_2**NH), 2.95 (s, 3H, S(O)C**H_3**), 2.92 (s, 3H, C**H_3**), 1.66–1.57 (m, 2H, NHCH_2C**H_2**), 1.56–1.48 (m, 2H, CH_2C**H_2**OH), 1.42-1.31 (m, 8H, C**H_2**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 167.1 (**C**(O)NH), 159.1 (**C**-6), 159.0 (**C_q**), 148.1 (**C_q**), 146.1 (**C**-4), 130.0 (**C_q**), 126.8 (**C_q**), 105.3 (**C_q**), 63.1 (**C**H_2OH), 40.9 (**C**H_2NH), 39.4 (S(O)**C**H_3), 33.8 (**C**H_2CH_2OH), 30.9 (**C**H_2CH_2NH), 30.7 (**C**H_2), 30.6 (**C**H_2), 28.2 (**C**H_2), 27.0 (**C**H_2), 16.3 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{18}H_{27}ClN_3O_3S_2^+$: 432.1. Found: 432.1.

3-Amino-5-chloro-N-(10-hydroxydecyl)-4-methyl-6-(methylsulfinyl)thieno[2,3-b]pyridine-2-carboxamide, ***33c*** *(FG_B_101)*



$C_{20}H_{30}ClN_3O_3S_2$

M_r = 460.05 g/mol

A solution of compound **32c** (250.0 mg, 563 µmol) in glacial acetic acid (5 ml) was treated with an aqueous solution of hydrogen peroxide (35 % wt., 56 µl, 647 µmol) and stirred at 35 °C for 29 h. The reaction mixture was worked up according to **G.11** to obtain **33c** as yellow solid.

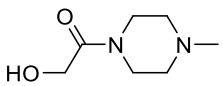
yield:	153 mg (59 %)
reaction control:	R_f = 0.35 (silica; EtOAc/Cy 2/1)
melting point [°C]:	143-144
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3308, 2918, 2848, 1592, 1538, 1277, 1072

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 3.89 (t, ^3J = 6.4 Hz, 2H, C**H$_2$**OH), 3.72–3.66 (m, 2H, NHC**H$_2$**), 3.28 (bs, 6H, S(O)C**H$_3$**, C**H$_3$**), 2.00–1.80 (m, 4H, C**H$_2$**CH_2NH, C**H$_2$**CH_2OH), 1.75-1.60 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 166.2 (**C**(O)NH), 158.4 (**C**-6), 158.3 (**C$_q$**), 146.8 (**C$_q$**), 144.9 (**C**-4), 129.4 (**C$_q$**), 125.8 (**C$_q$**), 105.1 (**C$_q$**), 62.8 (**C**H_2OH), 40.5 (**C**H_2NH) 39.5 (S(O)**C**H_3), 33.1 (**C**H_2CH_2OH), 30.2 (**C**H_2CH_2NH), 30.1 (**C**H_2), 30.1 (**C**H_2), 30.0 (**C**H_2), 29.9 (**C**H_2), 27.6 (**C**H_2), 26.4 (**C**H_2), 16.1 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{20}H_{31}ClN_3O_3S_2^+$: 460.2. Found: 460.1.

2-Hydroxy-1-(4-methylpiperazin-1-yl)ethan-1-one, ***34*** *(FG_B_12)*



$C_7H_{14}N_2O_2$

M_r = 158.20 g/mol

According to Castro *et al.*[120], *N*-methylpiperazine (1.11 ml, 9.98 mmol) and ethyl-2-hydroxy-acetate (1.18 ml, 12.48 mmol) were dissolved in dioxane (5 ml) in a sealed pressure tube and heated at 120 °C for 24 h. After cooling to rt, the solvent was removed *in vacuo* and the residue was purified by column chromatography (silica, DCM/CH_3OH 50/1 to 100/7) to give **34** as yellow oil.

yield:	956 mg (61 %)
reaction control:	R_f = 0.18 (silica, DCM/CH_3OH 50/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3399, 2938, 2850, 2795, 1641, 1442, 1290

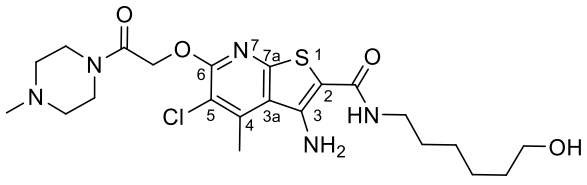
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.13 (s, 2H, C**H$_2$**OH), 3.67-3.64 (m, 2H, piperazinyl-C**H**$_2$), 3.59 (s, 1H, O**H**), 3.28-3.24 (m, 2H, piperazinyl-C**H**$_2$), 2.39 (dd, ^3J = 5.3 Hz, ^3J = 10.4 Hz, 4H, piperazinyl-C**H$_2$**), 2.29 (s, 3H, NC**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 170.2 (**C**(O)N), 59.9 (**C**H_2OH), 54.9 (piperazinyl-**C**H_2), 54.7 (piperazinyl-**C**H_2), 46.3 (N**C**H_3), 43.5 (piperazinyl-**C**H_2), 42.5 (piperazinyl-**C**H_2).

*General procedure **G.12** for nucleophilic attack of **34** at sulfoxide substituted thienopyridine ring*

Compound **34** (4 equiv) was dissolved in dry benzene under argon atmosphere and the solution was cooled to 5 °C. A 1 M solution of LiHMDS in THF (2 equiv) was added dropwise and the mixture was stirred at 5 °C for 30 min. The required sulfoxide substituted thienopyridine compound (1 equiv) was added subsequently and the reaction mixture was stirred for 30 min at 5 °C. The mixture was warmed to rt and heated under reflux for 2 h. After cooling to rt, the reaction was quenched by the addition of water (15 ml), followed by extraction with DCM (3x20 ml). After phase separation, the combined organic layers were dried over sodium sulfate and the solvent was removed *in vacuo*. The residue was purified by column chromatography as indicated.

*3-Amino-5-chloro-N-(6-hydroxyhexyl)-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)-thieno[2,3-b]pyridine-2-carboxamide, **35a** (FG_B_82)*



$C_{22}H_{32}ClN_5O_4S$

M_r = 498.04 g/mol

A solution of **34** (360.3 mg, 2.28 mmol) in dry benzene (15 ml) at 5 °C was treated with LiHMDS (1 M, 1.14 ml, 1.14 mmol) under argon atmosphere and stirred for 30 min, followed by the addition of **33a** (230.0 mg, 569 µmol) and further stirring at 5 °C for 30 min. The mixture was heated under reflux for 2 h and worked up as described in **G.12**. The crude product was

purified by column chromatography (alox basic, $CHCl_3/CH_3OH$ 100/2 → 100/5) to give **35a** as yellow crystals.

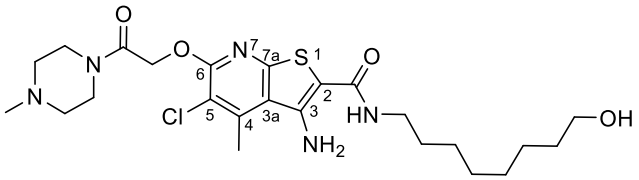
yield:	156 mg (55 %)
reaction control:	R_f = 0.48 (alox basic, $CHCl_3/CH_3OH$ 100/5, Dragendorff reagent)
melting point [°C]:	116-119
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3293, 2927, 2852, 1659, 1594, 1443, 1276

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.19 (s, 2H, N**H$_2$**), 5.67 (t, ^3J = 5.7 Hz, 1H, N**H**), 5.03 (s, 2H, OC**H$_2$**C(O)N), 3.65-3.50 (m, 6H, piperazinyl-C**H$_2$**, C**H$_2$**OH), 3.32 (dd, ^3J = 13.2 Hz, ^3J = 6.8 Hz, 2H, NHC**H$_2$**), 2.71 (s, 3H, C**H$_3$**), 2.50-2.44 (m, 2H, piperazinyl-C**H$_2$**), 2.43-2.38 (m, 2H, piperazinyl-C**H$_2$**), 2.30 (s, 3H, NC**H$_3$**), 1.59–1.48 (m, 4H, C**H$_2$**CH_2NH, C**H$_2$**CH_2OH), 1.40-1.30 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.8 (N**C**(O)CH_2), 165.7 (**C**(O)NH), 157.6 (**C**-6), 153.8 (**C$_q$**), 147.1 (**C$_q$**), 143.8 (**C**-4), 121.8 (**C$_q$**), 116.3 (**C$_q$**), 98.8 (**C$_q$**), 64.5 (O**C**H_2C(O)), 62.6 (**C**H_2OH), 55.1 (piperazinyl-**C**H_2), 54.7 (piperazinyl-**C**H_2), 46.1 (N**C**H_3), 44.9 (**C**H_2NH), 42.0 (piperazinyl-**C**H_2), 39.7 (piperazinyl-**C**H_2), 32.7 (**C**H_2CH_2OH), 29.9 (**C**H_2CH_2NH), 26.7 (**C**H_2), 25.5 (**C**H_2), 16.3 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{22}H_{33}ClN_5O_4S^+$: 498.2. Found: 498.1.

3-Amino-5-chloro-N-(8-hydroxyoctyl)-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)-thieno[2,3-b]pyridine-2-carboxamide, ***35b*** *(FG_B_103)*



$C_{24}H_{36}ClN_5O_4S$

M_r = 526.09 g/mol

A solution of **34** (322.3 mg, 2.04 mmol) in dry benzene (15 ml) at 5 °C was treated with LiHMDS (1 M, 1.02 ml, 1.02 mmol) under argon atmosphere and stirred for 30 min, followed by the addition of **33b** (220.0 mg, 509 µmol) and further stirring at 5 °C for 30 min. The mixture was heated under reflux for 2 h and worked up as described in **G.12**. The crude product was purified by column chromatography (alox basic, $CHCl_3/CH_3OH$ 100/2 → 100/5) to give **35b** as yellow crystals.

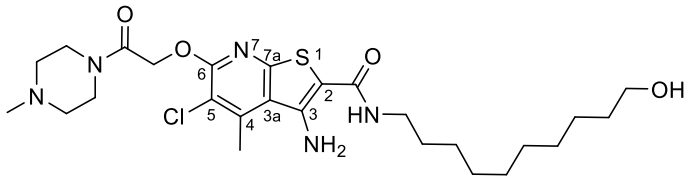
yield:	163 mg (61 %)
reaction control:	R_f = 0.51 (alox basic, $CHCl_3$/CH_3OH 100/5, Dragendorff reagent)
melting point [°C]:	114-118
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3410, 3325, 2926, 2850, 1651, 1531, 1446, 1285

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.25 (s, 2H, N**H$_2$**), 5.38 (t, ^3J = 5.5 Hz, 1H, N**H**), 5.09 (s, 2H, OC**H$_2$**C(O)N), 3.68-3.52 (m, 6H, piperazinyl-C**H$_2$**, C**H$_2$**OH), 3.36 (dd, ^3J = 13.2 Hz, ^3J = 6.8 Hz, 2H, NHC**H$_2$**), 2.82 (s, 3H, C**H$_3$**), 2.55-2.44 (m, 2H, piperazinyl-C**H$_2$**), 2.43-2.36 (m, 2H, piperazinyl-C**H$_2$**), 2.32 (s, 3H, NC**H$_3$**), 1.59–1.50 (m, 4H, C**H$_2$**CH_2NH, C**H$_2$**CH_2OH), 1.38-1.31 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.8 (N**C**(O)CH_2), 165.8 (**C**(O)NH), 158.0 (**C**-6), 154.0 (**C$_q$**), 147.3 (**C$_q$**), 143.8 (**C**-4), 122.1 (**C$_q$**), 116.6 (**C$_q$**), 98.8 (**C$_q$**), 64.8 (O**C**H_2C(O)), 63.2 (**C**H_2OH), 55.3 (piperazinyl-**C**H_2), 54.8 (piperazinyl-**C**H_2), 46.3 (N**C**H_3), 45.2 (**C**H_2NH), 42.2 (piperazinyl-**C**H_2), 39.9 (piperazinyl-**C**H_2), 33.0 (**C**H_2CH_2OH), 30.0 (**C**H_2CH_2NH), 29.5 (**C**H_2), 29.4 (**C**H_2), 27.0 (**C**H_2), 25.8 (**C**H_2), 16.4 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{24}H_{37}ClN_5O_4S^+$: 526.2. Found: 526.2.

3-Amino-5-chloro-N-(10-hydroxydecyl)-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)-thieno[2,3-b]pyridine-2-carboxamide, ***35c*** *(FG_B_102)*



$C_{26}H_{40}ClN_5O_4S$

M_r = 554.15 g/mol

A solution of **34** (178.8 mg, 1.13 mmol) in dry benzene (15 ml) at 5 °C was treated with LiHMDS (1 M, 565 µl, 565 µmol) under argon atmosphere and stirred for 30 min, followed by the addition of **33c** (130.0 mg, 283 µmol) and further stirring at 5 °C for 30 min. The mixture was heated under reflux for 2 h and worked up as described in **G.12**. The crude product was purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/2 → 100/5) to give **35c** as yellow crystals.

yield:	104 mg (66 %)

reaction control:	R_f = 0.55 (alox basic, $CHCl_3$/CH_3OH 100/5, Dragendorff reagent)
melting point [°C]:	107-108
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3378, 3299, 2922, 2848, 2790, 1659, 1531, 1346, 1273

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.25 (s, 2H, N**H$_2$**), 5.38 (t, ^3J = 5.6 Hz, 1H, N**H**), 5.09 (s, 2H, OC**H$_2$**C(O)N), 3.66-3.52 (m, 6H, piperazinyl-C**H$_2$**), C**H$_2$**OH), 3.36 (dd, ^3J = 13.1 Hz, ^3J = 6.9 Hz, 2H, NHC**H$_2$**), 2.81 (s, 3H, C**H$_3$**), 2.59-2.43 (m, 2H, piperazinyl-C**H$_2$**), 2.42-2.37 (m, 2H, piperazinyl-C**H$_2$**), 2.31 (s, 3H, NC**H$_3$**), 1.60–1.50 (m, 4H, C**H$_2$**CH_2NH,C**H$_2$**CH_2OH), 1.32-1.25 (m, 12H, C**H$_2$**).

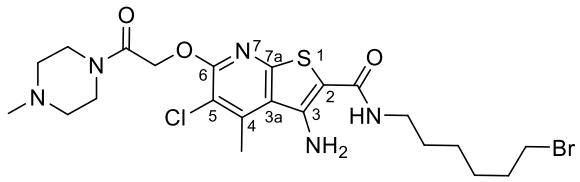
^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.8 (N**C**(O)CH_2), 165.8 (**C**(O)NH), 158.0 (**C**-6), 154.0 (**C$_q$**), 147.3 (**C$_q$**), 143.8 (**C**-4), 122.1 (**C$_q$**), 116.6 (**C$_q$**), 98.8 (**C$_q$**), 64.8 (O**C**H_2C(O)), 63.2 (**C**H_2OH), 55.3 (piperazinyl-**C**H_2), 54.8 (piperazinyl-**C**H_2), 46.3 (N**C**H_3), 45.2 (**C**H_2NH), 42.2 (piperazinyl-**C**H_2), 39.9 (piperazinyl-**C**H_2), 33.0 (**C**H_2CH_2OH), 30.0 (**C**H_2CH_2NH), 29.6 (**C**H_2), 29.6 (**C**H_2), 29.6 (**C**H_2), 25.4 (**C**H_2), 27.1 (**C**H_2), 26.0 (**C**H_2), 16.4 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{26}H_{41}ClN_5O_4S^+$: 554.3. Found: 554.2.

*General procedure **G.13** for introduction of a bromine substituent*

The appropriate thienopyridine intermediate (1 equiv) was added to dry DCM under argon atmosphere and the solution was cooled to 0 °C. After the addition of DIPEA (2 equiv), the mixture was stirred at 0 °C for 10 min, followed by the dropwise addition of methanesulfonyl chloride (1.6 equiv). The reaction mixture was stirred for 30 min at 0 °C and for 1 h at rt. The reaction was quenched with water (10 ml) and phases were separated. The aqueous layer was extracted with DCM (2x20 ml) and, after phase separation, the combined organics were dried over magnesium sulfate, concentrated *in vacuo*, and suspended in dry THF (10 ml) under argon atmosphere. The suspension was treated with lithium bromide (5 equiv) and heated under reflux for 2-3 h. After cooling to rt, the mixture was concentrated *in vacuo* and the residue was dissolved in $CHCl_3$ (15 ml). The solution was washed with water (10 ml) and phases were separated. The aqueous layer was further extracted with $CHCl_3$ (2x20 ml). After phase separation, the combined organic layers were dried over magnesium sulfate and concentrated *in vacuo* to give the product. The obtained compounds were directly used for the next reaction step without further purification due to instability under any storage conditions.

3-Amino-N-(6-bromohexyl)-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)-thieno[2,3-b]pyridine-2-carboxamide, ***36a*** *(FG_B_90)*



$C_{22}H_{31}BrClN_5O_3S$

M_r = 560.94 g/mol

According to general procedure **G.13**, methanesulfonyl chloride (14 µl, 177 µmol) was added dropwise to a solution of **35a** (55.0 mg, 110 µmol) and DIPEA (38 µl, 221 µmol) in dry DCM (10 ml) at 0 °C under argon atmosphere and stirred at rt for 1 h. The resulting sulfonate ester was reacted with lithium bromide (48.0 mg, 552 µmol) in dry THF (10 ml) under reflux for 2 h. After reaction work-up, **36a** was obtained as an orange solid.

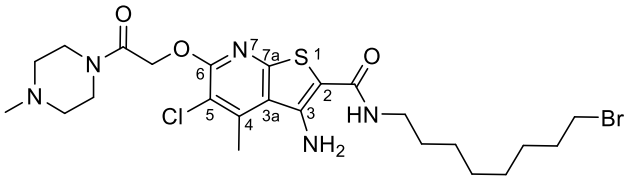
yield: 36 mg (58 %)

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 5.18 (s, 2H, OC**H$_2$**C(O)N), 3.72-3.63 (m, 4H, piperazinyl-C**H$_2$**), 3.48 (d, ^3J = 6.7 Hz, 2H, C**H$_2$**Br), 3.36-3.30 (m, 2H, NHC**H$_2$**), 2.83 (s, 3H, C**H$_3$**), 2.65-2.60 (m, 2H, piperazinyl-C**H$_2$**), 2.57-2.49 (m, 2H, piperazinyl-C**H$_2$**), 2.41 (s, 3H, NC**H$_3$**), 1.95-1.85 (m, 2H, C**H$_2$**CH_2Br), 1.65–1.57 (m, 2H, NHCH_2C**H$_2$**), 1.57-1.40 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 168.4 (N**C**(O)CH_2), 167.6 (**C**(O)NH), 159.1 (**C**-6), 155.8 (**C$_q$**), 149.0 (**C$_q$**), 145.8 (**C**-4), 123.1 (**C$_q$**), 117.0 (**C$_q$**), 100.0 (**C$_q$**), 65.3 (O**C**H_2C(O)), 62.6 (**C**H_2Br), 56.0 (piperazinyl-**C**H_2), 55.6 (piperazinyl-**C**H_2), 46.2 (N**C**H_3), 45.7 (**C**H_2NH), 42.8 (piperazinyl-**C**H_2), 40.7 (piperazinyl-**C**H_2), 34.5 (**C**H_2Br), 34.1 (**C**H_2CH_2Br), 30.8 (**C**H_2CH_2NH), 29.1 (**C**H_2), 27.4 (**C**H_2), 16.6 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{22}H_{32}BrClN_5O_3S^+$: 560.1. Found: 560.0.

3-Amino-N-(8-bromooctyl)-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)-thieno[2,3-b]pyridine-2-carboxamide, ***36b*** *(FG_B_107)*



$C_{24}H_{35}BrClN_5O_3S$

M_r = 588.99 g/mol

According to general procedure **G.13**, methanesulfonyl chloride (27 µl, 354 µmol) was added dropwise to a solution of **35b** (116.4 mg, 221 µmol) and DIPEA (77 µl, 443 µmol) in dry DCM

(10 ml) at 0 °C under argon atmosphere and stirred at rt for 1 h. The resulting sulfonate ester was reacted with lithium bromide (96.1 mg, 1.11 mmol) in dry THF (10 ml) under reflux for 2 h. After reaction work-up, **36b** was obtained as an orange solid.

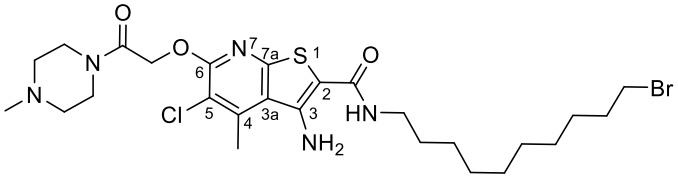
yield: 88 mg (68 %)

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.26 (s, 2H, N**H$_2$**), 5.43 (s, 1H, N**H**), 5.09 (s, 2H, OC**H$_2$**-C(O)N), 3.76-3.64 (m, 4H, piperazinyl-C**H$_2$**), 3.41-3.33 (m, 4H, C**H$_2$**Br, NHC**H$_2$**), 2.81 (s, 3H, C**H$_3$**), 2.68-2.54 (m, 4H, piperazinyl-C**H$_2$**), 2.43 (s, 3H, NC**H$_3$**), 1.87-1.79 (m, 2H, C**H$_2$**CH_2Br), 1.62–1.52 (m, 2H, NHCH_2C**H$_2$**), 1.45-1.32 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.9 (N**C**(O)CH_2), 165.7 (**C**(O)NH), 157.8 (**C**-6), 153.9 (**C$_q$**), 147.3 (**C$_q$**), 143.9 (**C**-4), 122.1 (**C$_q$**), 116.5 (**C$_q$**), 98.8 (**C$_q$**), 64.8 (O**C**H_2C(O)), 54.9 (piperazinyl-**C**H_2), 54.2 (piperazinyl-**C**H_2), 45.7 (N**C**H_3), 44.6 (**C**H_2NH), 41.5 (piperazinyl-**C**H_2), 39.9 (piperazinyl-**C**H_2), 34.2 (**C**H_2Br), 33.0 (**C**H_2CH_2Br), 30.0 (**C**H_2CH_2NH), 29.3 (**C**H_2), 28.9 (**C**H_2), 28.3 (**C**H_2), 27.0 (**C**H_2), 16.5 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{24}H_{36}BrClN_5O_3S^+$: 588.1. Found: 588.1.

*3-Amino-N-(10-bromodecyl)-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)-thieno[2,3-b]pyridine-2-carboxamide, **36c** (FG_B_104)*



$C_{26}H_{39}BrClN_5O_3S$

M_r = 617.04 g/mol

According to general procedure **G.13**, methanesulfonyl chloride (13 µl, 167 µmol) was added dropwise to a solution of **35c** (58.0 mg, 105 µmol) and DIPEA (36 µl, 209 µmol) in dry DCM (10 ml) at 0 °C under argon atmosphere and stirred at rt for 1 h. The resulting sulfonate ester was reacted with lithium bromide (45.5 mg, 523 µmol) in dry THF (10 ml) under reflux for 3 h. After reaction work-up, **36c** was obtained as an orange solid.

yield: 40 mg (61 %)

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 5.19 (s, 2H, OC**H$_2$**C(O)N), 3.75-3.66 (m, 4H, piperazinyl-C**H$_2$**), 3.41 (d, ^3J = 6.8 Hz, 2H, C**H$_2$**Br), 3.35-3.31 (m, 2H, NHC**H$_2$**), 2.87 (s, 3H, C**H$_3$**), 2.86-2.76 (m, 2H, piperazinyl-C**H$_2$**), 2.75-2.66 (m, 2H, piperazinyl-C**H$_2$**), 2.54 (s, 3H, NC**H$_3$**), 1.89-1.81 (m, 2H, C**H$_2$**CH_2Br), 1.67–1.57 (m, 2H, NHCH_2C**H$_2$**), 1.50-1.32 (m, 12H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 168.5 (N**C**(O)CH_2), 167.7 (**C**(O)NH), 159.2 (**C**-6), 155.9 (**C_q**), 149.1 (**C_q**), 145.9 (**C**-4), 123.2 (**C_q**), 117.1 (**C_q**), 100.2 (**C_q**), 65.4 (O**C**H_2C(O)), 55.7 (piperazinyl-**C**H_2), 55.5 (piperazinyl-**C**H_2), 45.7 (N**C**H_3), 45.2 (**C**H_2NH), 42.3 (piperazinyl-**C**H_2), 40.7 (piperazinyl-**C**H_2), 34.6 (**C**H_2Br), 34.2 (**C**$H_2$$CH_2$Br), 30.9 (**C**$H_2$$CH_2$NH), 30.7 (**C**$H_2$), 30.6 (**C**$H_2$), 30.5 (**C**$H_2$), 30.0 (**C**$H_2$), 29.3 (**C**$H_2$), 28.2 (**C**$H_2$), 16.6 (**C**$H_3$).

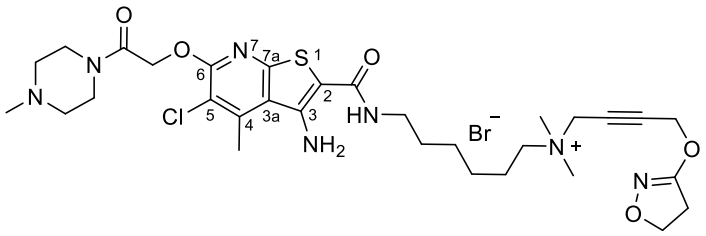
MS (ESI) m/z [M^+] Calcd for $C_{26}H_{40}BrClN_5O_3S^+$: 616.2. Found: 616.2.

IR spectra and melting points were not measured for compounds **36a-c** due to instability under storage conditions.

*General procedure **G.14** for linkage of iperoxo base with LY21-thienopyridine moieties*

Iperoxo base (**13**) (5 equiv) was added to a solution of the required thienopyridine compound (1 equiv) in acetonitrile. The reaction mixture was stirred at 35 °C for 8-10 days. After removal of solvent, the residue was purified by column chromatography as indicated.

*6-(3-Amino-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)thieno[2,3-b]pyridine-2-carboxamido)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethylhexan-1-aminium bromide, **LY21-A6-iper** (FG_B_92)*



$C_{31}H_{45}BrClN_7O_5S$

M_r = 743.16 g/mol

According to **G.14**, a solution of **13** (81.2 mg, 447 µmol) and **36a** (50.0 mg, 89 µmol) in acetonitrile (5 ml) was stirred at 35 °C for 8 days. Purification by column chromatography (alox basic, $CHCl_3$/CH_3OH 17/3) yielded **LY21-A6-iper** as colorless oil.

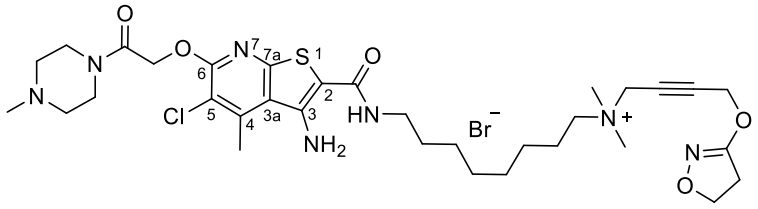
yield:	19 mg (28 %)
HPLC puritiy:	98 % (HPLC Method **IIb**)
reaction control:	R_f = 0.15 (alox basic, $CHCl_3$/ CH_3OH 100/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3404, 3310, 2928, 2857, 2800, 1658, 1594, 1341

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.28-6.20 (m, 3H, N**H**, N**H$_2$**), 5.07 (s, 2H, OC**H$_2$**C(O)N), 4.78 (m, 4H, OC**H$_2$**C≡C, $N^+(CH_3)_2$C**H$_2$**C≡C), 4.39 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 3.66-3.52 (m, 6H, piperazinyl-C**H$_2$**, $N^+(CH_3)_2$C**H$_2$**CH_2), 3.44-3.30 (m, 8H, N^+(C**H$_3$**)$_2$, C(O)NHC**H$_2$**), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-C**H$_2$**), 2.78 (s, 3H, C**H$_3$**), 2.48-2.35 (m, 4H, piperazinyl-C**H$_2$**), 2.30 (s, 3H, NC**H$_3$**), 1.82–1.70 (m, 2H, $N^+(CH_3)_2CH_2$C**H$_2$**), 1.64-1.56 (m, 2H, $NHCH_2$C**H$_2$**), 1.47-1.35 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.8 (isoxazolinyl-**C$_q$**), 165.7 (N**C**(O)CH_2), 165.7 (**C**(O)NH), 157.5 (**C**-6), 154.0 (**C$_q$**), 147.0 (**C$_q$**), 143.7 (**C**-4), 121.8 (**C$_q$**), 116.1 (**C$_q$**), 99.1 (**C$_q$**), 86.5 (C≡**C**CH_2O), 75.9 (C≡**C**CH_2N), 70.1 (isoxazolinyl-O**C**H_2), 64.6 (O**C**H_2C(O)), 64.2 ($N^+(CH_3)_2$**C**H_2CH_2), 57.3 (O**C**H_2C≡C), 55.0 (piperazinyl-**C**H_2), 54.8 (N^+**C**H_2C≡C), 54.6 (piperazinyl-**C**H_2), 50.6 (N^+(**C**H_3)$_2$), 46.1 (N**C**H_3), 44.9 (piperazinyl-**C**H_2), 42.0 (piperazinyl-**C**H_2), 39.2 (**C**H_2NH), 32.9 (isoxazolinyl-OCH_2**C**H_2), 29.1 (**C**H_2CH_2NH), 25.9 (**C**H_2), 25.5 (**C**H_2), 22.4 ($N^+(CH_3)_2CH_2$**C**H_2), 16.3 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{31}H_{46}ClN_7O_5S^{2+}$: 331.7. Found: 331.9.

8-(3-Amino-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)thieno[2,3-b]pyridine-2-carboxamido)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyloctan-1-aminium bromide, ***LY21-A8-iper*** *(FG_B_111)*



$C_{33}H_{49}BrClN_7O_5S$

M_r = 771.21 g/mol

According to **G.14**, a solution of **13** (133.0 mg, 730 µmol) and **36b** (86.0 mg, 146 µmol) in acetonitrile (5 ml) was stirred at 35 °C for 10 days. Purification by column chromatography (alox basic, $CHCl_3$/MeOH 17/3) yielded **LY21-A8-iper** as colorless oil.

yield:	49 mg (44 %)
HPLC puritiy:	97 % (HPLC Method **IIb**)
reaction control:	R_f = 0.19 (alox basic, $CHCl_3$/CH_3OH 17/3, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3397, 3312, 2926, 2855, 1654, 1593, 1278

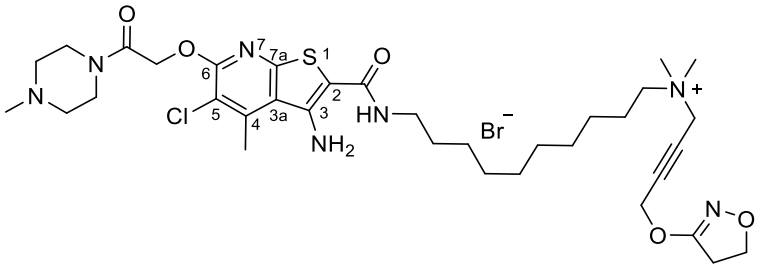
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.27 (s, 2H, N**H$_2$**), 5.83 (t, ^3J = 5.7 Hz, 1H, N**H**), 5.07 (s, 2H, OC**H$_2$**C(O)N), 4.88 (s, 2H, OC**H$_2$**C≡C), 4.77 (s, 2H, N^+C**H$_2$**C≡C), 4.38 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 3.62-3.50 (m, 6H, piperazinyl-C**H$_2$**, $N^+(CH_3)_2$C**H$_2$**CH_2), 3.45-3.30

(m, 8H, $N^+(CH_3)_2$, NHC**H₂**), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-C**H₂**), 2.79 (s, 3H, C**H₃**), 2.47-2.34 (m, 4H, piperazinyl-C**H₂**), 2.29 (s, 3H, NC**H₃**), 1.76–1.66 (m, 2H, $N^+(CH_3)_2CH_2CH_2$), 1.60-1.52 (m, 2H, $NHCH_2CH_2$), 1.40-1.26 (m, 8H, C**H₂**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.9 (isoxazolinyl-**C$_q$**), 165.8 (N**C**(O)CH_2), 165.7 (**C**(O)NH), 157.8 (**C**-6), 154.0 (**C$_q$**), 147.2 (**C$_q$**), 143.9 (**C**-4), 122.0 (**C$_q$**), 116.4 (**C$_q$**), 99.1 (**C$_q$**), 86.6 (C≡**C**CH_2O), 76.3 (C≡**C**CH_2N), 70.2 (isoxazolinyl-O**C**H_2), 64.8 (O**C**H_2C(O)), 64.3 ($N^+(CH_3)_2$**C**H_2CH_2), 57.4 (O**C**H_2C≡C), 55.2 (piperazinyl-**C**H_2), 54.9 (N^+**C**H_2C≡C), 54.8 (piperazinyl-**C**H_2), 50.6 (N^+(**C**H_3)$_2$), 46.3 (N**C**H_3), 45.2 (piperazinyl-**C**H_2), 42.2 (piperazinyl-**C**H_2), 39.6 (**C**H_2NH), 33.1 (isoxazolinyl-**C**H_2), 29.7 (**C**H_2CH_2NH), 28.8 (**C**H_2), 28.8 (**C**H_2), 26.6 (**C**H_2), 26.1 (**C**H_2), 22.9 ($N^+(CH_3)_2CH_2$**C**H_2), 16.5 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{33}H_{50}ClN_7O_5S^{2+}$: 345.7. Found: 346.0.

10-(3-Amino-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)thieno[2,3-b]pyridine-2-carboxamido)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyldecan-1-aminium bromide, ***LY21-A10-iper*** *(FG_B_105)*



$C_{35}H_{53}BrClN_7O_5S$

M_r = 799.27 g/mol

According to **G.14**, a solution of **13** (236.3 mg, 1.30 mmol) and **36c** (160.0 mg, 259 µmol) in acetonitrile (5 ml) was stirred at 35 °C for 8 days. Purification by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/17) yielded **LY21-A10-iper** as colorless oil.

yield:	39 mg (19 %)
HPLC puritiy:	95 % (HPLC Method **IIb**)
reaction control:	R_f = 0.20 (alox basic, $CHCl_3$/CH_3OH 17/3, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3315, 2924, 2853, 2800, 1660, 1595, 1340

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.26 (s, 2H, N**H₂**), 5.66 (t, ^3J = 5.7 Hz, 1H, N**H**), 5.07 (s, 2H, OC**H₂**C(O)N), 4.84 (s, 2H, OC**H₂**C≡C), 4.78 (s, 2H, N^+C**H₂**C≡C), 4.38 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H₂**), 3.63-3.51 (m, 6H, piperazinyl-C**H₂**), $N^+(CH_3)_2$C**H₂**CH_2), 3.45-3.30 (m, 8H, N^+(C**H₃**)$_2$, NHC**H₂**), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-C**H₂**), 2.79 (s, 3H, C**H₃**),

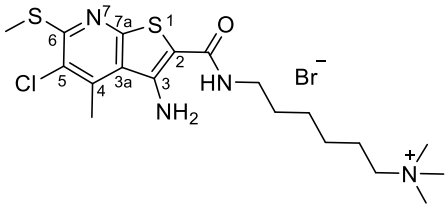
2.52-2.33 (m, 4H, piperazinyl-C**H**$_2$), 2.30 (s, 3H, NC**H**$_3$), 1.73–1.63 (m, 2H, $N^+(CH_3)_2CH_2C$**H**$_2$), 1.59-1.50 (m, 2H, $NHCH_2C$**H**$_2$), 1.37-1.21 (m, 12H, C**H**$_2$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.9 (isoxazolinyl-**C**$_q$), 165.8 (N**C**(O)CH_2), 165.7 (**C**(O)NH), 157.8 (**C**-6), 154.0 (**C**$_q$), 147.2 (**C**$_q$), 143.9 (**C**-4), 122.0 (**C**$_q$), 116.4 (**C**$_q$), 98.9 (**C**$_q$), 86.6 (C≡**C**CH_2O), 76.2 (C≡**C**CH_2N), 70.2 (isoxazolinyl-O**C**H_2), 64.8 (O**C**H_2C(O)), 64.4 ($N^+(CH_3)_2$**C**H_2CH_2), 57.4 (O**C**H_2C≡C), 55.2 (piperazinyl-**C**H_2), 54.9 (N**C**H_2C≡C), 54.8 (piperazinyl-**C**H_2), 50.7 (N^+(**C**$H_3)_2$), 46.2 (N**C**H_3), 45.1 (piperazinyl-**C**H_2), 42.2 (piperazinyl-**C**H_2), 39.8 (**C**H_2NH), 33.1 (isoxazolinyl-**C**H_2), 29.8 (**C**H_2CH_2NH), 29.3 (**C**H_2), 29.2 (**C**H_2), 29.2 (**C**H_2), 29.1 (**C**H_2), 26.9 (**C**H_2), 26.2 (**C**H_2), 23.0 ($N^+(CH_3)_2CH_2$**C**H_2), 16.5 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{35}H_{54}ClN_7O_5S^{2+}$: 359.7. Found: 360.0.

7.3.3 Preparation of LY21-An-TMA hybrids

6-(3-Amino-5-chloro-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethylhexan-1-aminium bromide, ***37a*** *(FG_B_116)*



$C_{19}H_{30}BrClN_4OS_2$

M_r = 509.95 g/mol

Carboxylic acid **27** (315.0 mg, 1.09 mmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (199 µl, 1.15 mmol) and PyBOP (567.7 mg, 1.09 mmol) were successively added. Compound **2-L6** (384.2 mg, 1.09 mmol) was dissolved in dry DMF (3 ml), treated with DIPEA (199 µl, 1.15 mmol) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/3 → 10/1) to give **37a** as yellowish crystals.

yield:	342 mg (61 %)
reaction control:	R_f = 0.10 (alox basic; $CHCl_3$/CH_3OH: 30/1; Dragendorff-reagent)
melting point [°C]:	136-144
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3480, 3316, 2928, 2860, 1523, 1263, 827

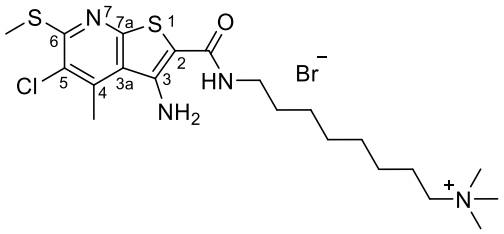
^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.38 (s, 2H, N**H**$_2$), 6.30 (t, ^3J = 5.2 Hz, 1H, N**H**), 3.34–3.27 (m, 2H, NHC**H**$_2$), 3.24-3.17 (m, 2H, C**H**$_2N^+(CH_3)_3$), 3.00 (s, 9H, N^+(C**H**$_3)_3$), 2.73 (s, 3H,

C**H₃**), 2.52 (s, 3H, SC**H₃**), 1.78-1.67 (m, 2H, C**H₂**$CH_2N^+(CH_3)_3$), 1.62-1.54 (m, 2H, $NHCH_2C$**H₂**), 1.46-1.35 (m, 4H, C**H₂**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.7 (**C**(O)NH), 160.0 (**C**-6), 157.9 (**C$_q$**), 148.6 (**C$_q$**), 142.5 (**C**-4), 126.8 (**C$_q$**), 124.1 (**C$_q$**), 100.5 (**C$_q$**), 67.9 (**C**$H_2N(CH_3)_3$), 54.2 (N^+(**C**$H_3)_3$), 40.2 (**C**H_2NH), 30.5 (**C**H_2CH_2NH), 27.4 (**C**H_2), 26.8 (**C**H_2), 23.8 (**C**$H_2CH_2N^+(CH_3)_3$), 17.0 (**C**H_3), 14.9 (S**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{19}H_{30}ClN_4OS_2^+$: 429.2. Found: 429.1.

8-(3-Amino-5-chloro-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyloctan-1-aminium bromide, ***37b*** *(FG_B_125)*



$C_{21}H_{34}BrClN_4OS_2$

M_r = 538.00 g/mol

Carboxylic acid **27** (250.0 mg, 866 µmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (158 µl, 910 µmol) and PyBOP (450.5 mg, 866 µmol) were successively added. Compound **2-L8** (330.1 mg, 866 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (158 µl, 910 µmol) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3/CH_3OH$ 100/3 → 10/1) to give **37b** as yellowish crystals.

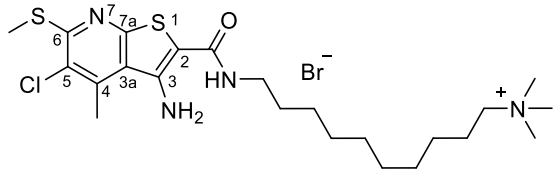
yield:	116 mg (25 %)
reaction control:	R_f = 0.12 (alox basic; $CHCl_3/CH_3OH$: 30/1; Dragendorff-reagent)
melting point [°C]:	172
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3486, 3414, 2927, 2850, 1600, 1509, 1254

^{1}H NMR (400 MHz, $(CD_3)_2CO$, δ [ppm]): 6.94 (t, ^3J = 5.7 Hz, 1H, N**H**), 6.78 (s, 2H, N**H₂**), 3.58-3.52 (m, 2H, NHC**H₂**), 3.24-3.17 (m, 11H, C**H₂**$N^+(CH_3)_3$, N^+(C**H₃**)$_3$), 2.89 (s, 3H, C**H₃**), 2.56 (s, 3H, SC**H₃**), 1.99-1.89 (m, 2H, C**H₂**$CH_2N^+(CH_3)_3$), 1.63-1.54 (m, 2H, $NHCH_2C$**H₂**), 1.45-1.35 (m, 8H, C**H₂**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.3 (**C**(O)NH), 159.5 (**C**-6), 157.8 (**C_q**), 148.5 (**C_q**), 142.2 (**C**-4), 126.3 (**C_q**), 124.0 (**C_q**), 100.5 (**C_q**), 67.6 (**C**$H_2N^+(CH_3)_3$), 56.7 (N^+(**C**H_3)$_3$), 40.1 (**C**H_2NH), 30.6 (NHCH_2**C**H_2), 30.1 (**C**H_2), 29.8 (**C**H_2), 27.6 (**C**H_2), 26.9 (**C**H_2), 23.6 (**C**$H_2CH_2N^+(CH_3)_3$), 16.5 (**C**H_3), 14.3 (S**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{21}H_{34}ClN_4OS_2^+$: 457.2. Found: 457.4

10-(3-Amino-5-chloro-4-methyl-6-(methylthio)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyldecan-1-aminium bromide, ***37c*** *(FG_B_141)*



$C_{23}H_{38}BrClN_4OS_2$

M_r = 566.06 g/mol

Carboxylic acid **27** (337.0 mg, 1.17 mmol) was dissolved in dry DMF (10 ml) under argon atmosphere. DIPEA (222 µl, 1.28 mmol) and PyBOP (607.3 mg, 1.17 µmol) were successively added. Compound **2-L10** (477.7 mg, 1.17 mmol) was dissolved in dry DMF (3 ml), treated with DIPEA (222 µl, 1.28 µmol) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 100/3 → 10/1) to give **37c** as yellowish crystals.

yield:	306 mg (46 %)
reaction control:	R_f = 0.12 (alox basic; $CHCl_3$/CH_3OH: 30/1; Dragendorff-reagent)
melting point [°C]:	138-146
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3316, 2924, 2853, 1661, 1523, 1480, 1215

^{1}H NMR (400 MHz, $(CD_3)_2SO$, δ [ppm]): 7.77 (t, ^3J = 5.3 Hz, 1H, N**H**), 6.83 (s, 2H, N**H$_2$**), 3.30-3.23 (m, 2H, NHC**H$_2$**), 3.23-3.16 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.04 (s, 9H, N^+(C**H$_3$**)$_3$), 2.80 (s, 3H, C**H$_3$**), 2.54 (s, 3H, SC**H$_3$**), 1.74-1.60 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.56-1.45 (m, 2H, NHCH_2C**H$_2$**), 1.35-1.20 (m, 12H, C**H$_2$**).

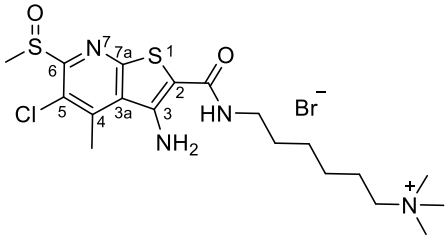
^{13}C NMR (100 MHz, $(CD_3)_2SO$, δ [ppm]): 164.8 (**C**(O)NH), 157.7 (**C**-6), 156.5 (**C_q**), 147.2 (**C_q**), 141.4 (**C**-4), 124.7 (**C_q**), 122.7 (**C_q**), 99.2 (**C_q**), 65.3 (**C**$H_2N^+(CH_3)_3$), 52.1 (N^+(**C**H_3)$_3$), 39.3 (**C**H_2NH), 29.3 (NHCH_2**C**H_2), 28.9 (**C**H_2), 28.8 (**C**H_2), 28.7 (**C**H_2), 28.4 (**C**H_2), 26.4 (**C**H_2), 25.7 (**C**H_2), 22.0 (**C**$H_2CH_2N^+(CH_3)_3$), 16.2 (**C**H_3), 13.7 (S**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{23}H_{38}ClN_4OS_2^+$: 485.2. Found: 485.3

*General procedure **G.15** for oxidation of organic sulfides to sulfoxides with mCPBA.*

The appropriate sulfide (1.0 equiv) was suspended in dry DCM under argon atmosphere and treated with $NaHCO_3$ (1.1 equiv). After cooling the reaction mixture to -78 °C, a solution of mCPBA (1.0 equiv) in DCM was added dropwise. The mixture was allowed to warm to rt within a 2 h period. An aqueous solution of $NaHCO_3$ was added and the mixture was extracted with $CHCl_3$ (10x30 ml). After phase separation, the combined organic layers were dried over sodium sulfate and the solvent was removed. The crude product was purified by column chromatography (alox basic, acetone/CH_3OH 100/3) and dried under high vacuum to remove diacetone alcohol which was formed in small amounts due to contact of acetone with basic alox.

*6-(3-Amino-5-chloro-4-methyl-6-(methylsulfinyl)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethylhexan-1-aminium bromide, **38a** (FG_B_121)*



$C_{19}H_{30}BrClN_4O_2S_2$

M_r = 525.95 g/mol

A solution of mCPBA (70 % wt., 120.6 mg, 490 µmol) in DCM (2 ml) was added dropwise to a suspension of compound **37a** (250.0 mg, 490 µmol) and $NaHCO_3$ (45.3 mg, 539 µmol) in DCM (5 ml) at -78 °C. The reaction mixture was worked up according to **G.15** to give **38a**.

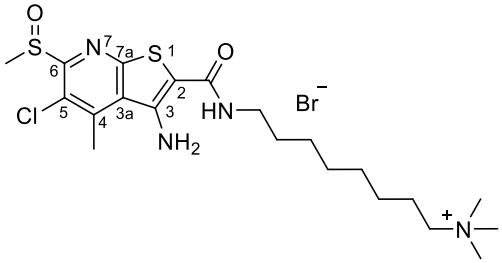
yield:	85 mg (33 %)
reaction control:	R_f = 0.35 (alox basic; acetone/CH_3OH: 100/5; Dragendorff-reagent)
melting point [°C]:	133-136
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3410, 3311, 2926, 2859, 1667, 1599, 1530, 1054

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.61-6.40 (m, 3H, N**H**, N**H$_2$**), 3.27-3.25 (m, 2H, NHC**H$_2$**), 3.23-3.18 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.00 (s, 9H, $N^+(C$**H$_3$**$)_3$), 2.86 (s, 3H, C**H$_3$**), 2.83 (s, 3H, S(O)C**H$_3$**), 1.77-1.69 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.65-1.56 (m, 2H, $NHCH_2C$**H$_2$**), 1.45-1.35 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.3 (**C**(O)NH), 161.1 (**C**-6), 158.4 (**C_q**), 148.0 (**C_q**), 145.6 (**C**-4), 129.6 (**C_q**), 127.1 (**C_q**), 104.4 (**C_q**), 67.9 (**C**$H_2N^+(CH_3)_3$), 54.2 (N^+(**C**H_3)$_3$), 40.3 (**C**H_2NH), 40.1 (S(O)**C**H_3), 30.4 (NHCH_2**C**H_2), 27.4 (**C**H_2), 26.8 (**C**H_2), 23.4 (**C**$H_2CH_2N^+(CH_3)_3$), 16.7 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{19}H_{30}ClN_4O_2S_2^+$: 445.2. Found: 445.2

8-(3-Amino-5-chloro-4-methyl-6-(methylsulfinyl)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyloctan-1-aminium bromide, ***38b*** *(FG_B_132)*



$C_{21}H_{34}BrClN_4O_2S_2$

M_r = 554.00 g/mol

A solution of mCPBA (70 % wt., 151.2 mg, 613 µmol) in DCM (2 ml) was added dropwise to a suspension of compound **37b** (330.0 mg, 613 µmol) and $NaHCO_3$ (56.7 mg, 674 µmol) in DCM (5 ml) at -78 °C. The reaction mixture was worked up according to **G.15** to give **38b**.

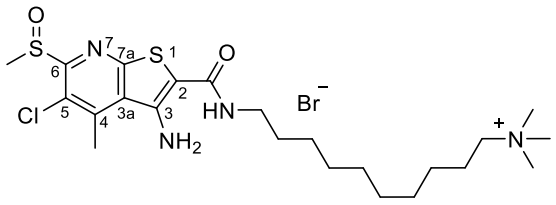
yield:	75 mg (22 %)
reaction control:	R_f = 0.48 (alox basic; acetone/ CH_3OH: 10/1; Dragendorff-reagent)
melting point [°C]:	137-138
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3303, 2925, 2856, 1669, 1593, 1538, 1058

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.57-6.45 (m, 3H, N**H**, N**H_2**), 3.36-3.27 (m, 2H, NHC**H_2**), 3.22-3.16 (m, 2H, C**H_2**$N^+(CH_3)_3$), 2.99 (s, 9H, N^+(C**H_3**)$_3$), 2.86 (s, 3H, C**H_3**), 2.83 (s, 3H, S(O)C**H_3**), 1.77-1.67 (m, 2H, C**H_2**$CH_2N^+(CH_3)_3$), 1.62-1.53 (m, 2H, NHCH_2C**H_2**), 1.41-1.25 (m, 8H, C**H_2**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.3 (**C**(O)NH), 161.0 (**C**-6), 158.4 (**C_q**), 147.9 (**C_q**), 145.6 (**C**-4), 129.6 (**C_q**), 127.1 (**C_q**), 104.5 (**C_q**), 67.9 (**C**$H_2N^+(CH_3)_3$), 54.2 (N^+(**C**H_3)$_3$), 40.6 (**C**H_2NH), 40.1 (S(O)**C**H_3), 30.1 (**C**H_2), 30.0 (**C**H_2), 29.9 (**C**H_2), 27.8 (**C**H_2), 27.6 (**C**H_2), 23.8 (**C**$H_2CH_2N^+(CH_3)_3$), 16.7 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{21}H_{34}ClN_4OS_2^+$: 473.2. Found: 473.2

10-(3-Amino-5-chloro-4-methyl-6-(methylsulfinyl)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyldecan-1-aminium bromide, ***38c*** *(FG_B_142)*



$C_{23}H_{38}BrClN_4O_2S_2$

M_r = 582.06 g/mol

A solution of mCPBA (70 % wt., 95.8 mg, 389 µmol) in DCM (2 ml) was added dropwise to a suspension of compound **37c** (220.0 mg, 389 µmol) and $NaHCO_3$ (35.9 mg, 428 µmol) in DCM (5 ml) at -78 °C. The reaction mixture was worked up according to **G.15** to give **38c**.

yield:	56 mg (25 %)
reaction control:	R_f = 0.44 (alox basic; acetone/ CH_3OH: 10/1; Dragendorff-reagent)
melting point [°C]:	126-129
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3418, 3303, 2925, 2854, 1593, 1531, 1064

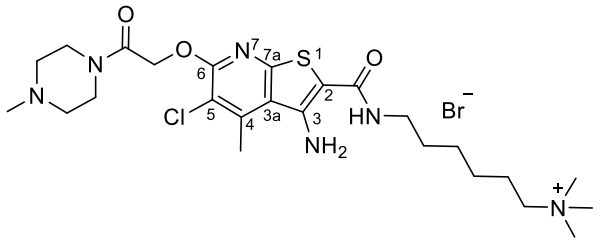
^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.54-6.45 (m, 3H, N**H**, N**H$_2$**), 3.36-3.27 (m, 2H, NHC**H$_2$**), 3.22-3.16 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 2.99 (s, 9H, $N^+(CH_3)_3$), 2.86 (s, 3H, C**H$_3$**), 2.83 (s, 3H, S(O)C**H$_3$**), 1.75-1.65 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.61-1.52 (m, 2H, $NHCH_2$C**H$_2$**), 1.38-1.29 (m, 6H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.2 (**C**(O)NH), 161.0 (**C**-6), 158.4 (**C$_q$**), 147.9 (**C$_q$**), 145.6 (**C**-4), 129.6 (**C$_q$**), 127.1 (**C$_q$**), 104.5 (**C$_q$**), 68.0 (**C**$H_2N^+(CH_3)_3$), 54.2 (N^+(**C**H_3)$_3$), 40.6 (**C**H_2NH), 40.1 (S(O)**C**H_3), 30.7 ($NHCH_2$**C**H_2), 30.4 (**C**H_2), 30.3 (**C**H_2), 30.2 (**C**H_2), 30.0 (**C**H_2), 27.9 (**C**H_2), 27.1 (**C**H_2), 23.8 (**C**$H_2CH_2N^+(CH_3)_3$), 16.7 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{23}H_{38}ClN_4O_2S_2^+$: 501.2. Found: 501.1

Compounds **LY21-An-TMA** were prepared according to a modified version of **G.12** which is further referred to as general procedure **G.12m**. Therein, after quenching of the reaction mixture with water, the mixture was concentrated *in vacuo* and the residue was purified by column chromatography (alox basic, acetone/CH_3OH 100/5). 2-Methoxy-2-propanol, formed during column chromatography, was removed under high vacuum. The remaining colorless oil was recrystallized in a mixture of acetonitrile and diethyl ether (acetonitrile/diethyl ether 1/50) at -10 °C to give **LY21-An-TMA** hybrids

6-(3-Amino-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethylhexan-1-aminium bromide, ***LY21-A6-TMA*** *(FG_B_122)*



$C_{25}H_{40}BrClN_6O_3S$

M_r = 620.05 g/mol

A solution of **34** (96.3 mg, 608 µmol) in dry benzene (10 ml) at 5 °C was treated with LiHMDS (1 M, 304 µl, 304 µmol) under argon atmosphere and stirred for 30 min, followed by the addition of **38a** (80.0 mg, 152 µmol) and further stirring at 5 °C for 30 min. The mixture was heated under reflux for 5 h. Purification according to **G.12m** gave **LY21-A6-TMA** as a white powder.

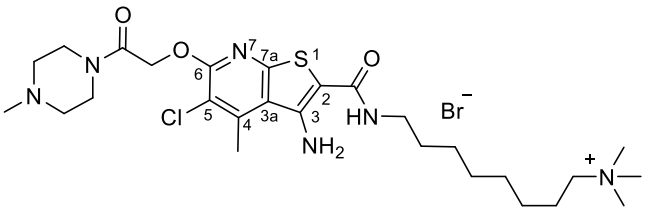
yield:	12 mg (13 %)
HPLC purity:	99 % (HPLC Method **IIb**)
reaction control:	R_f = 0.36 (alox basic; acetone/MeOH: 100/5; Dragendorff-reagent)
melting point [°C]:	137-143
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3494, 3355, 2932, 2791, 1646, 1519, 1442, 1283

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.42 (s, 2H, N**H$_2$**), 6.28 (t, ^3J = 6.0 Hz, 1H, N**H**), 5.12 (s, 2H, OC**H$_2$**C(O)N), 3.54-3.46 (m, 4H, piperazinyl-C**H$_2$**), 3.30 (m, 2H, C**H$_2$**NH), 3.22-3.17 (m, 2H, C**H$_2$**N$^+$(CH$_3$)$_3$), 2.99 (s, 9H, N$^+$(C**H$_3$**)$_3$), 2.80 (s, 3H, C**H$_3$**), 2.46-2.41 (m, 2H, piperazinyl-C**H$_2$**), 2.35-2.31 (m, 2H, piperazinyl-C**H$_2$**), 2.26 (s, 3H, NC**H$_3$**), 1.78-1.68 (m, 2H, C**H$_2$**CH$_2$N$^+$(CH$_3$)$_3$), 1.61-1.53 (m, 2H, NHCH$_2$C**H$_2$**), 1.43-1.41 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.5 (N**C**(O)CH$_2$), 166.5 (**C**(O)NH), 159.0 (**C**-6), 155.0 (**C$_q$**), 148.6 (**C$_q$**), 145.6 (**C**-4), 122.7 (**C$_q$**), 116.8 (**C$_q$**), 99.1 (**C$_q$**), 67.7 (**C**H$_2$N$^+$(CH$_3$)$_3$), 65.2 (O**C**H$_2$C(O)), 55.8 (piperazinyl-**C**H$_2$), 55.5 (piperazinyl-**C**H$_2$), 53.9 (N$^+$(**C**H$_3$)$_3$), 46.3 (N**C**H$_3$), 45.5 (piperazinyl-**C**H$_2$), 42.7 (piperazinyl-**C**H$_2$), 39.2 (**C**H$_2$NH), 30.3 (**C**H$_2$CH$_2$NH), 27.0 (**C**H$_2$), 26.5 (**C**H$_2$), 23.5 (**C**H$_2$CH$_2$N$^+$(CH$_3$)$_3$), 16.8 (**C**H$_3$).

MS (ESI) m/z [M$^+$] Calcd for $C_{25}H_{41}ClN_6O_3S^{2+}$: 270.1. Found: 270.3.

8-(3-Amino-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyloctan-1-aminium bromide, ***LY21-A8-TMA*** *(FG_B_140)*



$C_{27}H_{44}BrClN_6O_3S$

M_r = 648.10 g/mol

A solution of **34** (91.4 mg, 577 µmol) in dry benzene (10 ml) at 5 °C was treated with LiHMDS (1 M, 289 µl, 289 µmol) under argon atmosphere and stirred for 30 min, followed by the addition of **38b** (80.0 mg, 144 µmol) and further stirring at 5 °C for 30 min. The mixture was heated under reflux for 5 h. Purification according to **G.12m** gave **LY21-A8-TMA** as a white powder.

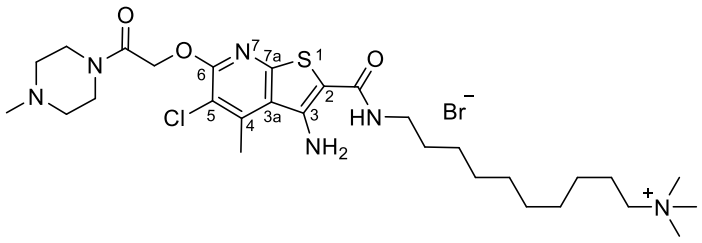
yield:	13 mg (14 %)
HPLC purity:	100 % (HPLC Method **IIb**)
reaction control:	R_f = 0.34 (alox basic; acetone/MeOH: 100/5; Dragendorff-reagent)
melting point [°C]:	145-150
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3417, 3320, 2928, 2856, 1659, 1596, 1449, 1288

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.40 (s, 2H, N**H$_2$**), 6.25 (t, ^3J = 5.6 Hz, 1H, N**H**), 5.12 (s, 2H, OC**H$_2$**C(O)N), 3.55-3.47 (m, 4H, piperazinyl-C**H$_2$**), 3.29 (m, 2H, C**H$_2$**NH), 3.20-3.14 (m, 2H, C**H$_2$**N$^+$(CH$_3$)$_3$), 2.98 (s, 9H, N$^+$(C**H$_3$**)$_3$), 2.79 (s, 3H, C**H$_3$**), 2.49-2.42 (m, 2H, piperazinyl-C**H$_2$**), 2.39-2.32 (m, 2H, piperazinyl-C**H$_2$**), 2.28 (s, 3H, NC**H$_3$**), 1.75-1.66 (m, 2H, C**H$_2$**CH$_2$N$^+$(CH$_3$)$_3$), 1.59-1.50 (m, 2H, NHCH$_2$C**H$_2$**), 1.42-1.30 (m, 8H, C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.8 (N**C**(O)CH$_2$), 166.7 (**C**(O)NH), 159.3 (**C**-6), 155.2 (**C$_q$**), 148.8 (**C$_q$**), 145.8 (**C**-4), 123.0 (**C$_q$**), 117.0 (**C$_q$**), 99.5 (**C$_q$**), 70.4 (**C**H$_2$N$^+$(CH$_3$)$_3$), 65.4 (O**C**H$_2$C(O)), 56.1 (piperazinyl-**C**H$_2$), 55.8 (piperazinyl-**C**H$_2$), 54.2 (N$^+$(**C**H$_3$)$_3$), 46.7 (N**C**H$_3$), 45.8 (piperazinyl-**C**H$_2$), 43.0 (piperazinyl-**C**H$_2$), 40.4 (**C**H$_2$NH), 30.8 (**C**H$_2$CH$_2$NH), 30.0 (**C**H$_2$), 29.9 (**C**H$_2$), 27.9 (**C**H$_2$), 27.0 (**C**H$_2$), 23.8 (**C**H$_2$CH$_2$N$^+$(CH$_3$)$_3$), 17.1 (**C**H$_3$).

MS (ESI) m/z [M$^+$] Calcd for $C_{27}H_{45}ClN_6O_3S^{2+}$: 284.2. Found: 284.3.

10-(3-Amino-5-chloro-4-methyl-6-(2-(4-methylpiperazin-1-yl)-2-oxoethoxy)thieno[2,3-b]pyridine-2-carboxamido)-N,N,N-trimethyldecan-1-aminium, ***LY21-A10-TMA*** *(FG_B_145)*



$C_{27}H_{44}BrClN_6O_3S$

M_r = 676.16 g/mol

A solution of **34** (101.1 mg, 639 µmol) in dry benzene (10 ml) at 5 °C was treated with LiHMDS (1 M, 320 µl, 320 µmol) under argon atmosphere and stirred for 30 min, followed by the addition of **38c** (93.0 mg, 160 µmol) and further stirring at 5 °C for 30 min. The mixture was heated under reflux for 5 h. Purification according to **G.12m** gave **LY21-A10-TMA** as a white powder.

yield:	11 mg (10 %)
HPLC purity:	96 % (HPLC Method **IIb**)
reaction control:	R_f = 0.33 (alox basic; acetone/MeOH: 100/5; Dragendorff-reagent)
melting point [°C]:	154-159
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3328, 2954, 2925, 2857, 1647, 1594, 1457, 1285

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.42 (s, 2H, N**H$_2$**), 6.23 (t, ^3J = 5.6 Hz, 1H, N**H**), 5.13 (s, 2H, OC**H$_2$**C(O)N), 3.56-3.48 (m, 4H, piperazinyl-C**H$_2$**), 3.28 (m, 2H, C**H$_2$**NH), 3.19-3.13 (m, 2H, C**H$_2$**N$^+$(CH$_3$)$_3$), 2.98 (s, 9H, N$^+$(C**H$_3$**)$_3$), 2.81 (s, 3H, C**H$_3$**), 2.51-2.44 (m, 2H, piperazinyl-C**H$_2$**), 2.40-2.34 (m, 2H, piperazinyl-C**H$_2$**), 2.29 (s, 3H, NC**H$_3$**), 1.73-1.63 (m, 2H, C**H$_2$**CH$_2$N$^+$(CH$_3$)$_3$), 1.58-1.50 (m, 2H, C**H$_2$**), 1.38-1.27 (m, 12H, C**H$_2$**).

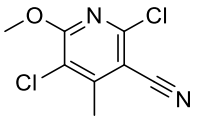
^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 166.8 (N**C**(O)CH$_2$), 166.7 (**C**(O)NH), 159.3 (**C**-6), 155.2 (**C$_q$**), 148.8 (**C$_q$**), 145.9 (**C**-4), 123.0 (**C$_q$**), 117.0 (**C$_q$**), 99.6 (**C$_q$**), 68.0 (**C**H$_2$N$^+$(CH$_3$)$_3$), 65.5 (O**C**H$_2$C(O)), 56.0 (piperazinyl-**C**H$_2$), 55.7 (piperazinyl-**C**H$_2$), 54.2 (N$^+$(**C**H$_3$)$_3$), 46.6 (N**C**H$_3$), 45.7 (piperazinyl-**C**H$_2$), 42.9 (piperazinyl-**C**H$_2$), 40.4 (**C**H$_2$NH), 30.8 (**C**H$_2$CH$_2$NH), 30.4 (**C**H$_2$), 30.3 (**C**H$_2$), 30.3 (**C**H$_2$), 30.0 (**C**H$_2$), 28.0 (**C**H$_2$), 27.1 (**C**H$_2$), 23.8 (**C**H$_2$CH$_2$N$^+$(CH$_3$)$_3$), 17.1 (**C**H$_3$).

MS (ESI) m/z [M$^+$] Calcd for $C_{29}H_{49}ClN_6O_3S^{2+}$: 298.2. Found: 298.5.

7.4 Synthesis of LY20-MQn hybrids

7.4.1 Preparation of thienopyridine carboxylic acid scaffold 39

*2,5-Dichloro-6-methoxy-4-methylnicotinonitrile, **40** (FG_B_8)*



$C_8H_6Cl_2N_2O$

M_r = 217.05 g/mol

According to Rubio *et al.*[67], **29** (1.0 g, 4.5 mmol) was suspended in dry methanol (35 ml) under argon atmosphere and cooled to 0 °C in an ice bath. After dropwise addition of a methanolic sodium methoxide solution (25 % wt., 1.14 ml, 5.0 mmol), the mixture was stirred for 1 h at 0 °C before the ice bath was removed and stirring continued at rt for 2 h. The suspension was quenched with water (50 ml) and stirred in an ice bath for 30 min. The precipitated white solid was filtered and dried *in vacuo* to give product **40**.

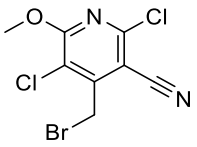
yield:	940 mg (96 %, Lit.:[67] 93 %)
reaction control:	R_f = 0.26 (silica gel; EtOAc/*n*-hexane: 1/30)
melting point [°C]:	144-150
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2961, 2226, 1579, 1476, 1367, 1168, 1083

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.08 (s, 3H, OC**H$_3$**), 2.59 (s, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 160.7 (**C$_q$**), 152.3 (**C$_q$**), 149.4 (**C$_q$**), 117.5 (**C$_q$**), 114.3 (**C$_q$**), 104.9 (**C$_q$**), 56.1 (O**C**H$_3$), 19.3 (**C**H$_3$).

Rubio *et al.* did not provide a melting point.

*4-(Bromomethyl)-2,5-dichloro-6-methoxynicotinonitrile, **41** (FG_B_49)*



$C_8H_5BrCl_2N_2O$

M_r = 295.95 g/mol

Compound **40** (1.5 g, 6.9 mmol) was dissolved in CCl_4 (40 ml) in a sealed pressure tube. CCl_4 was pre-dried over barium carbonate prior to use. After the addition of *N*-bromosuccinimide (3.7 g, 21.0 mmol) and benzoyl peroxide (167.4 mg, 0.7 mmol), the mixture was heated at 110 °C for a total of 3 d. The reaction mixture was cooled to rt, quenched with water (30 ml), and extracted with DCM (3x20 ml). After phase separation, the combined organic layers were

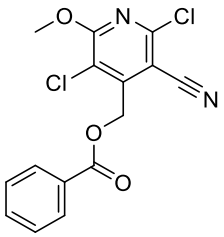
dried over magnesium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (silica, Cy/EtOAc 100/3 to 10/1) to give compound **41** as pale white solid.

yield:	1.2 g (59 %)
reaction control:	R_f = 0.15 (silica gel; EtOAc/*n*-hexane: 1/30)
melting point [°C]:	78-79
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2999, 2933, 2228, 1573, 1475, 1382, 1174, 1070

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.61 (s, 2H, C**H$_2$**Br), 4.11 (s, 3H, OC**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 161.3 (**C$_q$**), 150.3 (**C$_q$**), 150.2 (**C$_q$**), 117.7 (**C$_q$**), 113.3 (**C$_q$**), 103.4 (**C$_q$**), 56.6 (O**C**H$_3$), 24.7 (**C**H$_2$Br).

*(2,5-Dichloro-3-cyano-6-methoxypyridin-4-yl)methyl benzoate, **43** (FG_B_71)*



$C_{15}H_{10}Cl_2N_2O_3$

M_r = 337.16 g/mol

A solution of compound **41** (700.0 mg, 2.4 mmol) and sodium benzoate (340.8 mg, 2.4 mmol) in DMF (10 ml) was stirred at rt for 2 h. After addition of diethyl ether (20 ml), the mixture was washed with water (2x10 ml). After phase separation, the organic layer was dried over sodium sulfate and the solvent was removed to give product **43** as pale white solid.

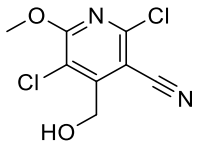
yield:	750 mg (94 %)
reaction control:	R_f = 0.52 (silica gel; EtOAc/Cy: 1/9)
melting point [°C]:	97–98
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3066, 3034, 2960, 2935, 2226, 1721, 1567, 1363

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 8.06-8.02 (m, 2H, ortho-bn-C**H**), 7.61-7.54 (dd, ^3J = 10.5 Hz, ^4J = 4.4 Hz, 1H, para-bn-C**H**), 7.46-7.40 (m, 2H, meta-bn-C**H**), 5.54 (s, 2H, C**H$_2$**), 4.10 (s, 3H, OC**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.9 (**C**(O)OCH_2), 161.2 (pyridinyl-**C$_q$**), 150.5 (pyridinyl-**C$_q$**), 148.4 (pyridinyl-**C$_q$**), 133.9 (para-bn-**C**), 130.2 (ortho-bn-**C**), 128.9 (benzylic-

C$_q$), 128.8 (meta-bn-**C**), 118.6 (pyridinyl-**C$_q$**), 113.7 (**C**N), 104.6 (pyridinyl-**C$_q$**), 61.4 (**C**H$_2$), 56.6 (O**C**H$_3$).

*2,5-Dichloro-4-(hydroxymethyl)-6-methoxynicotinonitrile, **44** (FG_B_78)*



$C_8H_6Cl_2N_2O_2$

M_r = 233.05 g/mol

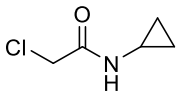
A suspension of compound **43** (730.0 mg, 2.2 mmol) in dry methanol (20 ml) was cooled to 0 °C in an ice bath under argon atmosphere. After dropwise addition of methanolic sodium methoxide solution (25 % wt., 495 µl, 2.2 mmol), the reaction mixture was warmed to rt and stirred for 15 min to give a clear, green solution. The solution was treated with an excess of ammonium chloride and stirred for further 10 min. The mixture was concentrated *in vacuo*, suspended in a 9/1 mixture of DCM/methanol (20 ml) and filtered. The filtrate was concentrated *in vacuo* and purified by column chromatography (silica, $CHCl_3$/CH_3OH 50/1) to give product **44** as orange solid.

yield:	414 mg (82 %)
reaction control:	R_f = 0.63 (silica gel; $CHCl_3$/CH_3OH: 50/1)
melting point [°C]:	127-132
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3335, 3208, 2963, 2935, 2224, 1766, 1673, 1607, 1472.

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 5.20 (s, 2H, C**H$_2$**OH), 4.10 (s, 3H, OC**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 160.4 (**C$_q$**) 156.9 (**C$_q$**), 155.2 (**C$_q$**), 142.7 (**C$_q$**), 116.5 (**C**N), 110.5 (**C$_q$**), 69.0 (**C**H$_2$OH), 56.4 (O**C**H$_3$).

*2-Chloro-N-cyclopropylacetamide, **45** (FG_B_1)*



C_5H_8ClNO

M_r = 133.58 g/mol

According to Rubio *et al.*[67], 2-chloroacetyl chloride (5.1 ml, 64.6 mmol) was added dropwise to an ice cooled solution of cyclopropylamine (9.0 ml, 129.3 mmol) in DCM (60 ml) and stirred for 4 h at 0 °C. The mixture was filtered through a pad of kieselgur and the filtrate was concentrated *in vacuo*. The resulting pinkish solid was slurried in *n*-hexane (100 ml), filtered, and air-dried to give **45** as pinkish solid.

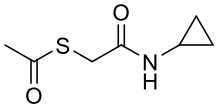
yield:	6.0 g (85 %, Lit.:[67] 99 %)
melting point [°C]:	71-76 °C (Lit.:[147] 74-78 °C)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3267, 3064, 3017, 2877, 1645, 1546, 1246

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.63 (s, 1H, **N**H), 3.99 (s, 2H, C**H$_2$**), 2.76-2.68 (m, 1H, cyclopropyl-C**H**), 0.83-0.76 (m, 2H, cyclopropyl-C**H$_2$**), 0.57-0.52 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 167.5 (**C**(O)NH), 42.7 (**C**H$_2$), 23.0 (cyclopropyl-**C**H), 6.6 (cyclopropyl-**C**H$_2$).

The obtained physical and spectroscopic data are consistent with that found in literature.[147]

S-(2-(Cyclopropylamino)-2-oxoethyl) ethanethioate, ***45*** *(FG_B_2)*



$C_7H_{11}NO_2S$

M_r = 173.23 g/mol

According to Rubio *et al.*[67], compound **45** (5.2 g, 38.9 mmol) was dissolved in DCM (85 ml), cooled to 0 °C in an ice bath, and treated with thiolactic acid (2.9 ml, 40.9 mmol). TEA (10.8 ml, 77.9 mmol) was added dropwise and the reaction mixture was stirred at 0 °C for 2 h. The mixture was poured into water (120 ml), followed by stirring at rt for 10 min. The pH of the aqueous phase was adjusted to 2 by addition of 2 M hydrochloric acid. The phases were separated and the aqueous phase was extracted with DCM (2x50 ml). After phase separation, the combined organic layers were washed with brine (30 ml). Phases were again separated and the organic layer was dried over sodium sulfate and concentrated *in vacuo*. The brownish residue was treated with cyclohexane (30 ml) and the resulting beige solid was filtered and air-dried to give **46** as beige solid.

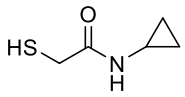
yield:	5.8 g (86 %, Lit.:[67] 99 %)
melting point [°C]:	77
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3301, 3060, 3011, 2982, 2875, 1645, 1548

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.21 (s, 1H, N**H**), 3.45 (s, 2H, SC**H$_2$**), 2.68-2.64 (m, 1H, cyclopropyl-C**H**), 2.38 (s, 3H, C**H$_3$**), 0.77-0.71 (m, 2H, cyclopropyl-C**H$_2$**), 0.49-0.45 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 195.7 (**C**(O)S), 169.6 (**C**(O)NH), 33.0 (S**C**H_2), 30.3 (**C**H_3), 22.9 (cyclopropyl-**C**H), 6.5 (cyclopropyl-**C**H_2).

Rubio *et al.* did not provide NMR data, IR data, or a melting point.

N-Cyclopropyl-2-mercaptoacetamide, ***42*** *(FG_B_4)*



C_5H_9NOS

M_r = 131.19 g/mol

Following a modified procedure from Rubio *et al.*[67], dry methanol (20 ml) was degassed for 30 min and treated with compound **46** (3.0 g, 17.3 mmol). After the addition of aqueous ammonia solution (25 % wt., 5.4 ml, 72.1 mmol), the reaction mixture was stirred at rt for 10 min. The mixture was poured into water (100 ml) and pH 2 was adjusted with 3 M hydrochloric acid, followed by extraction with DCM (2x30 ml). The combined organic layers were dried over sodium sulfate and concentrated *in vacuo* to give **42** as orange oil. The product is sensible to air and should be used directly.

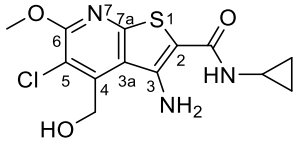
yield: 1.2 g (53 %, Lit.:[67] 99 %)

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.79 (s, 1H, N**H**), 3.19 (d, ^{3}J = 9.0 Hz, 2H, SC**H$_2$**), 2.74-2.69 (m, 1H, cyclopropyl-C**H**), 1.87 (t, ^{3}J = 9.0 Hz, 1H, S**H**), 0.82-0.76 (m, 2H, cyclopropyl-C**H$_2$**), 0.55-0.51 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 170.8 (**C**(O)NH), 28.4 (S**C**H_2), 23.1 (cyclopropyl-**C**H), 6.7 (cyclopropyl-**C**H_2).

Rubio *et al.* did not provide NMR data, IR data, or a melting point.

3-Amino-5-chloro-N-cyclopropyl-4-(hydroxymethyl)-6-methoxythieno[2,3-b]pyridine-2-carboxamide, ***47*** *(FG_B_79)*



$C_{13}H_{14}ClN_3O_3S$

M_r = 327.78 g/mol

A solution of **44** (250.0 mg, 1.1 mmol) in dry ethanol (30 ml) was treated with sodium carbonate (227.4 mg, 2.2 mmol) under argon atmosphere, followed by the addition of compound **42** (168.9 mg, 1.3 mmol). The reaction mixture was heated under reflux for 3 h. After cooling to rt, the reaction mixture was treated with water (10 ml) and stirred at 0 °C for

20 min. The thereby formed precipitate was filtered and dried *in vacuo* to give compound **47** as white solid.

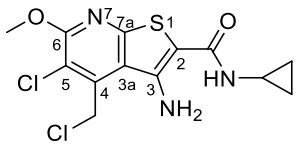
yield:	278 mg (78 %)
melting point [°C]:	105
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3324, 3289, 3070, 2953, 2834, 1641, 1524, 1471, 1345

^{1}H NMR (400 MHz, DMSO-d$_6$, δ [ppm]): 7.71 (d, ^{3}J = 3.2 Hz, 1H, N**H**), 7.28 (s, 2H, N**H$_2$**), 6.56 (s, 1H, O**H**), 4.99 (s, 2H, C**H$_2$**OH), 4.01 (s, 3H, OC**H$_3$**), 2.80 (m, 1H, cyclopropyl-C**H**), 0.69-0.63 (m, 2H, cyclopropyl-C**H$_2$**), 0.58-0.54 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, DMSO-d$_6$, δ [ppm]): 166.3 (**C**(O)NH), 158.3 (**C**-6), 154.7 (**C$_q$**), 147.1 (**C$_q$**), 144.7 (**C**-4), 121.1 (**C**-3a), 114.1 (**C**-5), 97.1 (**C$_q$**), 57.2 (**C**H$_2$OH), 55.0 (O**C**H$_3$), 22.8 (cyclopropyl-**C**H), 5.9 (cyclopropyl-**C**H$_2$).

MS (ESI) m/z [M$^+$] Calcd for $C_{13}H_{15}ClN_3O_3S^+$: 328.1. Found: 328.0.

*3-Amino-5-chloro-4-(chloromethyl)-N-cyclopropyl-6-methoxythieno[2,3-b]pyridine-2-carboxamide, **39** (FG_B_99)*



$C_{13}H_{13}Cl_2N_3O_2S$

M_r = 346.23 g/mol

A suspension of compound **47** (150.0 mg, 457.6 µmol) in dry DCM (10 ml) was cooled to 0 °C under argon atmosphere. TEA (104.7 µl, 755.1 µmol) and 4-dimethylaminopyridine (5.6 mg, 45.8 µmol) were subsequently added, followed by the addition of tosyl chloride (218.1 mg, 1.1 mmol) in small portions. The reaction mixture was stirred for 1 h at 0 °C, allowed to warm to rt, and heated under reflux for 3 d. After cooling to rt, the mixture was quenched with water (10 ml), stirred for 30 min, and extracted with DCM (3x20 ml). After phase separation, the combined organic layers were dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (silica, EtOAc/Cy 1/1) to give product **39** as yellow solid.

yield:	123 mg (78 %)
reaction control:	R_f = 0.83 (silica, EtOAc/Cy 1/1)
melting point [°C]:	decomposition >190

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3403, 3305, 2925, 1454, 1596, 1498, 1267

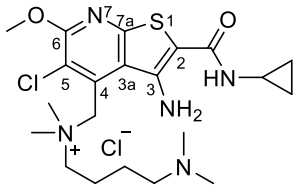
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 6.37 (s, 2H, N**H$_2$**), 5.60 (s, 1H, N**H**), 5.14 (s, 2H, C**H$_2$**Cl), 4.07 (s, 3H, OC**H$_3$**), 2.85-2.78 (m, 1H, cyclopropyl-C**H**), 0.88-0.82 (m, 2H, cyclopropyl-C**H$_2$**), 0.64-0.59 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 167.0 (**C**(O)NH), 159.4 (**C**-6), 155.1 (**C$_q$**), 146.7 (**C$_q$**), 140.9 (**C**-4), 120.7 (**C**-3a), 117.3 (**C**-5), 101.1 (**C$_q$**), 55.6 (O**C**H$_3$), 38.2 (**C**H$_2$Cl), 23.2 (cyclopropyl-**C**H), 7.2 (cyclopropyl-**C**H$_2$).

MS (ESI) m/z [M^+] Calcd for $C_{13}H_{13}Cl_2N_3O_2S^+$: 346.0. Found: 346.0.

7.4.2 Preparation of LY20-MQn-iper hybrids

N-((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)-methyl)-4-(dimethylamino)-N,N-dimethylbutan-1-aminium chloride, ***48a*** *(FG_B_156)*



$C_{21}H_{33}Cl_2N_5O_2S$

M_r = 490.49 g/mol

A solution of **39** (60.0 mg, 173 µmol) and 1,4-bis(dimethylamino)butane (378.8 µl, 2.1 mmol) in acetonitrile (5 ml) was stirred at 0 °C for 6 h. The solution was treated with cooled diethyl ether and the formed precipitate was filtered off, washed with diethyl ether, and dried *in vacuo* to give **48a** as yellow solid.

yield: 72 mg (84 %)

melting point [°C]: 161-168

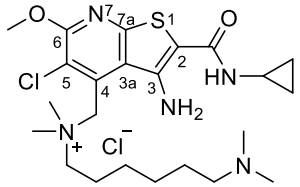
IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3186, 2945, 2860, 1593, 1521, 1370, 1274

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.86 (s, 2H, N**H$_2$**), 6.68 (s, 1H, N**H**), 4.09 (s, 3H, OC**H$_3$**), 3.57 (bs, 2H, C**H$_2$**N$^+$(CH$_3$)$_2$), 3.03 (bs, 6H, N$^+$(C**H$_3$**)$_2$), 2.83-2.75 (m, 1H, cyclopropyl-C**H**), 2.31-2.25 (m, 4H, CH$_2$C**H$_2$**N$^+$(CH$_3$)$_2$, C**H$_2$**N(CH$_3$)$_2$), 2.15 (s, 6H, N(C**H$_3$**)$_2$), 1.94-1.86 (m, 2H, C**H$_2$**CH$_2$N(CH$_3$)$_2$), 1.54-1.43 (m, 2H, C**H$_2$**CH$_2$N$^+$(CH$_3$)$_2$), 0.76-0.71 (m, 2H, cyclopropyl-C**H$_2$**), 0.66-0.61 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 167.5 (**C**(O)NH), 160.5 (**C**-6), 157.0 (**C$_q$**), 147.2 (**C$_q$**), 133.8 (**C$_q$**), 123.6 (**C$_q$**), 122.4 (**C$_q$**), 105.8 (**C$_q$**), 68.2 (**C**H$_2$N$^+$(CH$_3$)$_2$), 60.3 (**C**H$_2$N(CH$_3$)$_2$), 59.6 (**C**H$_2$N$^+$(CH$_3$)$_2$), 56.6 (O**C**H$_3$), 53.2 (N$^+$(**C**H$_3$)$_2$), 46.0 (N(**C**H$_3$)$_2$), 25.2 (**C**H$_2$), 24.2 (cyclopropyl-**C**H), 21.8 (**C**H$_2$CH$_2$N(CH$_3$)$_2$), 6.93 (cyclopropyl-**C**H$_2$).

MS (ESI) m/z [M^+] Calcd for $C_{21}H_{34}ClN_5O_2S^{2+}$: 227.6 Found: 227.8.

N-((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)-methyl)-6-(dimethylamino)-N,N-dimethylhexan-1-aminium chloride, ***48b*** *(AN_17)*



$C_{23}H_{37}Cl_2N_5O_2S$

M_r = 518.54 g/mol

A solution of **39** (70.0 mg, 202 µmol) and 1,6-bis(dimethylamino)hexane (518.7 µl, 2.4 mmol) in acetonitrile (5 ml) was stirred at 50 °C for 2 h. The solution was treated with cooled diethyl ether and the formed precipitate was filtered off, washed with diethyl ether, and dried *in vacuo* to give **48b** as yellow solid.

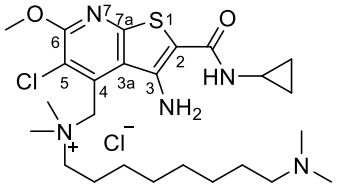
yield:	97 mg (92 %)
melting point [°C]:	152-157
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3187, 2937, 1597, 1367, 1276

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 6.88 (s, 2H, N**H$_2$**), 6.69 (s, 1H, N**H**), 4.09 (s, 3H, OC**H$_3$**), 3.55 (bs, 2H, C**H$_2$**$N^+(CH_3)_2$), 3.02 (bs, 6H, $N^+(CH_3)_2$), 2.82-2.75 (m, 1H, cyclopropyl-C**H**), 2.36-2.22 (m, 10H, $CH_2CH_2N^+(CH_3)_2$, $N(CH_3)_2$, $CH_2N(CH_3)_2$), 1.94-1.85 (m, 2H, $CH_2CH_2N(CH_3)_2$), 1.55-1.47 (m, 2H, $CH_2CH_2N^+(CH_3)_2$), 1.43-1.32 (m, 4H, C**H$_2$**), 0.76-0.71 (m, 2H, cyclopropyl-C**H$_2$**), 0.66-0.60 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 167.5 (**C**(O)NH), 160.5 (**C**-6), 157.0 (**C$_q$**), 147.3 (**C$_q$**), 133.8 (**C$_q$**), 123.6 (**C$_q$**), 122.4 (**C$_q$**), 105.8 (**C$_q$**), 68.5 ($CH_2N^+(CH_3)_2$), 60.1 ($CH_2N(CH_3)_2$), 60.1 ($CH_2N^+(CH_3)_2$), 56.6 (O**C**H_3), 51.5 ($N^+(CH_3)_2$), 45.6 ($N(CH_3)_2$), 27.8 (CH_2), 27.6 (CH_2), 27.0 (CH_2), 24.2 (cyclopropyl-**C**H), 23.7 ($CH_2CH_2N(CH_3)_2$), 6.93 (cyclopropyl-**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{23}H_{38}ClN_5O_2S^{2+}$: 241.6 Found: 241.8.

N-((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)-methyl)-6-(dimethylamino)-N,N-dimethylhexan-1-aminium chloride, ***48c*** *(FG_B_130)*



$C_{25}H_{41}Cl_2N_5O_2S$

M_r = 546.60 g/mol

A solution of **39** (20.0 mg, 58 µmol) and 1,8-bis(dimethylamino)octane (138.9 mg, 693 µmol) in acetonitrile (5 ml) was stirred at 50 °C for 2 h. The solution was treated with cooled diethyl ether and the formed precipitate was filtered off, washed with diethyl ether, and dried *in vacuo* to give **48c** as yellow solid.

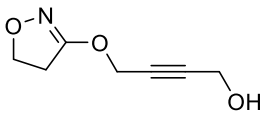
yield:	27 mg (84 %)
melting point [°C]:	158-167
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2928, 2855, 1597, 1522, 1466, 1367, 1269

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.28 (s, 2H, N**H$_2$**), 6.18 (s, 1H, N**H**), 4.07 (s, 3H, OC**H$_3$**), 3.76 (bs, 2H, C**H$_2$**N$^+$(CH$_3$)$_2$), 3.19 (bs, 6H, N$^+$(C**H$_3$**)$_2$), 2.83-2.75 (m, 1H, cyclopropyl-C**H**), 2.45-2.38 (m, 2H, C**H$_2$**N$^+$(CH$_3$)$_2$), 2.35-2.20 (s, 8H, N(C**H$_3$**)$_2$, C**H$_2$**N(CH$_3$)$_2$), 1.97-1.85 (m, 2H, C**H$_2$**CH$_2$N(CH$_3$)$_2$), 1.56-1.46 (m, 2H, C**H$_2$**CH$_2$N$^+$(CH$_3$)$_2$), 1.44-1.22 (m, 8H, C**H$_2$**), 0.85-0.78 (m, 2H, cyclopropyl-C**H$_2$**), 0.70-0.62 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.4 (**C**(O)NH), 158.8 (**C**-6), 156.0 (**C$_q$**), 146.0 (**C$_q$**), 132.3 (**C$_q$**), 122.8 (**C$_q$**), 120.7 (**C$_q$**), 104.7 (**C$_q$**), 67.9 (**C**H$_2$N$^+$(CH$_3$)$_2$), 59.8 (**C**H$_2$N(CH$_3$)$_2$), 59.5 (**C**H$_2$N$^+$(CH$_3$)$_2$), 55.8 (O**C**H$_3$), 50.9 (N$^+$(**C**H$_3$)$_2$), 45.2 (N(**C**H$_3$)$_2$), 29.0 (**C**H$_2$), 29.0 (**C**H$_2$), 27.0 (**C**H$_2$), 27.0 (**C**H$_2$), 26.3 (**C**H$_2$), 23.4 (**C**H$_2$CH$_2$N(CH$_3$)$_2$), 23.3 (cyclopropyl-**C**H), 7.0 (cyclopropyl-**C**H$_2$).

MS (ESI) m/z [M$^+$] Calcd for $C_{25}H_{42}ClN_5O_2S^{2+}$: 255.6 Found: 255.9.

4-((4,5-Dihydroisoxazol-3-yl)oxy)but-2-yn-1-ol, ***50*** *(AN10)*



$C_7H_9NO_3$

M_r = 155.15 g/mol

According to a previously published procedure[122, 123], 2-butyne-1,4-diol (1.5 g, 17.5 mmol) was suspended in dry THF (25 ml) under argon atmosphere. After the addition of sodium hydride (769.1 mg, 19.2 mmol), the reaction mixture was stirred at rt for 1 h. A solution of **11**

(2.1 g, 17.8 mmol) in dry THF (10 ml) was added dropwise, followed by heating under reflux for 3 h. After cooling to rt, water (20 ml) was added and the mixture was extracted with chloroform (3x30 ml). After phase separation, the combined organic layers were dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude product was purified by column chromatography (silica gel, $CHCl_3/CH_3OH$ 100/1 → 1/100) to give **50**.

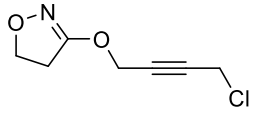
appearance:	yellow oil
yield:	1.3 g (49 %, Lit.:[122, 123] 51 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3395, 2935, 2887, 1625, 1334, 1254, 1136

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.81 (t, ^5J = 1.9 Hz, 2H, OC**H$_2$**C), 4.43 (t, ^3J = 9.5 Hz, 2H, NOC**H$_2$**), 4.34 (dt, ^5J = 1.9 Hz, ^3J = 6.3 Hz, C**H$_2$**OH), 3.00 (t, ^3J = 9.5 Hz, 2H, OCH_2C**H$_2$**), 1.67 (t, ^3J = 6.3 Hz, 1H, OH).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 168.5 (N**C**O), 77.4 (**C**≡**C**), 70.1 (NC**C**H_2), 58.2 (O**C**H_2C), 51.3 (C**C**H_2OH), 33.3 (OCH_2**C**H_2).

The obtained physical and spectroscopic data are consistent with that found in literature.[122, 123]

3-((4-Chlorobut-2-yn-1-yl)oxy)-4,5-dihydroisoxazole, ***49*** *(AN11)*



$C_7H_8ClNO_2$

M_r = 173.60 g/mol

According to Di Amici *et al.*[123], **50** (1.2 g, 8.0 mmol) was dissolved in a mixture of benzene (2 ml) and dichloromethane (2 ml). Then, the solution was cooled to 0 °C and pyridine (701.1 mg, 8.9 mmol) and thionyl chloride (620 µl, 8.5 mmol) were subsequently added. The reaction mixture was stirred at rt for 20 h and quenched with ice cold water (10 ml). The mixture was extracted with ethyl acetate (3x20 ml). After phase separation, the combined organic layers were washed with saturated $NaHCO_3$ solution (20 ml), water (20 ml), and brine (20 ml), respectively. Phases were separated and the organic layer was dried over Na_2SO_4. The solvent was removed and the obtained crude product was purified by column chromatography (silica gel, Cy/EtOAc 1/1) to give **49**.

appearance:	colorless oil
yield:	480 mg (35 %, Lit.:[122, 123] 36 %)
reaction control:	R_f = 0.63 (silica gel; Cy/EtOAc: 1/1)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 2994, 2953, 2887, 1262, 1154.

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.83 (t, ^{5}J = 1.8 Hz, 2H, OC**H$_2$**C), 4.43 (t, ^{3}J = 9.6 Hz, 2H, NOC**H$_2$**), 4.19 (t, ^{5}J = 1.8 Hz, C**H$_2$**Cl), 3.00 (t, ^{3}J = 9.6 Hz, 2H, OCH_2C**H$_2$**).

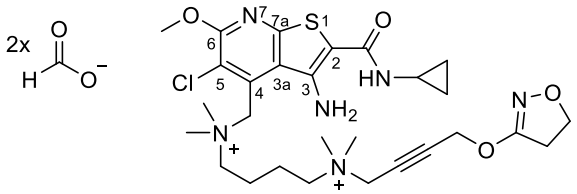
^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.8 (N**C**O), 82.8 ($ClCH_2$**C**≡C), 77.4 (OCH_2**C**≡C), 70.2 (NC**C**H_2), 58.0 (O**C**H_2C), 33.2 (OCH_2**C**H_2), 30.2 (**C**H_2Cl).

The obtained physical and spectroscopic data are consistent with that found in literature.[123]

*General procedure **G.16** for synthesis of **LY20-MQn-iper hybrids***

Compound **49** (5.0 equiv) and the appropriate orthosteric linker (12.0 equiv) were dissolved in acetonitrile and stirred at 50 °C for 4 h. After cooling to rt, the solvent was removed and the residue was diluted in only a few drops of acetonitrile. The addition of diethyl ether (20 ml) caused precipitation of the crude product. The solvent was decanted and the residue was dried under vacuum. Product was obtained after purification by flash chromatography (reversed phase, H_2O + 0.1 % FA/MeOH + 0.1 % FA 10/3).

*N^1-((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)methyl)-N^4-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N^1,N^1,N^4,N^4-tetramethylbutane-1,4-diaminium chloride, **LY20-MQ4-iper** (FG_B_164)*



$C_{30}H_{43}ClN_6O_8S$

M_r = 683.22 g/mol

A solution of **48a** (55.0 mg, 122 µmol) and **49** (518.7 µl, 561 µmol) in acetonitrile (5 ml) was stirred at 50 °C for 4 h and worked up according to **G.16** to give **LY20-MQ4-iper** as colorless solid.

yield:	16 mg (21 %)
HPLC purity:	98 % (HPLC Method **IIb**)
melting point [°C]:	decomposition >160
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3229, 2962, 2875, 1587, 1538, 1484, 1343, 966

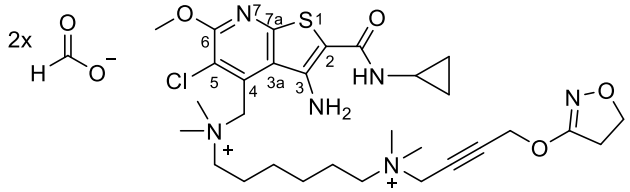
^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 8.52 (s, 2H, **H**COO^-), 5.50 (bs, 1H, C**H**$_2N^+(CH_3)_2$), 5.04 (bs, 1H, C**H**$_2N^+(CH_3)_2$), 4.93 (s, 2H, OC**H$_2$**C≡C), 4.42-4.34 (m, 4H, isoxazolinyl-OC**H$_2$**, $N^+(CH_3)_2$C**H$_2$**-C≡C), 4.14 (s, 3H, OC**H$_3$**), 3.78–3.60 (m, 2H, $N^+(CH_3)_2$C**H$_2$**$CH_2N^+(CH_3)_2$-CH_2C≡C), 3.55–3.48 (m, 2H, C**H$_2$**$N^+(CH_3)_2CH_2$C≡C), 3.20 (s, 12H, N^+(C**H$_3$**)$_2$), 3.05 (t, 2H, ^3J = 9.6 Hz, isoxazolinyl-OCH_2C**H$_2$**), 2.82-2.75 (m, 1H, cyclopropyl-C**H**), 2.08-1.98 (m, 2H, $N^+(CH_3)_2CH_2$C**H$_2$**$(CH_2)_2N^+(CH_3)_2CH_2$C≡C), 1.93-1.84 (bs, 2H, C**H$_2$**$CH_2N^+(CH_3)_2CH_2$C≡C), 0.83-0.78 (m, 2H, cyclopropyl-C**H$_2$**), 0.66-0.61 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 166.9, 166.8, 165.0, 158.6, 146.3, 132.9, 122.0, 120.5, 103.2, 84.1, 76.2, 69.6, 64.5, 57.3, 55.4, 49.9, 49.8, 45.4, 33.3, 29.2, 23.0, 23.0, 19.2, 5.8.

Due to a too low concentration of the NMR sample and solubility problems, some signals were not detected. Assignment of ^{13}C signals could neither be performed.

MS (ESI) m/z [M^+] Calcd for $C_{28}H_{41}ClN_6O_4S^{2+}$: 296.1 Found: 296.5.

N^1-((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)methyl)-N^6-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N^1,N^1,N^6,N^6-tetramethylhexane-1,6-diaminium chloride, ***LY20-MQ6-iper*** *(AN_18)*



$C_{32}H_{47}ClN_6O_8S$

M_r = 711.27 g/mol

A solution of **48b** (70.0 mg, 202 µmol) and **49** (518.7 µl, 2.4 mmol) in acetonitrile (5 ml) was stirred at 50 °C for 4 h and worked up according to **G.16** to give **LY20-MQ6-iper** as colorless solid.

yield:	7.6 mg (6 %)
HPLC purity:	97 % (HPLC Method **IIb**)
melting point [°C]:	decomposition >160
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3406, 2950, 2865, 1670, 1627, 1542, 1364

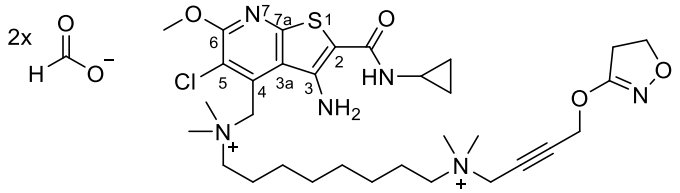
^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 8.54 (s, 2H, **H**COO^-), 5.62-5.45 (m, 1H, C**H$_2$**$N^+(CH_3)_2$), 5.20-5.02 (m, 1H, C**H$_2$**$N^+(CH_3)_2$), 4.92 (s, 2H, OC**H$_2$**C≡C), 4.43-4.36 (m, 4H, isoxazolinyl-OC**H$_2$**, $N^+(CH_3)_2$C**H$_2$**C≡C), 4.13 (s, 3H, OC**H$_3$**), 3.74 – 3.54 (m, 2H,

$N^+(CH_3)_2C\mathbf{H_2}(CH_2)_5N^+(CH_3)_2CH_2C{\equiv}C$), 3.51-3.41 (m, 2H, $C\mathbf{H_2}N^+(CH_3)_2CH_2C{\equiv}C$), 3.20 (s, 6H, $N^+(C\mathbf{H_3})_2$), 3.16 (s, 6H, $N^+(C\mathbf{H_3})_2$), 3.04 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OCH_2C\mathbf{H_2}$), 2.81-2.75 (m, 1H, cyclopropyl-C**H**), 2.00 (bs, 2H, $N^+(CH_3)_2CH_2C\mathbf{H_2}(CH_2)_4N^+(CH_3)_2CH_2C{\equiv}C$), 1.85 (bs, 2H, $C\mathbf{H_2}CH_2N^+(CH_3)_2CH_2C{\equiv}C$), 1.52 (bs, 4H, $N^+(CH_3)_2(CH_2)_2(C\mathbf{H_2})_2(CH_2)_2N^+(CH_3)_2$), 0.83-0.77 (m, 2H, cyclopropyl-C$\mathbf{H_2}$), 0.66-0.62 (m, 2H, cyclopropyl-C$\mathbf{H_2}$).

^{13}C NMR (100 MHz, MeOD, δ [ppm]): 168.9 (isoxazolinyl-$\mathbf{C_q}$), 168.6 (**C**(=O)), 160.8 (**C**-6), 157.6 ($\mathbf{C_q}$), 147.3 ($\mathbf{C_q}$), 135.8 (**C**-4), 123.6 ($\mathbf{C_q}$), 122.5 ($\mathbf{C_q}$), 107.5 ($\mathbf{C_q}$), 87.9 ($C{\equiv}\mathbf{C}CH_2O$), 76.7 ($C{\equiv}\mathbf{C}CH_2N^+(CH_3)_2$), 71.4 (isoxazolinyl-O**C**H_2), 68.1 ($N^+(CH_3)_2\mathbf{C}H_2(CH_2)_5N^+(CH_3)_2$-$CH_2C{\equiv}C$), 65.3 ($\mathbf{C}H_2N^+(CH_3)_2CH_2C{\equiv}C$), 60.0 ($\mathbf{C}H_2N^+(CH_3)_2$), 58.4 (O**C**$H_2C{\equiv}C$), 56.2 (O**C**$H_3$), 55.3 ($N^+(CH_3)_2\mathbf{C}H_2C{\equiv}C$), 51.4 ($N^+(\mathbf{C}H_3)_2$), 49.9 ($N^+(\mathbf{C}H_3)_2$), 33.8 (isoxazolinyl-$OCH_2\mathbf{C}H_2$), 27.0 ($\mathbf{C}H_2$), 26.9 ($\mathbf{C}H_2$), 24.0 (cyclopropyl-C**H**), 23.8 ($N^+(CH_3)_2CH_2\mathbf{C}H_2$-$(CH_2)_4N^+(CH_3)_2CH_2C{\equiv}C$), 23.6 ($\mathbf{C}H_2CH_2N^+(CH_3)_2CH_2C{\equiv}C$), 6.9 (cyclopropyl-C$\mathbf{H_2}$).

MS (ESI) m/z [M^+] Calcd for $C_{30}H_{45}ClN_6O_4S^{2+}$: 310.2 Found: 310.4.

N^1-((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)methyl)-N^8-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N^1,N^1,N^8,N^8-tetramethyloctane-1,8-diaminium chloride, ***LY20-MQ8-iper*** *(FG_B_138)*



$C_{34}H_{51}ClN_6O_8S$

M_r = 739.33 g/mol

A solution of **48c** (88.0 mg, 161 µmol) and **39** (139.7 mg, 805 µmol) in acetonitrile (5 ml) was stirred at 50 °C for 4 h and worked up according to **G.16** to give **LY20-MQ8-iper** as colorless solid.

yield:	9.1 mg (8 %)
HPLC purity:	95 % (HPLC Method **IIb**)
melting point [°C]:	decomposition >150
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3363, 2984, 2875, 1591, 1354, 1105

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 8.31 (s, 2H, **H**COO^-), 5.27-5.24 (m, 2H, $C\mathbf{H_2}N^+(CH_3)_2$), 4.94 (s, 2H, O$C\mathbf{H_2}C{\equiv}C$), 4.43 (s, 2H, $N^+(CH_3)_2C\mathbf{H_2}C{\equiv}C$), 4.32 (t, ^3J = 9.6 Hz 2H, isoxazolinyl-O$C\mathbf{H_2}$), 4.07 (s, 3H, O$C\mathbf{H_3}$), 3.38–3.27 (m, 2H, $C\mathbf{H_2}N^+(CH_3)_2CH_2C{\equiv}C$), 3.10-

2.96 (m, 14H, N(C**H**$_3$)$_2$, isoxazolinyl-OCH$_2$C**H**$_2$), 2.78-2.72 (m, 1H, cyclopropyl-C**H**), 2.10-2.08 (m, 2H, N$^+$(CH$_3$)$_2$CH$_2$C**H**$_2$(CH$_2$)$_4$N$^+$(CH$_3$)$_2$CH$_2$C≡C), 1.72-1.62 (m, 2H, C**H**$_2$CH$_2$N$^+$(CH$_3$)$_2$CH$_2$C≡C), 1.42-1.27 (m, 8H, N$^+$(CH$_3$)$_2$(CH$_2$)$_2$(C**H**$_2$)$_4$(CH$_2$)$_2$N$^+$(CH$_3$)$_2$), 0.71-0.63 (m, 2H, cyclopropyl-C**H**$_2$), 0.61-0.54 (m, 2H, cyclopropyl-C**H**$_2$).

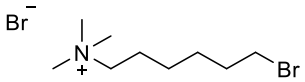
^{13}C NMR (100 MHz, DMSO-d$_6$, δ [ppm]): 166.8, 165.9, 165.8, 158.6, 146.2, 132.9, 125.5, 122.0, 120.6, 86.0, 76.2, 69.7, 66.0, 63.2, 59.2, 57.2, 55.5, 53.3, 49.9, 46.3, 35.1, 31.3, 28.7, 26.6, 26.6, 25.1, 22.1, 5.9.

Due to a too low concentration of the NMR sample and solubility problems, some signals were not detected. Assignment of the ^{13}C signals could neither be performed.

MS (ESI) m/z [M$^+$] Calcd for $C_{32}H_{49}ClN_6O_4S^{2+}$: 324.2 Found: 324.4.

7.4.3 Preparation of LY20-MQ6-TMA

6-Bromo-N,N,N-trimethylhexan-1-aminium bromide, ***51*** *(FG_B_123)*



$C_9H_{21}Br_2N$

M_r = 303.08 g/mol

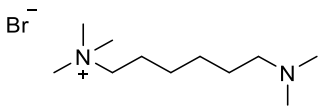
According to Bock *et al.*[124], 1,6-dibromohexane (10.2 ml, 67.0 mmol) and trimethylamine hydrochloride (4.00 g, 41.9 mmol) were dissolved in ethanol (50 ml). A solution of potassium hydroxide (3.05 g, 54.4 mmol) in ethanol (50 ml) was added dropwise and the mixture was stirred at rt for 4 days. The white precipitate was filtered off. The filtrate was concentrated to a few ml and added dropwise to ice cooled diethyl ether. The precipitate was filtered and extracted with acetone with a soxhlet extractor for 24 h. The extract was concentrated to a few ml and poured into ice cooled diethyl ether. The white precipitate was filtered and dried *in vacuo* to give **51** as colorless oil.

yield:	2.4 g (12 %, Lit.:[124] 78 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3012, 2935, 2860, 1479, 1251, 970.

^{1}H NMR (400 MHz, (CD$_3$)$_2$CO, δ [ppm]): 3.75-3.69 (m, 2H, C**H**$_2$N$^+$(CH$_3$)$_3$), 3.51 (d, ^3J = 6.7 Hz, 2H, C**H**$_2$Br), 3.42 (s, 9H, N$^+$(C**H**$_3$)$_3$), 1.97-1.86 (m, 4H, C**H**$_2$CH$_2$N$^+$(CH$_3$)$_3$, C**H**$_2$CH$_2$Br), 1.61-1.41 (m, 4H, C**H**$_2$).

^{13}C NMR (100 MHz, (CD$_3$)$_2$CO, δ [ppm]): 67.0 (N$^+$(CH$_3$)$_3$**C**H$_2$), 53.5 (N$^+$(**C**H$_3$)$_3$), 34.7 (**C**H$_2$Br), 33.3 (**C**H$_2$CH$_2$Br), 28.3 (**C**H$_2$), 26.1 (**C**H$_2$), 23.4 (N$^+$(CH$_3$)$_2$CH$_2$**C**H$_2$).

6-(Dimethylamino)-N,N,N-trimethylhexan-1-aminium bromide, ***52*** *(FG_B_127)*



$C_{11}H_{27}BrN_2$

M_r = 267.26 g/mol

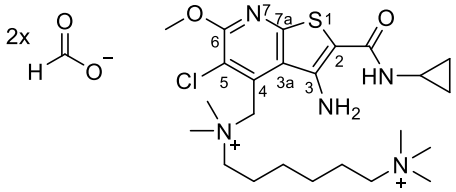
A solution of **51** (187.7 mg, 374 µmol) in ethanol (5 ml) was treated with a 5.2 M ethanolic solution of dimethyl amine (334 µl, 1.87 mmol) and stirred at 70 °C for 24 h in a sealed pressure tube. After cooling to rt, the solvent was removed and the residue was purified by column chromatography (alox basic, $CHCl_3$/CH_3OH 10/2) to give TMA linker **52** as yellowish solid.

yield:	91 mg (91 %)
reaction control:	R_f = 0.26 (alox basic; $CHCl_3$/CH_3OH: 10/2, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2930, 2859, 2762, 1483, 1466

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 3.63-3.58 (m, 2H, C**H$_2$**$N^+(CH_3)_3$), 3.43 (s, 9H, $N^+(C$**H$_3$**$)_3$), 2.41-2.36 (m, 2H, C**H$_2$**$N(CH_3)_2$), 2.29 (s, 6H, $N(C$**H$_3$**$)_2$), 1.81-1.72 (m, 2H, C**H$_2$**$CH_2N^+(CH_3)_3$), 1.56-1.46 (m, 2H, C**H$_2$**$CH_2N(CH_3)_2$), 1.45-1.36 (m, 4H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 67.0 ($N^+(CH_3)_3$**C**H_2), 59.2 ($N(CH_3)_2$**C**H_2), 53.6 (N^+(**C**$H_3)_3$), 45.2 (N(**C**$H_3)_2$), 26.9 (**C**H_2), 26.8 (**C**$H_2CH_2N(CH_3)_2$), 26.0 (**C**H_2), 23.2 (**C**$H_2CH_2N^+(CH_3)_3$).

N^1-((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)methyl)-N^1,N^1,N^6,N^6,N^6-pentamethylhexane-1,6-diaminium bromide chloride, ***LY20-MQ6-TMA*** *(FG_B_143)*



$C_{26}H_{42}ClN_5O_6S$

M_r = 588.16 g/mol

A solution of **39** (30.0 mg, 87 µmol) and **52** (46.4 mg, 174 µmol) in acetonitrile (5 ml) was stirred at 50 °C for 4 h. The solution was treated with cooled diethyl ether and the formed precipitate was filtered off, washed with diethyl ether, and dried *in vacuo*. Product **LY20-MQ6-TMA** was obtained after purification by flash chromatography (reversed phase, H_2O+0.1 % FA/MeOH+0.1 % FA 10/3).

yield:	13 mg (24 %)
HPLC purity:	98 % (HPLC Method **IIb**)

melting point [°C]:	decomposition > 160 °C
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3380, 2952, 2871, 1591, 1537, 1480, 1369, 952

^{1}H NMR (400 MHz, MeOD, δ [ppm]): 8.45 (s, 2H, **H**COO$^-$), 5.66-5.45 (m, 1H, C**H**$_2$N$^+$(CH$_3$)$_2$), 5.25-5.10 (m, 1H, C**H**$_2$N$^+$(CH$_3$)$_2$), 4.16 (s, 3H, OC**H$_3$**), 3.77-3.55 (m, 2H, N$^+$(CH$_3$)$_2$C**H$_2$**(CH$_2$)$_5$N$^+$(CH$_3$)$_3$), 3.45-3.39 (m, 2H, C**H$_2$**N$^+$(CH$_3$)$_3$), 3.21-3.15 (m, 15H, N$^+$(C**H$_3$**)$_2$(CH$_2$)$_6$N$^+$(C**H$_3$**)$_3$), 2.84-2.78 (m, 1H, cyclopropyl-C**H**), 2.08-1.98 (m, 2H, N$^+$(CH$_3$)$_2$CH$_2$C**H$_2$**(CH$_2$)$_4$N$^+$(CH$_3$)$_3$), 1.94-1.84 (m, 2H, C**H$_2$**CH$_2$N$^+$(CH$_3$)$_3$), 1.58-1.48 (m, 4H, N$^+$(CH$_3$)$_2$(CH$_2$)$_2$(C**H$_2$**)$_2$(CH$_2$)$_2$N$^+$(CH$_3$)$_3$), 0.86-0.80 (m, 2H, cyclopropyl-C**H$_2$**), 0.70-0.64 (m, 2H, cyclopropyl-C**H$_2$**).

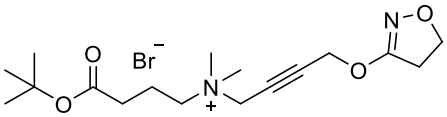
^{13}C NMR (100 MHz, MeOD, δ [ppm]): 168.6 (**C**(=O)), 160.8 (**C**-6), 157.6 (**C$_q$**), 147.3 (**C$_q$**), 133.8 (**C**-4), 123.6 (**C$_q$**), 122.5 (**C$_q$**), 107.4 (**C$_q$**), 68.2 (N$^+$(CH$_3$)$_2$**C**H$_2$(CH$_2$)$_5$N$^+$(CH$_3$)$_3$), 67.8 (**C**H$_2$N$^+$(CH$_3$)$_3$), 60.0 (**C**H$_2$N$^+$(CH$_3$)$_2$), 56.2 (O**C**H$_3$), 53.8 (N$^+$(**C**H$_3$)$_3$), 49.3 (N$^+$(**C**H$_3$)$_2$), 27.0 (**C**H$_2$), 26.9 (**C**H$_2$), 24.0 (cyclopropyl-C**H**), 23.9 (N$^+$(CH$_3$)$_2$CH$_2$**C**H$_2$(CH$_2$)$_4$N$^+$(CH$_3$)$_3$), 23.8 (**C**H$_2$CH$_2$N$^+$(CH$_3$)$_3$), 6.9 (cyclopropyl-C**H$_2$**).

MS (ESI) m/z [M$^+$] Calcd for $C_{24}H_{40}ClN_5O_2S^{2+}$: 248.6 Found: 248.9.

7.5 Synthesis of LY20-MAn hybrids

7.5.1 Preparation of orthosteric iperoxo linkers 5-Ln

4-(tert-Butoxy)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyl-4-oxobutyl-1-aminium bromide, ***53a*** *(FG_B_166)*



$C_{17}H_{29}BrN_2O_4$

M_r = 405.33 g/mol

A solution of *tert*-butyl 4-brombutanoate (250.0 mg, 1.12 mmol) and **13** (306.3 mg, 1.68 mmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 7 h. The reaction mixture was worked up as described in general procedure **G.3**. Further purification by column chromatography (alox basic; DCM/CH$_3$OH 20/1 to 10/1) yielded **53a** as an orange oil.

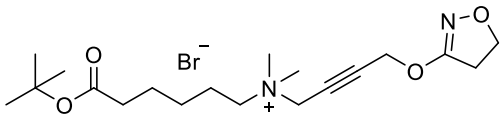
yield:	308 mg (68 %)
reaction control:	R_f = 0.40 (alox basic; CHCl$_3$/CH$_3$OH 10/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3423, 2978, 2912, 1733, 1626, 1337, 1150

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.89 (s, 2H, $N^+(CH_3)_2C\mathbf{H_2}C{\equiv}C$), 4.79 (s, 2H, $OC\mathbf{H_2}C{\equiv}C$), 4.37 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OC\mathbf{H_2}$), 3.71-3.65 (m, 2H, $N^+(CH_3)_2C\mathbf{H_2}CH_2$), 3.42 (s, 6H, $N^+(C\mathbf{H_3})_2$), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OCH_2C\mathbf{H_2}$), 2.36 (t, 2H, ^3J = 6.9 Hz, $C\mathbf{H_2}C(O)$), 2.03-1.94 (m, 2H, $N^+(CH_3)_2CH_2C\mathbf{H_2}$), 1.38 (s, 9H, $C(C\mathbf{H_3})_3$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 171.3 (**C**(O)-*tert*-butyl), 166.9 (isoxazolinyl-$\mathbf{C_q}$), 86.8 ($OCH_2\mathbf{C}{\equiv}C$), 81.5 ($\mathbf{C}(CH_3)_3$), 76.0 ($C{\equiv}\mathbf{C}CH_2N^+(CH_3)_2$), 70.2 (isoxazolinyl-$O\mathbf{C}H_2$), 63.3 ($N^+(CH_3)_2\mathbf{C}H_2CH_2$), 57.5 ($O\mathbf{C}H_2C{\equiv}C$), 55.0 ($N^+(CH_3)_2\mathbf{C}H_2C{\equiv}C$), 50.7 ($N^+(\mathbf{C}H_3)_2$), 33.1 (isoxazolinyl-$OCH_2\mathbf{C}H_2$), 31.3 ($\mathbf{C}H_2C(O)$), 28.2 ($C(\mathbf{C}H_3)_3$), 18.5 ($N^+(CH_3)_2CH_2\mathbf{C}H_2$).

MS (ESI) m/z [M^+] Calcd for $C_{17}H_{29}N_2O_4^+$: 325.2. Found: 325.4.

6-(tert-Butoxy)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyl-6-oxohexan-1-aminium bromide, ***53b*** *(FG_B_128)*



$C_{19}H_{33}BrN_2O_4$

M_r = 433.39 g/mol

A solution of *tert*-butyl 6-bromhexanoate (200.0 mg, 796 µmol) and **13** (217.7 mg, 1.19 mmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 7 h. The reaction mixture was worked up as described in general procedure **G.3**. Further purification by column chromatography (alox basic; DCM/CH_3OH 20/1 to 10/1) yielded **53b** as an orange oil.

yield:	296 mg (86 %)
reaction control:	R_f = 0.38 (alox basic; $CHCl_3$/CH_3OH 10/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3367, 2930, 1720, 1626, 1339, 1154.

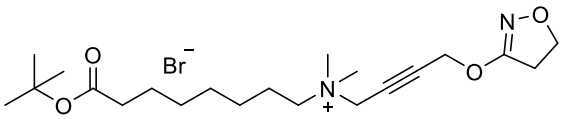
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.94 (s, 2H, $N^+(CH_3)_2C\mathbf{H_2}C{\equiv}C$), 4.80 (s, 2H, $OC\mathbf{H_2}C{\equiv}C$), 4.41 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OC\mathbf{H_2}$), 3.65-3.59 (m, 2H, $N^+(CH_3)_2C\mathbf{H_2}$-CH_2), 3.44 (s, 6H, $N^+(C\mathbf{H_3})_2$), 3.00 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OCH_2C\mathbf{H_2}$), 2.22 (t, ^3J = 7.2 Hz, 2H,$C\mathbf{H_2}C(O)$), 1.80-1.71 (m, 2H, $N^+(CH_3)_2CH_2C\mathbf{H_2}$), 1.66-1.58 (m, 2H, $C\mathbf{H_2}CH_2C(O)$), 1.46-1.37 (m, 11H, $C\mathbf{H_2}$, $C(C\mathbf{H_3})_3$).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 172.8 (**C**(O)-*tert*-butyl), 166.9 (isoxazolinyl-$\mathbf{C_q}$), 87.0 ($OCH_2\mathbf{C}{\equiv}C$), 80.6 ($\mathbf{C}(CH_3)_3$), 76.1 ($C{\equiv}\mathbf{C}CH_2N^+(CH_3)_2$), 70.3 (isoxazolinyl-$O\mathbf{C}H_2$), 64.3 ($N^+(CH_3)_2\mathbf{C}H_2CH_2$), 57.4 ($O\mathbf{C}H_2C{\equiv}C$), 55.1 ($N^+(CH_3)_2\mathbf{C}H_2C{\equiv}C$), 50.8 ($N^+(\mathbf{C}H_3)_2$), 35.2

(**C**H_2C(O)), 33.1 (isoxazolinyl-OCH_2**C**H_2), 28.3 (C(**C**H_3)$_3$), 25.8 (**C**H_2), 24.5 (**C**H_2CH_2C(O)), 22.9 (N^+(CH_3)$_2$CH_2**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{19}H_{33}N_2O_4^+$: 353.2. Found: 353.3.

8-(tert-Butoxy)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyl-8-oxooctan-1-aminium bromide, ***53c*** *(FG_B_159)*



$C_{21}H_{37}BrN_2O_4$

M_r = 460.19 g/mol

A solution of *tert*-butyl 8-bromoctanoate (200.0 mg, 716 µmol) and **13** (195.8 mg, 1.07 mmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 7 h. The reaction mixture was worked up as described in general procedure **G.3**. Further purification by column chromatography (alox basic; DCM/CH_3OH 20/1 to 10/1) yielded **53c** as a white solid.

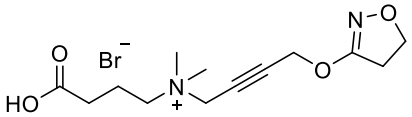
yield:	238 mg (72 %)
reaction control:	R_f = 0.39 (alox basic; CHCl_3/CH_3OH 10/1, Dragendorff reagent)
Melting point [°C]:	119-125
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3367, 2914, 2856, 1726, 1627, 1338, 1148.

^{1}H NMR (400 MHz, CDCl_3, δ [ppm]): 4.78 (s, 2H, N^+(CH_3)$_2$C**H$_2$**C≡C), 4.76 (s, 2H, OC**H$_2$**C≡C), 4.38 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 3.56-3.50 (m, 2H, N^+(CH_3)$_2$C**H$_2$**-CH_2), 3.40 (s, 6H, N^+(C**H$_3$**)$_2$), 2.97 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.18-2.12 (m, 2H, C**H$_2$**C(O)), 1.75-1.65 (m, 2H, N^+(CH_3)$_2$CH_2C**H$_2$**), 1.56-1.46 (m, 2H, C**H$_2$**CH_2C(O)), 1.38 (s, 9H, C(C**H$_3$**)$_3$), 1.36-1.18 (m, 6H, C**H$_2$**).

^{13}C NMR (100 MHz, CDCl_3, δ [ppm]): 173.4 (**C**(O)-*tert*-butyl), 166.9 (isoxazolinyl-**C$_q$**), 86.6 (OCH_2**C**≡C), 80.3 (**C**(CH_3)$_3$), 76.1 (C≡**C**C$H_2$$N^+$(C$H_3$)$_2$), 70.2 (isoxazolinyl-O**C**H_2), 64.3 (N^+(CH_3)$_2$**C**H_2CH_2), 57.4 (O**C**H_2C≡C), 54.9 (N^+(CH_3)$_2$**C**H_2C≡C), 50.8 (N^+(**C**H_3)$_2$), 35.5 (**C**H_2C(O)), 33.1 (isoxazolinyl-OCH_2**C**H_2), 28.9 (**C**H_2), 28.8 (**C**H_2), 28.3 (C(**C**H_3)$_3$), 26.1 (**C**H_2), 24.9 (**C**H_2CH_2C(O)), 22.9 (N^+(CH_3)$_2$CH_2**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{21}H_{37}N_2O_4^+$: 381.3. Found: 381.5.

N-(3-Carboxypropyl)-4-((4,5-dihydroisoxazol-3-yl)oxy)-N,N-dimethylbut-2-yn-1-aminium bromide, ***5-L3*** *(FG_B_167)*



$C_{13}H_{21}BrN_2O_4$

M_r = 349.23 g/mol

TFA (376 µl, 4.91 mmol) was added dropwise to a solution of **53a** (166.0 mg, 410 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 3 d at rt, the reaction mixture was worked up according to **G.4** to give **5-L3** as orange oil.

yield: 132 mg (92 %)

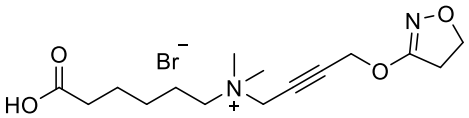
IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3414, 2964, 1730, 1627, 1342, 1142.

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 4.86 (t, ^3J = 1.6 Hz, 2H, OC**H$_2$**C≡C), 4.36 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 4.16 (t, ^3J = 1.6 Hz, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 3.41-3.35 (m, 2H, $N^+(CH_3)_2$C**H$_2$**CH_2), 3.08 (s, 6H, N^+(C**H$_3$**)$_2$), 2.99 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.41 (t, ^3J = 7.0 Hz, 2H, C**H$_2$**C(O)OH), 2.03-1.96 (m, 2H, $N^+(CH_3)_2CH_2$C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 174.0 (**C**(O)-*tert*-butyl), 168.5 (isoxazolinyl-**C$_q$**), 88.2 (OCH_2**C**≡C), 76.1 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 64.6 ($N^+(CH_3)_2$**C**H_2CH_2), 58.5 (O**C**H_2C≡C), 55.8 ($N^+(CH_3)_2$**C**H_2C≡C), 51.8 (N^+(**C**H_3)$_2$), 33.8 (**C**H_2C(O)OH), 30.9 (isoxazolinyl-OCH_2**C**H_2), 19.3 ($N^+(CH_3)_2CH_2$**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{13}H_{21}N_2O_4^+$: 269.2. Found: 269.1.

5-Carboxy-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethylpentan-1-aminium bromide, ***5-L5*** *(FG_B_133)*



$C_{15}H_{25}BrN_2O_4$

M_r = 377.28 g/mol

TFA (288 µl, 3.77 mmol) was added dropwise to a solution of **53b** (136.0 mg, 314 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 3 d at rt, the reaction mixture was worked up according to **G.4** to give **5-L5** as orange oil.

yield: 69 mg (58 %)

IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3422, 2951, 2875, 1721, 1627, 1341

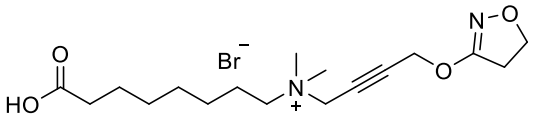
^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 4.86 (t, ^3J = 1.7 Hz, 2H, OC**H$_2$**C≡C), 4.36 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 4.16 (t, ^3J = 1.7 Hz, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 3.34-3.29 (m, 2H, $N^+(CH_3)_2$C**H$_2$**CH_2), 3.05 (s, 6H, N^+(C**H$_3$**)$_2$), 2.98 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-

$OCH_2C\mathbf{H_2}$), 2.32 (t, ^3J = 7.4 Hz, 2H, $C\mathbf{H_2}C(O)OH$), 1.76-1.69 (m, 2H, $N^+(CH_3)_2CH_2C\mathbf{H_2}$), 1.67-1.58 (m, 2H, $C\mathbf{H_2}CH_2C(O)OH$), 1.42-1.34 (m, 2H, $C\mathbf{H_2}$).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 175.0 (**C**(O)-*tert*-butyl), 168.1 (isoxazolinyl-$\mathbf{C_q}$), 87.8 ($OCH_2\mathbf{C}\equiv C$), 75.9 ($C\equiv\mathbf{C}CH_2N^+(CH_3)_2$), 71.0 (isoxazolinyl-$O\mathbf{C}H_2$), 65.1 ($N^+(CH_3)_2\mathbf{C}H_2CH_2$), 58.2 ($O\mathbf{C}H_2C\equiv C$), 55.4 ($N^+(CH_3)_2\mathbf{C}H_2C\equiv C$), 51.5 ($N^+(\mathbf{C}H_3)_2$), 33.9 ($\mathbf{C}H_2C(O)OH$), 33.6 (isoxazolinyl-$OCH_2\mathbf{C}H_2$), 26.2 ($\mathbf{C}H_2$), 25.0 ($\mathbf{C}H_2CH_2C(O)$), 23.1 ($N^+(CH_3)_2CH_2\mathbf{C}H_2$).

MS (ESI) m/z [M^+] Calcd for $C_{15}H_{25}N_2O_4^+$: 297.2. Found: 297.2.

*7-Carboxy-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethylheptan-1-aminium bromide, **5-L7** (FG_B_162)*



$C_{17}H_{29}BrN_2O_4$

M_r = 405.33 g/mol

TFA (289 µl, 3.77 mmol) was added dropwise to a solution of **53c** (145.0 mg, 314 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 3 d at rt, the reaction mixture was worked up according to **G.4** to give **5-L7** as orange oil.

yield:	110 mg (86 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3412, 2937, 2864, 1719, 1627, 1341, 1154

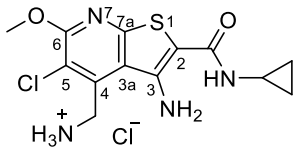
^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 4.86 (t, ^3J = 1.6 Hz, 2H, $OC\mathbf{H_2}C\equiv C$), 4.35 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OC\mathbf{H_2}$), 4.17-4.14 (m, 2H, $N^+(CH_3)_2C\mathbf{H_2}C\equiv C$), 3.34-3.28 (m, 2H, $N^+(CH_3)_2C\mathbf{H_2}CH_2$), 3.05 (s, 6H, $N^+(C\mathbf{H_3})_2$), 2.98 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OCH_2C\mathbf{H_2}$), 2.28 (t, ^3J = 7.4 Hz, 2H, $C\mathbf{H_2}C(O)OH$), 1.75-1.65 (m, 2H, $N^+(CH_3)_2CH_2C\mathbf{H_2}$), 1.62-1.54 (m, 2H, $C\mathbf{H_2}CH_2C(O)OH$), 1.38-1.31 (m, 6H, $C\mathbf{H_2}$).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 175.6 (**C**(O)-*tert*-butyl), 168.4 (isoxazolinyl-$\mathbf{C_q}$), 88.0 ($OCH_2\mathbf{C}\equiv C$), 76.2 ($C\equiv\mathbf{C}CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-$O\mathbf{C}H_2$), 65.6 ($N^+(CH_3)_2\mathbf{C}H_2$-$CH_2$), 58.5 ($O\mathbf{C}H_2C\equiv C$), 55.6 ($N^+(CH_3)_2\mathbf{C}H_2C\equiv C$), 51.8 ($N^+(\mathbf{C}H_3)_2$), 34.6 ($\mathbf{C}H_2C(O)OH$), 33.8 (isoxazolinyl-$OCH_2\mathbf{C}H_2$), 29.6 ($\mathbf{C}H_2$), 29.5 ($\mathbf{C}H_2$), 26.8 ($\mathbf{C}H_2$), 25.7 ($\mathbf{C}H_2CH_2C(O)$), 23.5 ($N^+(CH_3)_2CH_2\mathbf{C}H_2$).

MS (ESI) m/z [M^+] Calcd for $C_{17}H_{29}N_2O_4^+$: 325.2. Found: 325.4.

7.5.2 Preparation of LY20-MAn-iper hybrids

3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)methan-aminium chloride, ***54*** *(FG_B_106)*



$C_{13}H_{16}Cl_2N_4O_2S$

M_r = 363.28 g/mol

A suspension of compound **39** (110.0 mg, 317.7 µmol) in methanol (5 ml) was treated with a 7 M methanolic ammonia solution (11.4 ml, 79.4 mmol) in a sealed pressure tube and heated at 95 °C for 2 h. After cooling to rt, the solvent was removed to give compound **54** as a yellow solid.

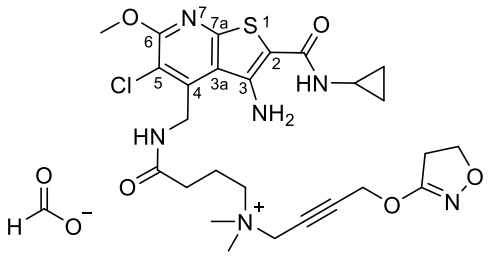
yield:	115 mg (100 %)
melting point [°C]:	decomposition >170
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3621, 3353, 3293, 2926, 2860, 1591, 1499, 1363

^{1}H NMR (400 MHz, MeOD, δ [ppm]): 4.47 (s, 2H, C**H$_2$**NH$_3^+$), 4.07 (s, 3H, OC**H$_3$**), 2.78-2.73 (m, 1H, cyclopropyl-C**H**), 0.83-0.76 (m, 2H, cyclopropyl-C**H$_2$**), 0.66-0.62 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, MeOD, δ [ppm]): 169.5 (**C**(O)NH), 160.7 (**C**-6), 158.8 (**C$_q$**), 157.0 (**C$_q$**), 149.5 (**C$_q$**), 146.3 (**C$_q$**), 122.9 (**C**-3a), 117.3 (**C**-5), 55.6 (O**C**H$_3$), 40.3 (**C**H$_2$NH$_3^+$), 23.8 (cyclopropyl-**C**H), 7.0 (cyclopropyl-**C**H$_2$).

MS (ESI) m/z [M$^+$] Calcd for $C_{13}H_{16}ClN_4O_2S^+$: 327.1. Found: 327.0.

N-(4-(((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)-methyl)amino)-4-oxobutyl)-4-((4,5-dihydroisoxazol-3-yl)oxy)-N,N-dimethylbut-2-yn-1-aminium bromide, ***LY20-MA3-iper*** *(FG_B_168)*



$C_{27}H_{35}ClN_6O_7S$

M_r = 623.12 g/mol

Carboxylic acid **5-L3** (96.1 mg, 275 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (48 µl, 275 µmol) and PyBOP (143.3 mg, 275 µmol) were successively

added. Compound **54** (100.0 mg, 275 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (48 µl, 275 µmol) and the solution was added to the reaction mixture. After stirring at rt for 5 h, the mixture was worked up as described in **G.10** and purified by flash chromatography (reversed phase, H_2O+0.1 % FA/MeOH+0.1 % FA 10/3) to give **LY20-MA3-iper** as pale-yellow solid.

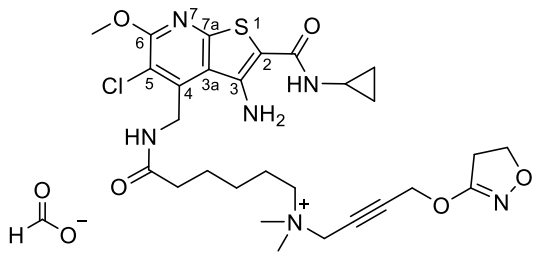
yield:	22 mg (12 %)
HPLC purity:	95 % (HPLC Method **IIb**)
reaction control:	R_f = 0.22 (alox basic; $CHCl_3$/CH_3OH: 20/1; Dragendorff-reagent)
Melting point:	decomposition >170
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3273, 2892, 2871, 1578, 1388, 1349, 1065

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 8.74 (t, ^3J = 5.3, 1H, CH_2N**H**C(O)), 8.44 (s, 1H, **H**COO^-), 7.82 (d, ^3J = 3.4, 1H, CHN**H**C(O)), 7.29 (s, 2H, N**H$_2$**), 4.91 (s, 2H, OC**H$_2$**C≡C), 4.82 (d, ^3J = 5.2, 2H, C**H$_2$**NHC(O)), 4.43 (s, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 4.30 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OC**H$_2$**), 4.01 (s, 3H, OC**H$_3$**), 3.33-3.27 (m, 2H, $N^+(CH_3)_2$C**H$_2$**CH_2), 3.06 (s, 6H, N^+(C**H$_3$**)$_2$), 3.00 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.78-2.72 (m, 1H, cyclopropyl-C**H**), 2.27 (t, ^3J = 7.1 Hz, 2H, C**H$_2$**C(O)NH), 1.94-1.85 (m, 2H, C**H$_2$**CH_2C(O)NH), 0.69-0.64 (m, 2H, cyclopropyl-C**H$_2$**), 0.58-0.54 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 172.5 (CH_2NH**C**(O)), 167.2 (**C**(O)NH), 166.8 (isoxazolinyl-**C$_q$**), 159.4 (**C**-6), 158.8 (**C$_q$**), 147.8 (**C$_q$**), 143.3 (**C**-4), 120.9 (**C$_q$**), 117.2 (**C$_q$**), 99.2 (**C$_q$**), 86.5 (C≡**C**CH_2O), 76.6 (C≡**C**$CH_2N^+(CH_3)_2$), 70.1 (isoxazolinyl-O**C**H_2), 65.4 ($N^+(CH_3)_2$**C**H_2CH_2), 57.7 (O**C**H_2C≡C), 55.5 (O**C**H_3), 50.3 ($N^+(CH_3)_2$**C**H_2C≡C), 49.1 (N^+(**C**H_3)$_2$), 36.5 (**C**H_2NHC(O)), 32.7 (**C**H_2C(O)NH), 31.4 (isoxazolinyl-OCH_2**C**H_2), 23.4 (cyclopropyl-**C**H), 18.5 (**C**H_2CH_2C(O)NH), 6.4 (cyclopropyl-**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{26}H_{35}ClN_6O_5S^{2+}$: 289.1. Found: 289.3.

6-(((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)methyl)amino)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyl-6-oxohexan-1-aminium bromide, ***LY20-MA5-iper*** *(FG_B_134)*



$C_{29}H_{39}ClN_6O_7S$

M_r = 651.18 g/mol

Carboxylic acid **5-L5** (109.2 mg, 289 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (50 µl, 289 µmol) and PyBOP (150.4 mg, 289 µmol) were successively added. Compound **54** (105.0 mg, 289 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (50 µl, 289 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by flash chromatography (reversed phase, H_2O+0.1 % FA/MeOH+0.1 % FA 10/3) to give **LY20-MA5-iper** as pale-yellow solid.

yield:	25 mg (11 %)
HPLC purity:	99 % (HPLC Method **IIb**)
reaction control:	R_f = 0.25 (alox basic; $CHCl_3$/CH_3OH: 20/1; Dragendorff-reagent)
Melting point:	decomposition >170
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3282, 2924, 2873, 1593, 1470, 1365, 1073

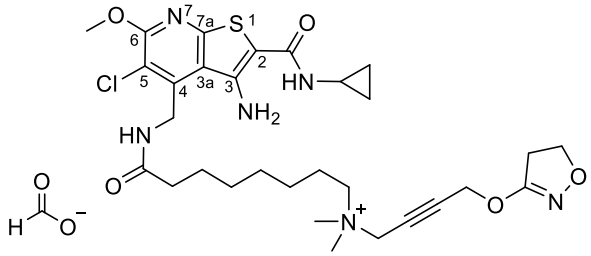
^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 8.45 (s, 1H, **H**COO^-), 7.52-7.36 (m, 3H, N**H**, N**H$_2$**), 6.36 (s, 1H, N**H**), 4.88 (d, ^3J = 6.1 Hz, 2H, C**H$_2$**NHC(O)), 4.83 (s, 2H, OC**H$_2$**C≡C), 4.33 (t, ^3J = 9.6 Hz, 2H,isoxazolinyl-OC**H$_2$**), 4.14 (s, 2H, $N^+(CH_3)_2$C**H$_2$**C≡C), 4.03 (s, 3H, OC**H$_3$**), 3.29-3.23 (m, 2H, $N^+(CH_3)_2$C**H$_2$**CH_2), 3.02 (s, 6H, N^+(C**H$_3$**)$_2$), 2.96 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-OCH_2C**H$_2$**), 2.80-2.73 (m, 1H, cyclopropyl-C**H**), 2.22 (t, ^3J = 7.2 Hz, 2H, C**H$_2$**C(O)NH), 1.72-1.55 (m, 4H, $N^+(CH_3)_2CH_2$C**H$_2$**, C**H$_2$**CH_2C(O)NH), 1.33-1.28 (m, 2H, C**H$_2$**), 0.75-0.70 (m, 2H, cyclopropyl-C**H$_2$**), 0.61-0.56 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 175.4 (CH_2NH**C**(O)), 168.4 (**C**(O)NH), 168.2 (isoxazolinyl-**C$_q$**), 160.4 (**C**-6), 156.7 (**C$_q$**), 149.4 (**C$_q$**), 144.3 (**C**-4), 121.9 (**C$_q$**), 118.8 (**C$_q$**), 99.9 (**C$_q$**), 88.1 (C≡**C**CH_2O), 76.2 (C≡**C**$CH_2N^+(CH_3)_2$), 71.3 (isoxazolinyl-O**C**H_2), 65.4 ($N^+(CH_3)_2$**C**H_2CH_2), 58.5 (O**C**H_2C≡C), 56.1 (O**C**H_3), 55.6 ($N^+(CH_3)_2$**C**H_2C≡C), 51.8

($N^+(\mathbf{C}H_3)_2$), 38.2 ($\mathbf{C}H_2NHC(O)$), 36.0 ($\mathbf{C}H_2C(O)NH$), 33.8 (isoxazolinyl-$OCH_2\mathbf{C}H_2$), 31.3 ($\mathbf{C}H_2C(O)NH$)), 26.5 ($\mathbf{C}H_2$), 25.8 ($\mathbf{C}H_2CH_2C(O)NH$), 24.1 (cyclopropyl-**C**H), 23.2 ($N^+(CH_3)_2CH_2\mathbf{C}H_2$), 7.0 (cyclopropyl-$\mathbf{C}H_2$).

MS (ESI) m/z [M^+] Calcd for $C_{28}H_{39}ClN_6O_5S^{2+}$: 303.1. Found: 303.3.

8-(((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)methyl)amino)-N-(4-((4,5-dihydroisoxazol-3-yl)oxy)but-2-yn-1-yl)-N,N-dimethyl-8-oxooctan-1-aminium bromide, ***LY20-MA7-iper*** *(FG_B_165)*



$C_{31}H_{43}ClN_6O_7S$

M_r = 679.23 g/mol

Carboxylic acid **5-L7** (120.0 mg, 296 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (52 µl, 296 µmol) and PyBOP (154.1 mg, 296 µmol) were successively added. Compound **54** (107.5 mg, 296 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (52 µl, 296 µmol) and the solution was added to the reaction mixture. After stirring at rt for 4 h, the mixture was worked up as described in **G.10** and purified by flash chromatography (reversed phase, H_2O+0.1 % FA/MeOH+0.1 % FA 10/3) to give **LY20-MA7-iper** as pale-yellow solid.

yield:	21 mg (10 %)
HPLC purity:	97 % (HPLC Method **IIb**)
reaction control:	R_f = 0.26 (alox basic; $CHCl_3$/CH_3OH: 20/1; Dragendorff-reagent)
Melting point:	decomposition >170
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2892, 2871, 1578, 1351, 1069

^{1}H NMR (400 MHz, DMSO-d_6, δ [ppm]): 8.66 (t, ^3J = 6.2, 1H, $CH_2N\mathbf{H}C(O)$), 8.40 (s, 1H, $\mathbf{H}COO^-$), 7.83 (d, ^3J = 3.2, 1H, $CHN\mathbf{H}C(O)$), 7.33 (s, 2H, $N\mathbf{H_2}$), 4.93 (s, 2H, $OC\mathbf{H_2}C{\equiv}C$), 4.78 (d, ^3J = 5.2, 2H, $C\mathbf{H_2}NHC(O)$), 4.44 (s, 2H, $N^+(CH_3)_2C\mathbf{H_2}C{\equiv}C$), 4.30 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OC\mathbf{H_2}$), 4.01 (s, 3H, $OC\mathbf{H_3}$), 3.36-3.27 (m, 2H, $N^+(CH_3)_2C\mathbf{H_2}CH_2$), 3.17 (s, 6H, $N^+(C\mathbf{H_3})_2$), 3.00 (t, ^3J = 9.6 Hz, 2H, isoxazolinyl-$OCH_2C\mathbf{H_2}$), 2.71-2.79 (m, 1H, cyclopropyl-

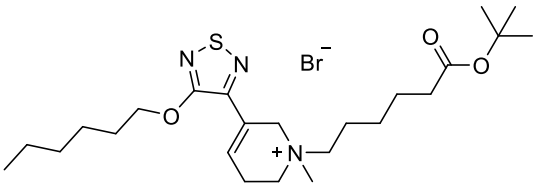
C**H**), 2.16 (t, ^3J = 7.2 Hz, 2H, C**H₂**C(O)NH), 1.69-1.58 (m, 2H, $N^+(CH_3)_2CH_2C\mathbf{H_2}$), 1.54-1.52 (m, 2H, C**H₂**CH_2C(O)NH), 1.32-1.18 (m, 6H, C**H₂**), 0.69-0.63 (m, 2H, cyclopropyl-C**H₂**), 0.59-0.53 (m, 2H, cyclopropyl-C**H₂**).

^{13}C NMR (100 MHz, DMSO-d_6, δ [ppm]): 173.7 (CH_2NH**C**(O)), 166.7 (**C**(O)NH), 166.3 (isoxazolinyl-**C$_q$**), 158.3 (**C**-6), 154.9 (**C$_q$**), 147.3 (**C$_q$**), 143.0 (**C**-4), 120.4 (**C$_q$**), 116.6 (**C$_q$**), 98.6 (**C$_q$**), 86.0 (C≡**C**CH_2O), 76.1 (C≡**C**$CH_2N^+(CH_3)_2$), 69.6 (isoxazolinyl-O**C**H_2), 63.2 ($N^+(CH_3)_2$**C**H_2CH_2), 57.2 (O**C**H_2C≡C), 55.0 (O**C**H_3), 53.3 ($N^+(CH_3)_2$**C**H_2C≡C), 49.8 (N^+(**C**$H_3)_2$), 36.6 (**C**H_2NHC(O)), 34.6 (**C**H_2C(O)NH), 32.2 (isoxazolinyl-OCH_2**C**H_2), 28.2 (**C**H_2), 28.1 (**C**H_2), 25.5 (**C**H_2), 24.9 (**C**H_2CH_2C(O)NH), 22.9 (cyclopropyl-**C**H), 21.7 ($N^+(CH_3)_2CH_2$**C**H_2), 5.9 (cyclopropyl-**C**H_2).

MS (ESI) m/z [M^+] Calcd for $C_{30}H_{43}ClN_6O_5S^{2+}$: 317.1. Found: 317.4.

7.5.3 Preparation of LY20-MA5-XanA

1-(6-(tert-Butoxy)-6-oxohexyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium bromide, ***55*** *(FG_XA_25)*



$C_{24}H_{42}BrN_3O_3S$

M_r = 532.58 g/mol

According to general procedure **G.9**, a solution of *tert*-butyl-6-bromohexanoate (291.0 mg, 1.2 mmol) and **22** (163.0 mg, 579 µmol) in acetonitrile (40 ml) was heated under microwave irradiation at 78 °C for 20 h to give **55** as an orange oil.

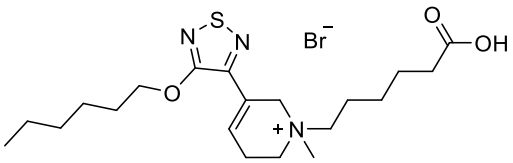
yield:	293 mg (95 %)
reaction control:	R_f = 0.26 (alox basic; $CHCl_3$/CH_3OH 10/1, Dragendorff reagent)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2953, 2930, 2860, 1723, 1509, 1366, 1149

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.22-7.18 (m, 1H, tetrahydropyridinyl-C**H**), 4.52 (s, 2H, tetrahydropyridinyl-N^+C**H₂**C(C)=C), 4.44 (t, ^3J = 6.7 Hz, 2H, OC**H₂**), 4.34-4.26 (m, 1H, tetrahydropyridinyl-N^+C**H**$_2CH_2$), 3.97-3.89 (m, 1H, tetrahydropyridinyl-N^+C**H**$_2CH_2$), 3.88-3.79 (m, 1H, $N^+(CH_3)$C**H**$_2$), 3.69-3.59 (m, 1H, $N^+(CH_3)$C**H**$_2$), 3.43 (s, 3H, N^+C**H₃**), 2.90-2.67 (m, 2H, tetrahydropyridinyl-N^+CH_2C**H₂**), 2.20 (t, ^3J = 7.2 Hz, 2H, C**H₂**C(=O)O), 1.90-1.77 (m, 4H, $N^+(CH_3)CH_2$C**H₂**, OCH_2C**H₂**), 1.66-1.58 (m, 2H, C**H₂**CH_2C(=O)O), 1.46-1.37 (m, 13H, C**H₂** , C(C**H₃**)$_3$), 1.35-1.29 (m, 4H, C**H₂**), 0.87 (t, ^3J = 6.8 Hz, 3H, C**H₃**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 172.8 (**C**(=O)O), 162.8 (thiadiazolyl-N**C**O), 143.8 (thiadiazolyl-**C**$_q$), 126.1 (tetrahydropyridinyl-**C**H), 123.2 (tetrahydropyridinyl-**C$_q$**), 80.6 (**C**$(CH_3)_3$), 71.8 (O**C**H_2), 63.5 ($N^+(CH_3)$**C**H_2), 58.9 (tetrahydropyridinyl-N^+**C**H_2C(C)=C), 56.9 (tetrahydropyridinyl-N^+**C**H_2CH_2), 48.7 (N^+**C**H_3), 35.1 (**C**H_2C(=O)O), 31.5 (**C**H_2), 29.0 (**C**H_2), 28.3 (C(**C**$H_3)_3$), 26.0 (**C**H_2), 25.8 (**C**H_2), 24.6 (**C**H_2), 22.7 (**C**H_2), 22.4 (**C**H_2), 22.1 (tetrahydropyridinyl-N^+CH_2**C**H_2), 14.2 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{24}H_{42}N_3O_3S^+$: 452.3. Found: 452.4.

1-(5-Carboxypentyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridin-1-ium bromide, ***6-L5*** *(FG_XA_26)*



$C_{20}H_{34}BrN_3O_3S$

M_r = 476.47 g/mol

TFA (350 µl, 4.57 mmol) was added dropwise to a solution of **55** (203.0 mg, 381 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 3 d at rt, the reaction mixture was worked up according to **G.4** to give **6-L5** as dark orange oil.

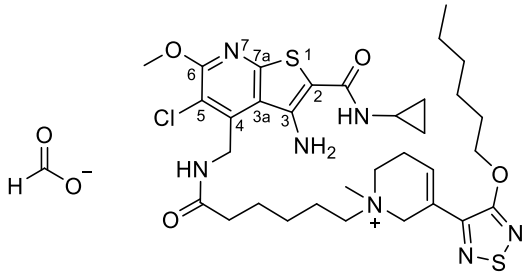
yield:	176 mg (97 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2952, 2930, 2859, 1722, 1510, 1376, 1174.

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 7.26-7.22 (m, 1H, tetrahydropyridinyl-C**H**), 4.39-4-24 (s, 2H, tetrahydropyridinyl-N^+C**H$_2$**C(C)=C), 4.48 (t, ^3J = 6.5 Hz, 2H, OC**H$_2$**), 3.54-3.40 (m, 2H, tetrahydropyridinyl-N^+C**H**$_2$$CH_2$), 3.39-3.31 (m, 2H, $N^+(CH_3)$C**H$_2$**), 3.06 (s, 3H, N^+C**H$_3$**), 2.74-2.68 (m, 2H, tetrahydropyridinyl-N^+CH_2C**H$_2$**), 2.33 (t, ^3J = 7.4 Hz, 2H, C**H$_2$**C(O)O), 1.88-1.79 (m, 4H, $N^+(CH_3)CH_2$C**H$_2$**, OCH_2C**H$_2$**), 1.68-1.60 (m, 2H, C**H$_2$**CH_2C(O)O), 1.52-1.44 (m, 2H, C**H$_2$**), 1.43-1.33 (m, 6H, C**H$_2$**), 0.91 (t, ^3J = 7.3 Hz, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, CD_3CN, δ [ppm]): 175.3 (**C**(O)O), 164.1 (thiadiazolyl-N**C**O), 145.9 (thiadiazolyl-**C**$_q$), 128.2 (tetrahydropyridinyl-**C**H), 124.1 (tetrahydropyridinyl-**C$_q$**), 72.8 (O**C**H_2), 65.2 ($N^+(CH_3)$**C**H_2), 60.5 (tetrahydropyridinyl-N^+**C**H_2C(C)=C), 57.4 (tetrahydropyridinyl-N^+**C**H_2CH_2), 49.5 (N^+**C**H_3), 34.4 (**C**H_2C(O)O), 32.5 (**C**H_2), 29.8 (**C**H_2), 26.7 (**C**H_2), 26.6 (**C**H_2), 25.3 (**C**H_2), 23.6 (**C**H_2), 22.8 (**C**H_2), 22.6 (tetrahydropyridinyl-N^+CH_2**C**H_2), 14.7 (**C**H_3).

MS (ESI) m/z [M^+] Calcd for $C_{20}H_{34}N_3O_3S^+$: 396.2. Found: 396.3.

1-(6-(((3-Amino-5-chloro-2-(cyclopropylcarbamoyl)-6-methoxythieno[2,3-b]pyridin-4-yl)-methyl)amino)-6-oxohexyl)-5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetra-hydropyridin-1-ium, ***LY20-MA5-XanA*** *(FG_XA_27)*



$C_{34}H_{48}ClN_7O_6S_2$

M_r = 750.37 g/mol

Carboxylic acid **6-L5** (83.8 mg, 176 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (31 µl, 180 µmol) and PyBOP (91.5 mg, 176 µmol) were successively added. Compound **54** (63.9 mg, 176 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (31 µl, 180 µmol) and the solution was added to the reaction mixture. After stirring at rt for 3 h, the mixture was worked up as described in **G.10** and purified by flash chromatography (reversed phase, H_2O+0.1 % FA/MeOH+0.1 % FA 2/3) to give **LY20-MA5-XanA** as pale-yellow solid. Remaining phosphorous oxide impurities were removed by recrystallisation in methanol and diethyl ether.

yield:	80 mg (58 %)
HPLC purity:	95 % (HPLC Method **IIb**)
reaction control:	R_f = 0.45 (alox basic; $CHCl_3$/CH_3OH: 200/1; Dragendorff-reagent)
Melting point:	125-133
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3291, 2932, 2867, 1652, 1512, 1368, 1271

^{1}H NMR (400 MHz, CD_3OD, δ [ppm]): 8.51 (s, 1H, **H**COO^-), 7.29-7.26 (m, 1H, tetrahydropyridinyl-C**H**), 4.92 (s, 2H, C**H$_2$**NH), 4.51 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 4.41 (s, 2H, tetrahydropyridinyl-N^+C**H$_2$**C(C)=C), 4.04 (s, 3H, OC**H$_3$**), 3.63-3.47 (m, 2H, tetrahydropyridinyl-N^+C**H$_2$**CH_2), 3.40 (t, ^3J = 6.6 Hz, 2H, $N^+(CH_3)$C**H$_2$**), 3.12 (s, 3H, N^+(C**H$_3$**)), 2.83-2.69 (m, 3H, tetrahydropyridinyl-N^+CH_2C**H$_2$**, cyclopropyl-C**H**), 2.30 (t, ^3J = 7.1 Hz, NHC(=O)C**H$_2$**), 1.91-1.81 (m, 4H, $N^+(CH_3)CH_2$C**H$_2$**, OCH_2C**H$_2$**), 1.74-1.64 (m, 2H, NHC(=O)CH_2C**H$_2$**), 1.52-1.32 (m, 8H, C**H$_2$**), 0.95-0.89 (m, 3H, C**H$_3$**), 0.79-0.74 (m, 2H, cyclopropyl-C**H$_2$**), 0.63-0.58 (m, 2H, cyclopropyl-C**H$_2$**).

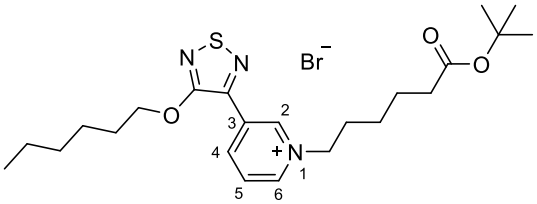
^{13}C NMR (100 MHz, CD_3OD, δ [ppm]): 176.5 (CH_2NH**C**(=O)), 169.4 (**C**ONHCH), 164.1 (thiadiazolyl-N**C**O), 160.6 (**C**-6), 157.4 (**C$_q$**), 149.3 (**C$_q$**), 145.6 (thiadiazolyl-**C$_q$**), 143.9 (**C**-4),

127.7 (tetrahydropyridinyl-**C**H), 124.4 (tetrahydropyridinyl-**C$_q$**), 121.8 (**C$_q$**), 118.9 (**C$_q$**), 100.5 (**C$_q$**), 72.8 (O**C**H$_2$), 65.6 (N$^+$(CH$_3$)**C**H$_2$), 60.5 (tetrahydropyridinyl-N$^+$**C**H$_2$C(C)=C), 57.4 (tetrahydropyridinyl-N$^+$**C**H$_2$CH$_2$), 55.7 (O**C**H$_3$), 48.4 (N$^+$(**C**H$_3$)), 38.3 (**C**H$_2$NH), 35.9 (NHC(=O)**C**H$_2$), 32.7 (**C**H$_2$), 30.0 (**C**H$_2$), 26.9 (**C**H$_2$), 26.9 (**C**H$_2$), 26.1 (NHC(=O)CH$_2$**C**H$_2$), 23.9 (cyclopropyl-**C**H), 23.7 (**C**H$_2$), 22.9 (**C**H$_2$), 22.7 (tetrahydropyridinyl-N$^+$CH$_2$**C**H$_2$), 14.5 (**C**H$_3$), 7.0 (cyclopropyl-C**H$_2$**).

MS (ESI) m/z [M$^+$] Calcd for $C_{33}H_{47}ClN_7O_4S_2^{2+}$: 352.6. Found: 352.9.

7.5.4 Preparation of LY20-MA5-XanC

1-(6-(tert-Butoxy)-6-oxohexyl)-3-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)pyridin-1-ium bromide, ***56*** *(FG_XA_28)*



$C_{23}H_{36}BrN_3O_3S$

M_r = 514.52 g/mol

A solution of **20** (1.21 g, 4.6 mmol) in DMF (10 ml) was added dropwise to a solution of *tert*-butyl-6-bromohexanoate (1.15 g, 4.6 mmol) in DMF (10 ml) at rt. The mixture was heated in a sealed tube to 110 °C for 2 d. The mixture was allowed to warm to rt and the solvent was removed. The residue was purified by column chromatography (silica, DCM/CH$_3$OH 10/1) to give **56** as a yellowish solid.

yield:	1.2 g (51 %)
reaction control:	R_f = 0.48 (silica; DCM/CH$_3$OH 10/1)
melting point [°C]:	81
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3441, 2927, 2857, 1670, 1508, 1365

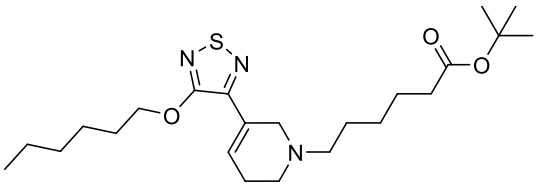
^{1}H NMR (400 MHz, CDCl$_3$, δ [ppm]): 9.87 (d, ^3J = 6.1 Hz, 1H, **H**-4), 9.46 (s, 1H, **H**-2), 9.03 (d, ^3J = 8.3 Hz, 1H, **H**-6), 8.26 (m, 1H, **H**-5), 5.05 (t, ^3J = 7.4 Hz, 2H, N$^+$C**H$_2$**), 4.50 (t, ^3J = 6.8 Hz, 2H, OC**H$_2$**), 2.12 (t, ^3J = 7.2 Hz, C(=O)C**H$_2$**), 2.08-2.00 (m, 2H, N$^+$CH$_2$C**H$_2$**), 1.88-1.78 (m, 2H OCH$_2$C**H$_2$**), 1.58-1.50 (m, 2H, C(O)CH$_2$C**H$_2$**), 1.41-1.33 (m, 4H, N$^+$CH$_2$CH$_2$C**H$_2$**, OCH$_2$CH$_2$C**H$_2$**), 1.32-1.21 (m, 13H, C**H$_2$**, C(C**H$_3$**)$_3$), 0.80 (t, ^3J = 7.0 Hz, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, CDCl$_3$, δ [ppm]): 172.7 (**C**(=O)O), 163.1 (thiadiazolyl-N**C**O), 145.8 (**C**-4), 142.0 (**C**-2), 142.0 (**C**-6), 139.8 (thiadiazolyl-**C$_q$**), 131.6 (**C**-3), 129.1 (**C**-5), 80.3 (**C**(CH$_3$)$_3$), 72.4 (O**C**H$_2$), 62.5 (N$^+$**C**H$_2$), 34.9 (C(=O)**C**H$_2$), 31.5 (**C**H$_2$), 31.4 (N$^+$CH$_2$**C**H$_2$), 28.7

($OCH_2\mathbf{C}H_2$), 28.1 ($C(\mathbf{C}H_3)_3$), 25.6 ($\mathbf{C}H_2$), 25.3 ($\mathbf{C}H_2$), 24.1 ($C(O)CH_2\mathbf{C}H_2$), 22.5 ($\mathbf{C}H_2$), 14.0 ($\mathbf{C}H_3$).

MS (ESI) m/z [M^+] Calcd for $C_{23}H_{36}N_3O_3S^+$: 434.3. Found: 434.7.

*tert-Butyl 6-(5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-3,6-dihydropyridin-1(2H)-yl)hexanoate, **57** (FG_XA_29)*



$C_{23}H_{39}N_3O_3S$

M_r = 437.64 g/mol

A solution of sodium borohydride (235.3 mg, 6.20 mmol) in methanol (5 ml) was added dropwise to a solution of compound **56** (400.0 mg, 777 µmol) in methanol (10 ml) at 0 °C. The mixture was allowed to warm to rt and stirred for 7 h. The reaction mixture was treated with saturated $NaHCO_3$ solution (10 ml) and extracted with DCM (3x20 ml). After phase separation, the combined organic layers were dried over Na_2SO_4 and the solvent was removed. The crude product was purified by column chromatography (silica, DCM/CH_3OH 19/1) to give **57** as a yellow oil.

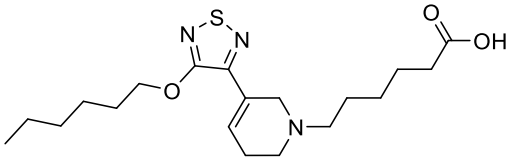
yield:	211 mg (62 %)
reaction control:	R_f = 0.31 (silica; DCM/CH_3OH 19/1)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2927, 2858, 1729, 1507, 1445, 1365, 1151, 989

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.06-7.02 (m, 1H, tetrahydropyridinyl-C**H**), 4.42 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 3.47 (s, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 2.59 (t, ^3J = 5.4 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.53-2.47 (m, 2H, NC**H$_2$**), 2.43-2.38 (m, 2H, tetrahydropyridinyl-NCH_2C**H$_2$**), 2.20 (t, ^3J = 7.5 Hz, C(=O)C**H$_2$**), 1.84-1.77 (m, 2H, OCH_2C**H$_2$**), 1.65-1.56 (m, 6H, C**H$_2$**), 1.42 (s, 9H, $C(C\mathbf{H_3})_3$), 1.38-1.30 (m, 6H, C**H$_2$**), 0.90-0.87 (m, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 173.4 (**C**(=O)O), 162.8 (thiadiazolyl-N**C**O), 143.7 (thiadiazolyl-**C$_q$**), 130.5 (tetrahydropyridinyl-**C$_q$**), 129.0 (tetrahydropyridinyl-**C**H), 80.2 ($\mathbf{C}(CH_3)_3$), 71.3 ($O\mathbf{C}H_2$), 58.6 ($N\mathbf{C}H_2$), 53.5 (tetrahydropyridinyl-N**C**H_2C(C)=C), 49.7 (tetrahydropyridinyl-N**C**H_2CH_2), 35.8 (C(=O)**C**H_2), 31.6 ($\mathbf{C}H_2$), 29.1 ($\mathbf{C}H_2$), 29.1 ($\mathbf{C}H_2$), 28.4 ($C(\mathbf{C}H_3)_3$), 28.4 ($\mathbf{C}H_2$), 27.3 (tetrahydropyridinyl-$NCH_2\mathbf{C}H_2$), 25.9 ($\mathbf{C}H_2$), 25.3 ($\mathbf{C}H_2$), 22.8 ($\mathbf{C}H_2$), 14.2 ($\mathbf{C}H_3$).

MS (ESI) m/z [M^+] Calcd for $C_{23}H_{40}N_3O_3S^+$: 438.3. Found: 438.6.

6-(5-(4-(Hexyloxy)-1,2,5-thiadiazol-3-yl)-3,6-dihydropyridin-1(2H)-yl)hexanoic acid, ***7-L5*** *(FG_XA_30)*



$C_{19}H_{31}N_3O_3S$

M_r = 381.54 g/mol

TFA (308 µl, 4.02 mmol) was added dropwise to a solution of **57** (176.0 mg, 402 µmol) in dry DCM (3 ml) at -20 °C under argon atmosphere. After 30 min of stirring at -20 °C and 3 d at rt, the reaction mixture was concentrated *in vacuo* to give orthosteric linker **7-L5** as yellow oil.

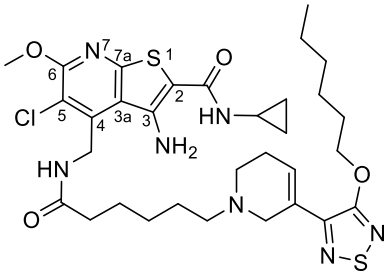
yield:	145 mg (95 %)
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2956, 2932, 2862,1713, 1658, 1447, 1377, 1148, 796

^{1}H NMR (400 MHz, CD_3CN, δ [ppm]): 9.84 (s, 1H, COO**H**), 7.21 (t, ^4J = 4.0 Hz, 1H, tetrahydropyridinyl-C**H**), 4.49-4.38 (m, 3H, OC**H$_2$**, tetrahydropyridinyl-NC**H**$_2$C(C)=C), 3.92 (d, ^2J = 15.6 Hz, 1H, tetrahydropyridinyl-NC**H**$_2$C(C)=C), 3.62-3.54 (m, 1H, tetrahydropyridinyl-NC**H**$_2$CH$_2$), 3.25-3.07 (m, 3H, tetrahydropyridinyl-NC**H**$_2$CH$_2$, NC**H$_2$**), 2.77-2.55 (m, 2H, tetrahydropyridinyl-NCH$_2$C**H$_2$**), 2.29 (t, 2H, ^3J = 7.4 Hz, C(=O)C**H$_2$**), 1.87-1.76 (m, 4H, C**H$_2$**), 1.65-1.56 (m, 2H, C**H$_2$**), 1.50-1.30 (m, 8H, C**H$_2$**), 0.93-0.87 (m, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 175.4 (**C**(=O)O), 164.0 (thiadiazolyl-N**C**O), 146.1 (thiadiazolyl-**C$_q$**), 129.1 (tetrahydropyridinyl-**C**H), 125.0 (tetrahydropyridinyl-**C$_q$**), 72.7 (O**C**H$_2$), 57.3 (N**C**H$_2$), 51.5 (tetrahydropyridinyl-N**C**H$_2$C(C)=C), 49.3 (tetrahydropyridinyl-N**C**H$_2$CH$_2$), 34.3 (C(=O)**C**H$_2$), 32.5 (**C**H$_2$), 29.8 (**C**H$_2$), 26.9 (**C**H$_2$), 25.9 (**C**H$_2$), 25.2 (**C**H$_2$), 24.7 (**C**H$_2$), 23.6 (**C**H$_2$), 23.6 (**C**H$_2$), 14.7 (**C**H$_3$).

MS (ESI) m/z [M^+] Calcd for $C_{19}H_{32}N_3O_3S^+$: 382.2. Found: 382.5.

3-Amino-5-chloro-N-cyclopropyl-4-((6-(5-(4-(hexyloxy)-1,2,5-thiadiazol-3-yl)-3,6-dihydropyridin-1(2H)-yl)hexanamido)methyl)-6-methoxythieno[2,3-b]pyridine-2-carboxamide, ***LY20-MA5-XanC*** *(FG_XA_31)*



$C_{32}H_{44}ClN_7O_4S_2$

M_r = 689.26 g/mol

Carboxylic acid **7-L5** (85.0 mg, 223 µmol) was dissolved in dry DMF (5 ml) under argon atmosphere. DIPEA (39 µl, 223 µmol) and PyBOP (115.9 mg, 223 µmol) were successively added. Compound **54** (80.9 mg, 223 µmol) was dissolved in dry DMF (3 ml), treated with DIPEA (39 µl, 223 µmol) and the solution was added to the reaction mixture. After stirring at rt for 5 h, the mixture was worked up as described in **G.10** and purified by column chromatography (alox basic, DCM/CH_3OH 250/1) to give **LY20-MA5-XanC** as yellow crystals.

yield:	45 mg (29 %)
HPLC purity:	95 % (HPLC Method **IIb**)
reaction control:	R_f = 0.14 (alox basic; DCM/CH_3OH 250/1; Dragendorff-reagent)
Melting point:	75-78
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	3288, 2926, 2856, 1652, 1505, 1445, 1270, 863

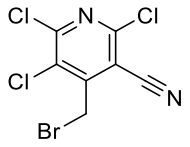
^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.46 (s, 2H, N**H$_2$**), 7.05-7.00 (m, 1H, tetrahydropyridinyl-C**H**), 6.42 (t, ^3J = 6.5 Hz, 1H, C(=O)N**H**CH_2), 5.55 (s, 1H, N**H**), 4.93 (d, ^3J = 6.6 Hz, 2H, C**H$_2$**NH), 4.40 (t, ^3J = 6.6 Hz, 2H, OC**H$_2$**), 4.04 (s, 3H, OC**H$_3$**), 3.42 (d, ^4J = 1.5 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**C(C)=C), 2.82-2.75 (m, 1H, cyclopropyl-C**H**), 2.55 (t, ^3J = 5.6 Hz, 2H, tetrahydropyridinyl-NC**H$_2$**CH_2), 2.47-2.34 (m, 4H, tetrahydropyridinyl-NCH_2C**H$_2$**, N(CH_3)C**H$_2$**), 2.19 (t, ^3J = 7.5 Hz, 2H, NHC(=O)C**H$_2$**), 1.95-1.75 (m, 2H, OCH_2C**H$_2$**,), 1.65-1.51 (m, 4H, NHC(=O)CH_2C**H$_2$**, N(CH_3)CH_2C**H$_2$**), 1.47-1.38 (m, 2H, C**H$_2$**), 1.36-1.26 (m, 6H, C**H$_2$**), 0.90-0.78 (m, 5H, C**H$_3$**, cyclopropyl-C**H$_2$**), 0.61-0.55 (m, 2H, cyclopropyl-C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 174.0 (CH_2NH**C**(=O)), 167.2 (**C**ONHCH), 162.8 (thiadiazolyl-N**C**O), 159.0 (**C**-6), 155.7 (**C$_q$**), 148.4 (**C$_q$**), 147.1 (thiadiazolyl-**C$_q$**), 142.3 (**C**-4), 129.5 (tetrahydropyridinyl-**C$_q$**), 129.0 (tetrahydropyridinyl-**C**H), 120.9 (**C$_q$**), 117.4 (**C$_q$**), 99.1 (**C$_q$**), 71.2 (O**C**H_2), 58.4 (N(CH_3)**C**H_2), 55.3 (O**C**H_3), 53.5 (tetrahydropyridinyl-N**C**H_2C(C)=C), 49.6 (tetrahydropyridinyl-N**C**H_2CH_2), 37.2 (**C**H_2NH), 36.4 (NHC(=O)**C**H_2), 31.6 (**C**H_2), 29.0 (**C**H_2), 27.2 (**C**H_2), 26.8 (**C**H_2), 26.7 (**C**H_2), 25.8 (**C**H_2), 25.5 (**C**H_2), 23.1 (cyclopropyl-**C**H), 22.7 (**C**H_2), 14.2 (**C**H_3), 7.1 (cyclopropyl-C**H$_2$**).

MS (ESI) m/z [M^+] Calcd for $C_{32}H_{45}ClN_7O_4S_2^+$: 690.3. Found: 690.2.

7.6 Attempted preparation of LY21-MAn hybrids

4-(Bromomethyl)-2,5,6-trichloronicotinonitrile, ***59*** *(FG_B_157)*



$C_7H_2BrCl_3N_2$

M_r = 300.36 g/mol

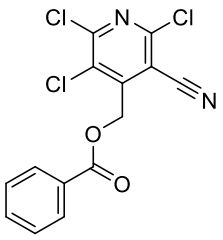
Compound **29** (1.1 g, 5.2 mmol) was dissolved in CCl_4 (40 ml) in a sealed pressure tube. CCl_4 was pre-dried over barium carbonate prior to use. After the addition of *N*-bromosuccinimide (1.8 g, 10.3 mmol) and benzoyl peroxide (62.3 mg, 0.3 mmol), the mixture was heated at 110 °C for a total of 2 d. The reaction mixture was cooled to rt, quenched with water (20 ml), and extracted with DCM (2x20 ml). After phase separation, the combined organic layers were dried over magnesium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (silica, Cy/EtOAc 20/1 → 5/1) to give compound **59** as white solid.

yield:	798 mg (52 %)
reaction control:	R_f = 0.36 (silica gel; EtOAc/*n*-hexane: 1/20)
melting point [°C]:	102
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2928, 2918, 2220, 1526, 1438, 1341, 1226

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.63 (s, 2H, C**H_2**Br).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 158.8 (**C_q**), 153.5 (**C_q**), 152.0 (**C_q**), 130.2 (**C_q**), 112.3 (**C_q**), 110.6 (**C_q**), 25.1 (**C**H_2Br).

(2,3,6-Trichloro-5-cyanopyridin-4-yl)methyl benzoate, ***60*** *(FG_B_161)*



$C_{14}H_7Cl_3N_2O_2$

M_r = 341.57 g/mol

A solution of compound **60** (775.0 mg, 2.6 mmol) in DMF (10 ml) was cooled to -50 °C and then treated with sodium benzoate (371.8 mg, 2.6 mmol). The solution was allowed to warm to rt and stirred for 2 h. After addition of diethyl ether (30 ml), the mixture was washed with water (2x10 ml). After phase separation, the organic layer was dried over sodium sulfate and the

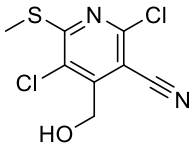
solvent was removed. The residue was purified by column chromatography (silica, Cy/EtOAc 20/1 → 10/1) to give compound **60** as white solid.

yield:	630 mg (72 %)
reaction control:	R_f = 0.34 (silica gel; EtOAc/Cy: 1/10)
melting point [°C]:	95
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2966, 2925, 1725, 1545, 1340, 1266

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 8.06-8.02 (m, 2H, ortho-bn-C**H**), 7.59 (m, 1H, para-bn-C**H**), 7.44 (t, ^3J = 7.8 Hz, 2H, meta-bn-C**H**), 5.59 (s, 2H, C**H$_2$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 165.8 (**C**(O)OCH_2), 150.6 (pyridinyl-**C$_q$**), 150.3 (pyridinyl-**C$_q$**), 149.5 (pyridinyl-**C$_q$**), 134.1 (para-bn-**C**), 130.3 (ortho-bn-**C**), 128.9 (benzylic-**C$_q$**), 128.6 (meta-bn-**C**), 113.6 (pyridinyl-**C$_q$**), 112.5 (**C**N), 111.6 (pyridinyl-**C$_q$**), 61.1 (**C**H_2).

2,5-Dichloro-4-(hydroxymethyl)-6-(methylthio)nicotinonitrile, ***61*** *(FG_B_163)*



$C_8H_6Cl_2N_2OS$

M_r = 249.11 g/mol

A suspension of compound **60** (533.0 mg, 1.6 mmol) in dry methanol (5 ml) was cooled to -78 °C in a dry ice bath under argon atmosphere. After addition of sodium methanethiolate (111.6 mg, 1.6 mmol), the reaction mixture was allowed to warm to 0 °C and stirred for 1 h. Then the mixture was warmed to rt, followed by further stirring for 1 h. The mixture was quenched with water (20 ml) and extracted with ethyl acetate (3x20 ml). The combined organic layers were dried over magnesium sulfate. After solvent removal, the residue was purified by column chromatography (silica, Cy/EtOAc 20/1) to give product **61** as white solid.

yield:	80 mg (21 %)
reaction control:	R_f = 0.27 (silica gel; Cy/EtOAc: 20/1)
melting point [°C]:	159
IR (ATR, $\tilde{\nu}$ [cm^{-1}]):	2928, 2848, 1763, 1595, 1342, 1168

^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 5.18 (s, 2H, C**H$_2$**OH), 2.65 (s, 3H, SC**H$_3$**).

^{13}C NMR (100 MHz, $CDCl_3$, δ [ppm]): 166.8 (**C$_q$**) 166.0 (**C$_q$**), 164.1 (**C$_q$**), 155.1 (**C$_q$**), 121.8 (**C**N), 117.2 (**C$_q$**), 66.8 (**C**H_2OH), 14.6 (S**C**H_3).

7.7 Solubility

A continuous shake flask protocol was followed for determination of thermodynamic solubility of **LY20-A6-iper**. Therefore, 1.00 mg of **LY20-A6-iper** was treated with 1 ml of PBS buffer (pH 7.4) in an Eppendorf vial, followed by 72 h of continuous shaking (800 rpm) und constant warming (37 °C). After centrifugation (13000 rpm, 1 min), samples of 100 µl were taken and analyzed by HPLC method **I** (B: 35 %). Solubility of **LY20-A6-iper** was determined in three independent measurements and is expressed as average. A solubility of 0.36 mg/ml was obtained. The calibration equation was created by dissolution of 2 mg of **LY20-A6-iper** in methanol and subsequent dilution to concentrations of 1.00 mg/ml, 0.75 mg/ml, 0.50 mg/ml, 0.25 mg/ml, 0.10 mg/ml, 0.05 mg/l, and 0.01 mg/l. The samples were analyzed by HPLC method **I** (B: 35 %) and the obtained peak areas were plotted against the corresponding concentrations to give the calibration curve.

8 Appendix

Processed data of mini-G protein BRET assay of **LY20-An-iper** series at receptor subtypes M_1-M_5

log [μM]	M1								M2							
	Acetylcholine		LY20-A6-iper		LY20-A8-iper		LY20-A10-iper		Acetylcholine		LY20-A6-iper		LY20-A8-iper		LY20-A10-iper	
	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]
-3.00	19.03	4.32	19.03	3.19	18.71	8.01	18.11	6.03	20.15	9.59	21.04	6.56	25.01	7.46	21.64	7.33
-2.00	22.64	15.65	/	/	/	/	/	/	20.06	12.19	/	/	/	/	/	/
-1.00	43.65	10.30	11.77	7.12	22.20	15.55	22.89	17.32	31.67	15.41	21.96	5.15	16.65	13.09	35.90	7.62
-0.52	50.10	18.60	28.28	9.05	28.71	5.47	29.43	16.36	49.80	10.46	32.13	6.39	30.91	15.80	37.10	10.82
0.00	63.14	9.54	40.85	11.62	34.45	13.47	32.71	14.48	66.84	12.23	37.74	6.68	39.37	10.92	40.61	8.58
0.48	73.40	16.62	39.94	10.70	41.63	16.30	24.34	7.44	69.99	15.06	37.24	12.99	44.44	16.96	48.99	4.48
1.00	70.15	10.78	37.09	11.81	36.28	17.64	39.37	13.72	78.97	20.09	40.56	8.08	35.84	5.66	54.91	13.11
2.00	/	/	36.27	7.76	55.18	13.41	37.54	20.37	/	/	48.14	8.47	44.46	18.59	57.07	21.98

log [μM]	M3								M4							
	Acetylcholine		LY20-A6-iper		LY20-A8-iper		LY20-A10-iper		Acetylcholine		LY20-A6-iper		LY20-A8-iper		LY20-A10-iper	
	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]
-3.00	27.49	7.36	29.43	6.33	29.32	7.40	27.05	10.69	13.82	6.13	13.60	5.24	13.17	4.12	12.39	5.45
-2.00	30.31	7.23	/	/	/	/	/	/	18.88	5.81	/	/	/	/	/	/
-1.00	41.60	9.88	28.31	8.11	34.73	10.23	21.60	7.01	37.02	9.92	13.89	6.92	14.02	2.52	12.05	5.65
-0.52	62.21	17.52	29.67	3.12	37.91	7.91	25.22	11.39	51.42	15.17	21.78	7.37	20.69	5.16	17.43	4.91
0.00	65.73	8.87	35.83	8.55	45.40	6.92	20.72	13.00	61.30	16.31	25.84	6.50	27.60	5.59	30.20	6.30
0.48	81.03	12.67	41.54	11.19	45.24	11.69	25.81	14.94	68.76	14.03	36.58	8.93	31.53	4.04	38.77	5.35
1.00	84.80	7.92	37.78	14.59	42.14	10.35	29.91	12.53	68.35	20.59	34.26	8.08	32.11	5.10	42.26	8.33
2.00	/	/	36.76	5.51	45.39	8.10	30.65	17.93	/	/	43.26	9.95	33.71	4.64	45.38	7.93

log [μM]	M5							
	Acetylcholine		LY20-A6-iper		LY20-A8-iper		LY20-A10-iper	
	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]	mean [%]	sdv [%]
-3.00	11.53	4.42	8.68	5.03	11.53	1.22	11.35	2.40
-2.00	20.26	6.98	/	/	/	/	/	/
-1.00	48.11	7.16	7.38	4.61	9.82	1.30	7.78	3.63
-0.52	66.47	7.06	8.15	9.00	12.36	1.59	12.01	4.82
0.00	78.05	5.83	11.10	4.51	14.76	3.90	12.54	5.41
0.48	82.62	6.09	12.20	8.49	14.43	4.29	17.35	5.31
1.00	88.62	7.77	12.85	9.51	16.82	4.21	17.15	3.66
2.00	/	/	13.55	7.61	13.65	2.10	15.13	2.53

FRET assay data of LY-hybrids at receptor subtypes M_2 and M_4

log [µM]	M2															
	Iperoxo		Xanomeline		LY20-A10-iper		LY20-A10-TMA		LY21-A10-iper		LY21-A10-TMA		LY20-A10-XanA		LY20-A10-XanB	
	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]
1.00	0.958	0.961	/	/	0.981	0.724	0.988	0.679	0.978	0.816	0.992	1.112	/	/	/	/
0.00	0.964	0.842	1.003	0.826	1.004	0.617	1.005	0.800	1.007	1.104	1.006	1.558	0.988	0.886	0.995	0.659
-0.52	0.963	0.871														
-1.00	0.967	0.925	1.007	1.021	1.008	0.841	1.008	0.579	1.010	1.020	1.013	2.077	0.994	0.966	1.005	0.711
-1.52	0.970	1.228														
-2.00	0.977	0.952	1.008	0.478	1.006	0.520	1.007	0.738	1.007	0.717	1.013	1.694	0.999	0.576	1.006	0.513
-2.52	0.990	0.721	1.006	0.760	1.006	0.481	1.007	0.614	1.003	0.670	1.012	1.587	1.000	0.679	1.004	0.384
-3.00	0.999	0.824	1.006	0.932	1.002	0.912	1.009	0.676	1.006	0.765	1.014	1.781	1.000	0.831	1.009	0.734
-4.00	1.002	0.000	1.005	0.574	1.008	0.804	1.005	0.741	1.002	1.278	1.008	1.050	0.999	0.615	1.005	0.578

log [µM]	M2						M4									
	LY20-MQ4-iper		LY20-MQ6-iper		LY20-MQ8-iper		Iperoxo		Xanomeline		LY20-A10-iper		LY20-A10-TMA		LY21-A10-iper	
	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]
1.00	0.991	0.861	0.984	0.569	0.996	0.575	0.977	1.085	/	/	0.965	1.170	0.974	1.221	0.956	1.665
0.00	0.998	0.839	1.001	0.537	1.004	0.233	0.986	0.873	1.014	2.397	1.001	1.420	1.004	0.851	0.989	1.047
-0.52	0.999	0.391	1.002	0.284	1.003	0.830	/	/	/	/	/	/	/	/	/	/
-1.00	0.998	0.645	1.005	0.449	1.006	0.405	0.987	0.786	1.015	2.239	1.002	1.206	1.003	1.023	1.003	0.990
-1.52	1.005	0.879	1.002	0.572	1.004	0.344	0.988	0.893	/	/	/	/	/	/	/	/
-2.00	1.000	0.785	1.002	0.647	1.008	0.519	0.984	0.900	1.021	2.308	1.006	0.900	1.001	0.698	0.997	0.646
-2.52	/	/	/	/	/	/	0.990	1.014	1.022	2.208	1.004	1.580	1.004	0.796	0.999	1.156
-3.00	0.999	2.144	1.009	0.677	1.007	0.521	0.996	0.941	1.025	2.276	1.004	1.642	1.003	0.735	1.005	0.930
-4.00	1.002	1.224	1.002	0.487	1.005	0.580	1.004	0.798	1.022	2.311	1.000	1.317	0.998	0.611	1.001	0.722

log [µM]	M4											
	LY21-A10-TMA		LY20-A10-XanA		LY20-A10-XanB		LY20-MQ4-iper		LY20-MQ6-iper		LY20-MQ8-iper	
	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]	mean	sdv [%]
1.00	0.970	0.691	/	/	/	/	0.996	0.745	0.980	2.278	0.996	0.575
0.00	0.990	0.635	0.994	0.984	/	/	1.003	0.731	0.995	0.866	1.004	0.233
-0.52	/	/	/	/	/	/	0.999	0.391	1.002	0.898	1.003	0.830
-1.00	0.996	0.671	1.000	1.058	0.990	1.774	1.006	1.040	0.996	0.422	1.006	0.405
-1.52	/	/	/	/	/	/	1.008	0.634	1.000	0.897	1.004	0.344
-2.00	0.999	0.745	1.000	0.935	0.990	2.027	1.005	1.222	0.999	0.975	1.008	0.519
-2.52	1.000	1.141	1.003	0.939	0.988	1.929	/	/	/	/	/	/
-3.00	1.003	0.748	0.999	0.461	0.991	2.460	1.009	0.419	0.998	0.602	1.007	0.521
-4.00	0.999	1.042	0.997	0.593	0.991	2.324	1.006	0.453	0.999	0.285	1.005	0.580

Processed data of mini-G protein BRET assay of LY-hybrids at receptor subtypes M_1, M_2 and M_4

log [µM]	M1															
	Iperoxo		Xanomeline		LY20-A10-iper		LY20-A10-TMA		LY21-A10-iper		LY21-A10-TMA		LY20-A10-XanA		LY20-A10-XanB	
	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]
1.00	79,45	6,50	41.26	6.16	17.69	4.43	11.07	5.76	20.87	3.53	16.65	4.67	/	/	/	/
0.00	81.36	7.55	45.96	4.84	22.89	2.93	13.62	6.52	22.59	6.91	20.47	2.92	21.50	4.29	23.82	3.85
-1.00	75.51	7.04	43.17	6.31	19.86	2.65	12.14	3.00	19.70	5.79	18.44	3.80	20.00	6.55	28.58	3.82
-2.00	62.37	5.93	32.30	2.99	12.46	2.87	8.47	4.79	14.47	2.66	14.10	2.42	20.07	2.94	22.58	3.33
-2.52	48.21	5.01	27.56	2.74	13.42	2.74	13.55	4.71	12.48	2.09	14.19	4.05	19.71	4.39	19.52	3.34
-3.00	34.39	5.35	26.97	6.02	12.31	2.08	10.69	5.65	12.80	2.87	11.97	2.47	16.69	7.47	19.89	1.64
-4.00	16.86	4.50	23.42	2.89	14.27	2.61	11.69	5.19	11.60	2.43	11.89	1.85	21.47	3.34	21.22	1.68

log [µM]	M1						M2									
	LY20-MQ4-iper		LY20-MQ6-iper		LY20-MQ8-iper		Iperoxo		Xanomeline		LY20-A10-iper		LY20-A10-TMA		LY21-A10-iper	
	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]
1.00	17.97	3.96	17.90	4.02	17.23	2.69	75.44	12.49	20.08	3.84	20.68	4.69	13.51	2.91	23.79	5.60
0.00	14.57	4.58	20.53	3.46	13.10	3.75	73.11	12.42	20.04	3.29	20.68	4.10	14.00	2.84	22.33	4.54
-1.00	8.98	3.29	14.74	2.14	10.03	3.58	73.41	7.87	16.82	3.51	18.77	3.08	13.81	3.97	19.15	3.63
-2.00	10.41	2.58	10.98	3.46	9.36	2.53	65.83	5.90	13.66	3.05	11.11	3.99	12.59	2.59	12.91	3.50
-2.52	10.42	4.43	11.40	4.04	9.29	2.70	55.81	6.30	13.31	3.17	13.63	2.16	12.21	2.84	10.63	4.98
-3.00	9.81	4.17	12.10	4.38	9.77	2.29	42.06	8.22	9.93	3.08	11.86	1.84	13.99	4.43	11.90	4.89
-4.00	10.01	4.79	9.20	3.56	9.25	4.00	21.39	4.43	13.10	3.18	14.03	4.07	11.06	2.46	11.60	5.80

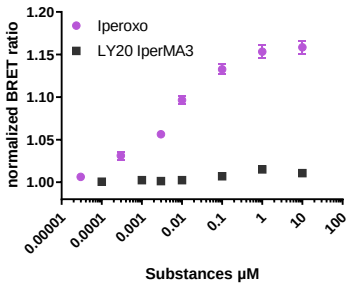
log [µM]	M2												M4			
	LY21-A10-TMA		LY20-A10-XanA		LY20-A10-XanB		LY20-MQ4-iper		LY20-MQ6-iper		LY20-MQ8-iper		Iperoxo		Xanomeline	
	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]
1.00	12.69	5.32	38.14	33.53	10.99	4.06	20.72	5.24	28.07	4.18	17.51	3.21	72.85	15.47	51.63	6.45
0.00	13.16	7.58	34.25	32.48	16.55	4.49	18.11	2.94	23.28	6.39	13.37	4.38	73.79	15.00	47.67	7.45
-1.00	12.90	5.84	39.26	33.31	12.84	3.07	13.26	4.10	19.07	6.03	13.30	5.71	72.96	14.55	45.24	4.38
-2.00	11.86	5.34	35.75	25.66	7.89	3.16	11.49	2.32	11.21	3.96	9.14	2.98	69.15	11.65	25.65	2.29
-2.52	12.55	3.70	29.41	21.38	8.86	4.47	11.51	2.43	12.41	5.37	12.04	2.87	58.84	8.18	16.04	1.73
-3.00	10.14	5.15	22.54	13.91	10.65	3.14	11.41	3.74	11.94	4.16	12.35	3.38	46.01	10.51	10.84	1.75
-4.00	14.93	4.84	13.10	6.00	9.54	1.82	13.12	5.11	11.06	4.07	12.62	4.00	15.92	3.73	6.72	2.74

log [µM]	M4															
	LY20-A10-iper		LY20-A10-TMA		LY21-A10-iper		LY21-A10-TMA		LY20-A10-XanA		LY20-A10-XanB		LY20-MQ4-iper		LY20-MQ6-iper	
	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]	sdv [%]
1.00	26.80	3.53	8.21	1.72	33.88	3.25	8.98	2.77	9.61	3.56	11.55	4.02	39.85	8.07	12.62	2.83
0.00	25.30	3.78	7.26	2.16	38.80	3.53	9.18	1.78	7.60	3.00	22.16	8.82	17.78	3.19	9.13	1.53
-1.00	16.90	2.90	5.94	3.08	27.46	3.51	7.07	2.06	6.62	3.65	14.15	3.83	9.14	2.08	8.63	1.87
-2.00	7.19	1.38	6.03	1.78	9.21	2.62	4.74	2.17	6.05	2.27	9.14	2.79	4.51	2.27	6.05	3.18
-2.52	7.56	2.84	8.02	1.87	8.47	1.71	5.12	2.91	7.49	2.95	7.11	2.14	5.86	2.30	5.68	2.02
-3.00	8.30	3.14	4.95	2.54	7.86	1.46	5.37	2.45	6.90	1.88	7.28	3.55	4.94	1.29	5.96	2.39
-4.00	7.16	2.24	5.91	2.83	8.31	1.97	5.51	3.12	5.36	1.66	6.22	2.79	5.97	2.20	6.97	1.33

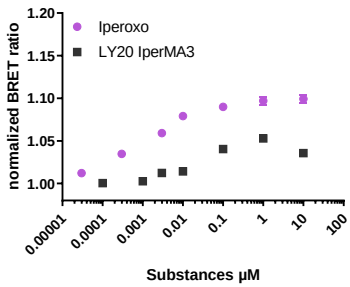
log [µM]	M4	
	LY20-MQ8-iper	
	sdv [%]	sdv [%]
1.00	12.15	3.29
0.00	9.38	2.83
-1.00	5.64	2.35
-2.00	3.73	1.70
-2.52	3.95	1.42
-3.00	5.67	2.17
-4.00	4.05	2.29

Preliminary results of LY20-MAn hybrid ligands

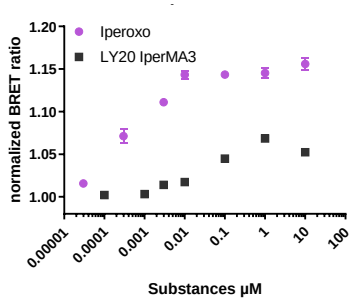
(Provided by Prof. Dr. Carsten Hoffmann)



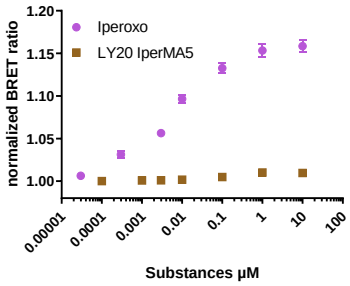
M_1 subtpye
LY20-MA3-iper



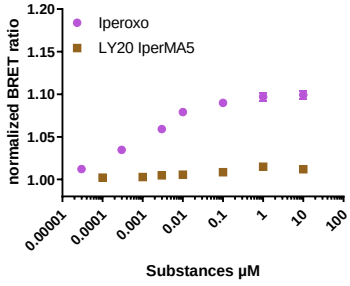
M_2 subtpye
LY20-MA3-iper



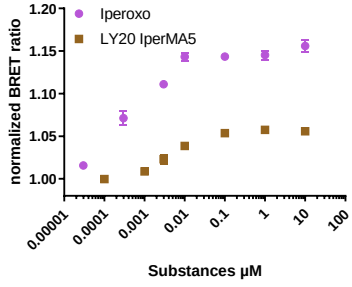
M_4 subtpye
LY20-MA3-iper



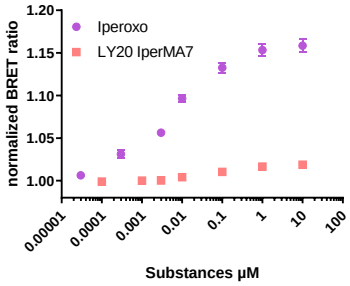
M_1 subtpye
LY20-MA5-iper



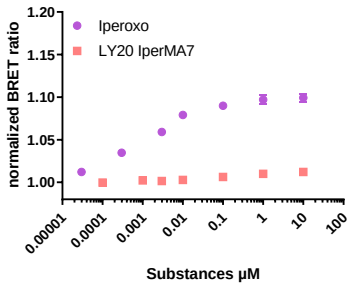
M_2 subtpye
LY20-MA5-iper



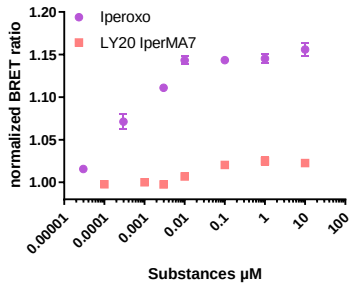
M_4 subtpye
LY20-MA5-iper



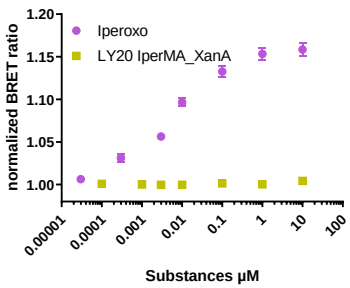
M_1 subtpye
LY20-MA7-iper



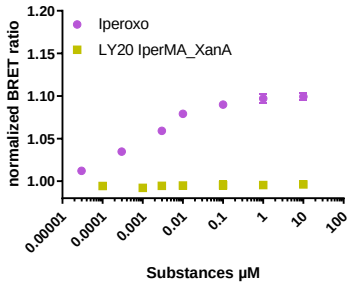
M_2 subtpye
LY20-MA7-iper



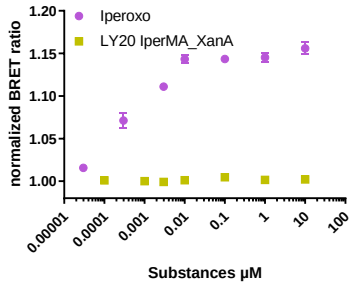
M_4 subtpye
LY20-MA7-iper



M_1 subtpye
LY20-MA5-XanA

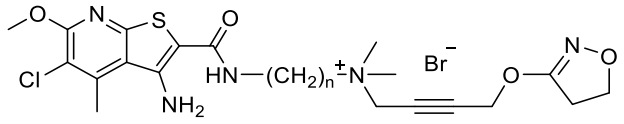


M_2 subtpye
LY20-MA5-XanA

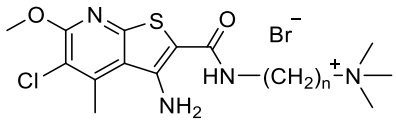


M_4 subtpye
LY20-MA5-XanA

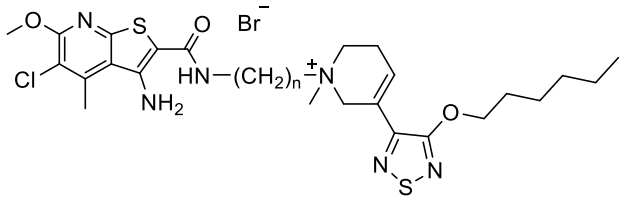
Overview of synthesized hybrid compounds:



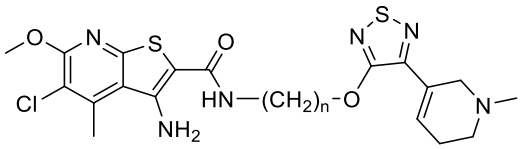
LY20-An-iper series
n = 6, 8, 10



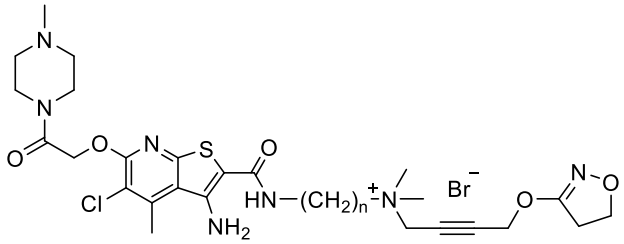
LY20-An-TMA series
n = 6, 8, 10



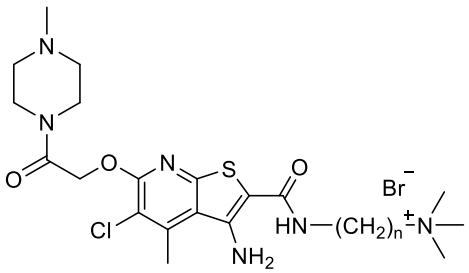
LY20-An-XanA series
n = 6, 8, 10



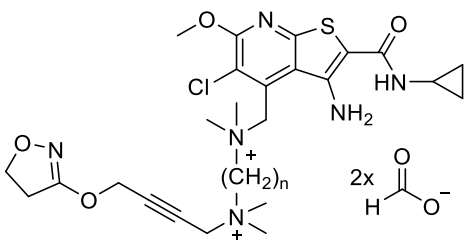
LY20-An-XanB series
n = 6, 8, 10



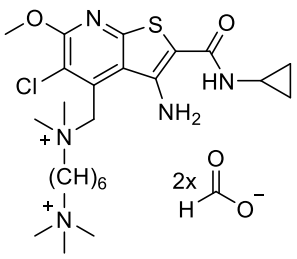
LY21-An-iper series
n = 6, 8, 10



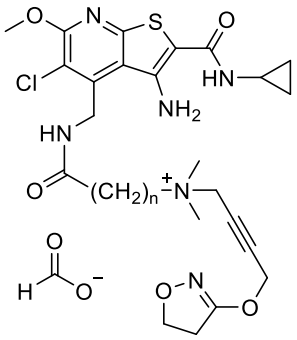
LY21-An-TMA series
n = 6, 8, 10



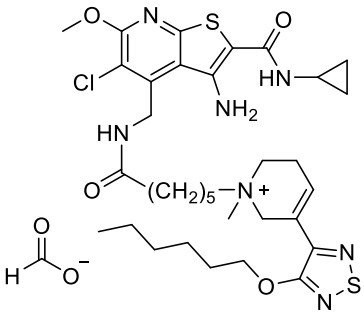
LY20-MQn-iper series
n = 4, 6, 8



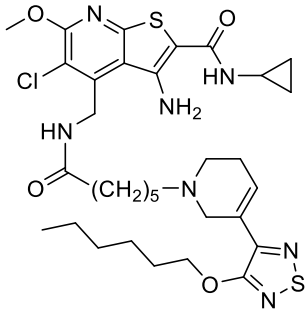
LY20-MQ6-TMA



LY20-MAn-iper series
n = 3, 5, 7



LY20-MA5-XanA



LY20-MA5-XanC

Abbreviations

AC	adenylate cyclase
ACh	acetylcholine
Boc	*tert*-butyloxycarbonyl protective group
Boc_2O	Boc anhydride
BQCA	benzyl quinolone carboxylic acid
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CFP	cyan fluorescent protein
CH_3CH_2OH	ethanol
$CHCl_3$	chloroform
CH_3OH	methanol
CNS	central nervous system
Cy	cyclohexane
DAG	diacylglycerol
DCM	dichloromethane
DMF	Dimethylformamide
DMSO	dimethylsulfoxide
EtOAc	ethyl acetate
FRET	Förster resonance energy transfer
ECL	extracellular loops
ESI	electrospray ionization
FlAsH	fluorescein arsenical hairpin binder
GDP	guanosine diphosphate
GPCR	G-protein-coupled receptor
G-protein	guanine nucleotide binding protein
GRK	G-protein coupled receptor kinase
GTP	guanosine triphosphate
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
ICL	intracellular loops
IP_3	inositol 1,4,5-trisphosphate
IR	infrared spectroscopy
LC/MS	liquid chromatography-mass spectrometry
LiHMDS	lithium bis trimethyl silyl amide
M_1-M_5	muscarinic acetylcholine subtype 1-5
mAChR	muscarinic acetylcholine receptors
mCPBA	*m*-chloroperoxybenzoic acid
MPLC	medium pressure liquid chromatography
nAChR	nicotinic acetylcholine receptor
NBS	*N*-bromo succinimide
NMR	nuclear magnetic resonance
PBS	phosphate-buffered saline
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PKC	protein kinase C
PyBOP	benzotriazol-1-yloxytripyrrolidinophos-phonium hexafluorophosphate
RP	reversed phase
rt	room temperature
SAR	structure activity relationship
SD	standard deviation

SEM	standard error of mean
TEA	triethylamine
TFA	trifluoro acetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
TM	transmembrane-spanning α-helical domain
TMA	trimethyl ammonium ion
UV-VIS	ultraviolet-visible
YFP	yellow fluorescent protein

9 Literature

[1] E. Mutschler, G. Geisslinger, H. Kroemer, P. Ruth, M. Schäfer-Körting, *Mutschler Arzneimittelwirkungen : Lehrbuch der Pharmakologie, der klinischen Pharmakologie und Toxikologie*, Wiss. Verlag-Ges. Stuttgart, **2013**.

[2] C. Matera, A. M. Tata, *Recent Pat. CNS Drug Discov.* **2014**, *9*, 85-100.

[3] J. M. Crook, E. Tomaskovic-Crook, D. L. Copolov, B. Dean, *Biol. Psychiatry* **2000**, *48*, 381-388.

[4] I. Ruiz de Azua, D. Gautam, J.-M. Guettier, J. Wess, *Trends Endocrinol. Metab.* **2011**, *22*, 74-80.

[5] A. C. Kruse, J. Hu, A. C. Pan, D. H. Arlow, D. M. Rosenbaum, E. Rosemond, H. F. Green, T. Liu, P. S. Chae, R. O. Dror, D. E. Shaw, W. I. Weis, J. Wess, B. K. Kobilka, *Nature* **2012**, *482*, 552-556.

[6] K. Haga, A. C. Kruse, H. Asada, T. Yurugi-Kobayashi, M. Shiroishi, C. Zhang, W. I. Weis, T. Okada, B. K. Kobilka, T. Haga, T. Kobayashi, *Nature* **2012**, *482*, 547-551.

[7] A. C. Kruse, A. M. Ring, A. Manglik, J. Hu, K. Hu, K. Eitel, H. Hübner, E. Pardon, C. Valant, P. M. Sexton, A. Christopoulos, C. C. Felder, P. Gmeiner, J. Steyaert, W. I. Weis, K. C. Garcia, J. Wess, B. K. Kobilka, *Nature* **2013**, *504*, 101-106.

[8] Z. Vuckovic, P. R. Gentry, A. E. Berizzi, K. Hirata, S. Varghese, G. Thompson, E. T. van der Westhuizen, W. A. C. Burger, R. Rahmani, C. Valant, C. J. Langmead, C. W. Lindsley, J. B. Baell, A. B. Tobin, P. M. Sexton, A. Christopoulos, D. M. Thal, *PNAS* **2019**, *116*, 26001-26007.

[9] J. Wess, R. M. Eglen, D. Gautam, *Nat. Rev. Drug Discov.* **2007**, *6*, 721-733.

[10] J. Wess, *Handb. Exp. Pharmacol.* **2012**, 95-117.

[11] K. Palczewski, T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, M. Yamamoto, M. Miyano, *Science* **2000**, *289*, 739-745.

[12] R. Fredriksson, M. C. Lagerström, L. G. Lundin, H. B. Schiöth, *Mol. Pharmacol.* **2003**, *63*, 1256-1272.

[13] M. Congreve, C. J. Langmead, J. S. Mason, F. H. Marshall, *J. Med. Chem.* **2011**, *54*, 4283-4311.

[14] M. F. Hibert, S. Trumpp-Kallmeyer, J. Hoflack, A. Bruinvels, *Trends Pharmacol. Sci.* **1993**, *14*, 7-12.

[15] T. I. Bonner, *Trends Neurosci.* **1989**, *12*, 148-151.

[16] M. P. Caulfield, *Pharmacol. Therap.* **1993**, *58*, 319-379.

[17] M. P. Caulfield, N. J. Birdsall, *Pharmacol. Rev.* **1998**, *50*, 279-290.

[18] E. C. Hulme, N. J. Birdsall, N. J. Buckley, *Annu. Rev. Pharmacol. Toxicol.* **1990**, *30*, 633-673.

[19] J. Wess, *Crit. Rev. Neurobiol.* **1996**, *10*, 69-99.

[20] E. Kostenis, B. R. Conklin, J. Wess, *Biochemistry* **1997**, *36*, 1487-1495.

[21] J. Wess, *Trends Pharmacol. Sci.* **1993**, *14*, 308-313.

[22] C. J. van Koppen, B. Kaiser, *Pharmacol. Ther.* **2003**, *98*, 197-220.

[23] P. Michal, E. El-Fakahany, V. Dolezal, *J. Pharmacol. Exp. Therap.* **2007**, *320*, 607-614.

[24] T. Oki, Y. Takagi, S. Inagaki, M. M. Taketo, T. Manabe, M. Matsui, S. Yamada, *Mol. Brain Res.* **2005**, *133*, 6-11.

[25] A. I. Levey, *Life. Sci.* **1993**, *52*, 441-448.

[26] M. T. Vilaró, G. Mengod, J. M. Palacios, *Progress in Brain Research, Vol. 98*, Elsevier, **1993**, pp. 95-101.

[27] R. M. Eglen, S. S. Hegde, N. Watson, *Pharmacol. Rev.* **1996**, *48*, 531-565.

[28] O. E. Brodde, M. C. Michel, *Pharmacol. Rev.* **1999**, *51*, 651-690.

[29] C. C. Felder, F. P. Bymaster, J. Ward, N. DeLapp, *J. Med. Chem.* **2000**, *43*, 4333-4353.

[30] T. A. Spalding, N. J. Birdsall, C. A. Curtis, E. C. Hulme, *J. Biol. Chem.* **1994**, *269*, 4092-4097.

[31] J. Wess, R. Maggio, J. R. Palmer, Z. Vogel, *J. Biol. Chem.* **1992**, *267*, 19313-19319.

[32] Y. Miao, S. E. Nichols, P. M. Gasper, V. T. Metzger, J. A. McCammon, *PNAS* **2013**, *110*, 10982-10987.

[33] P. Scheerer, J. H. Park, P. W. Hildebrand, Y. J. Kim, N. Krauss, H. W. Choe, K. P. Hofmann, O. P. Ernst, *Nature* **2008**, *455*, 497-502.

[34] J. H. Park, P. Scheerer, K. P. Hofmann, H. W. Choe, O. P. Ernst, *Nature* **2008**, *454*, 183-187.

[35] D. Steinhilber, M. Schubert-Zsilavecz, H. J. Roth, *Medizinische Chemie: Targets und Arzneistoffe*, Dt. Apotheker Verlag Stuttgart, **2005**.

[36] G. Milligan, E. Kostenis, *Br. J. Pharmacol* **2006**, *147*, 46-55.

[37] K. Palczewski, J. Buczyłko, M. W. Kaplan, A. S. Polans, J. W. Crabb, *J. Biol. Chem.* **1991**, *266*, 12949-12955.

[38] J. A. Pitcher, N. J. Freedman, R. J. Lefkowitz, *Annu. Rev. Bioch.* **1998**, *67*, 653-692.

[39] M. T. Drake, S. K. Shenoy, R. J. Lefkowitz, *Circ. Res.* **2006**, *99*, 570-582.

[40] L. M. Luttrell, S. S. G. Ferguson, Y. Daaka, W. E. Miller, S. Maudsley, G. J. Della Rocca, F.-T. Lin, H. Kawakatsu, K. Owada, D. K. Luttrell, M. G. Caron, R. J. Lefkowitz, *Science* **1999**, *283*, 655-661.

[41] R. H. Oakley, S. A. Laporte, J. A. Holt, M. G. Caron, L. S. Barak, *J. Biol. Chem.* **2000**, *275*, 17201-17210.

[42] Y. Werbonat, N. Kleutges, K. H. Jakobs, C. J. van Koppen, *J. Biol. Chem.* **2000**, *275*, 21969-21974.

[43] O. Vögler, G. S. Bogatkewitsch, C. Wriske, P. Krummenerl, K. H. Jakobs, C. J. van Koppen, *J. Biol. Chem.* **1998**, *273*, 12155-12160.

[44] Y. Lurie, S. P. Wasser, M. Taha, H. Shehade, J. Nijim, Y. Hoffmann, F. Basis, M. Vardi, O. Lavon, S. Suaed, B. Bisharat, Y. Bentur, *Clin. Toxicol.* **2009**, *47*, 562-565.

[45] K. J. Gregory, N. E. Hall, A. B. Tobin, P. M. Sexton, A. Christopoulos, *J. Biol. Chem.* **2010**, *285*, 7459-7474.

[46] D. A. Sykes, M. R. Dowling, S. J. Charlton, *Mol. Pharmacol.* **2009**, *76*, 543-551.

[47] R. Schrage, W. K. Seemann, J. Klöckner, C. Dallanoce, K. Racké, E. Kostenis, M. De Amici, U. Holzgrabe, K. Mohr, *Br. J. Pharmacol.* **2013**, *169*, 357-370.

[48] N. R. Mirza, D. Peters, R. G. Sparks, *CNS Drug Rev.* **2003**, *9*, 159-186.

[49] A. Shekhar, W. Potter, J. Lightfoot, J. Lienemann, S. Dubé, C. Mallinckrodt, F. P. Bymaster, D. L. McKinzie, C. C. Felder, *Am. J. Psychiatry* **2008**, *165*, 1033-1039.

[50] N. C. Bodick, W. W. Offen, A. I. Levey, N. R. Cutler, S. G. Gauthier, A. Satlin, H. E. Shannon, G. D. Tollefson, K. Rasmussen, F. P. Bymaster, D. J. Hurley, W. Z. Potter, S. M. Paul, *Arch. Neurol.* **1997**, *54*, 465-473.

[51] H. E. Shannon, F. P. Bymaster, D. O. Calligaro, B. Greenwood, C. H. Mitch, B. D. Sawyer, J. S. Ward, D. T. Wong, P. H. Olesen, M. J. Sheardown, *J. Pharmacol. Exp. Ther.* **1994**, *269*, 271-281.

[52] S. Brannan, S. Sawchak, A. Miller, S. M. Paul, A. Breier, *Biol. Psychiatry* **2020**, *87*, 169.

[53] S. K. Brannan, S. Sawchak, A. C. Miller, J. A. Lieberman, S. M. Paul, A. Breier, *N. Engl. J. Med.* **2021**, *384*, 717-726.

[54] F. J. Ehlert, *Mol. Pharmacol.* **1988**, *33*, 187-194.

[55] K. Mohr, J. Schmitz, R. Schrage, C. Tränkle, U. Holzgrabe, *Angew. Chem. Int. Ed.* **2013**, *52*, 508-516.

[56] D. Jäger, C. Schmalenbach, S. Prilla, J. Schrobang, A. Kebig, M. Sennwitz, E. Heller, C. Tränkle, U. Holzgrabe, H.-D. Höltje, K. Mohr, *J. Biol. Chem.* **2007**, *282*, 34968-34976.

[57] L. T. May, V. A. Avlani, C. J. Langmead, H. J. Herdon, M. D. Wood, P. M. Sexton, A. Christopoulos, *Mol. Pharmacol.* **2007**, *72*, 463-476.

[58] G. Milligan, N. J. Smith, *Trends Pharmacol. Sci.* **2007**, *28*, 615-620.

[59] C. J. Langmead, A. Christopoulos, *Trends Pharmacol. Sci.* **2006**, *27*, 475-481.

[60] D. M. Thal, B. Sun, D. Feng, V. Nawaratne, K. Leach, C. C. Felder, M. G. Bures, D. A. Evans, W. I. Weis, P. Bachhawat, T. S. Kobilka, P. M. Sexton, B. K. Kobilka, A. Christopoulos, *Nature* **2016**, *531*, 335-340.

[61] W. A. C. Burger, P. M. Sexton, A. Christopoulos, D. M. Thal, *J. Gen. Physiol.* **2018**, *150*, 1360-1372.

[62] H. Kords, H. Lüllmann, F. K. Ohnesorge, O. Wassermann, *Eur. J. Pharmacol.* **1968**, *3*, 341-346.

[63] H. Lüllmann, F. K. Ohnesorge, G. C. Schauwecker, O. Wassermann, *Eur. J. Pharmacol.* **1969**, *6*, 241-247.

[64] J. Jakubík, L. Bacáková, E. E. El-Fakahany, S. Tucek, *Mol. Pharmacol.* **1997**, *52*, 172-179.

[65] S. Lazareno, P. Gharagozloo, D. Kuonen, A. Popham, N. J. Birdsall, *Mol. Pharmacol.* **1998**, *53*, 573-589.

[66] T. A. Spalding, C. Trotter, N. Skjaerbaek, T. L. Messier, E. A. Currier, E. S. Burstein, D. Li, U. Hacksell, M. R. Brann, *Mol. Pharmacol.* **2002**, *61*, 1297-1302.

[67] E. A. Rubio, D. W. Hilliard, **2006**, *WO2006047124 A1*, WIPO.

[68] W. Y. Chan, D. L. McKinzie, S. Bose, S. N. Mitchell, J. M. Witkin, R. C. Thompson, A. Christopoulos, S. Lazareno, N. J. M. Birdsall, F. P. Bymaster, C. C. Felder, *Proceed. Nat. Acad. Sci.* **2008**, *105*, 10978-10983.

[69] R. L. Gannon, M. J. Millan, *Psychopharmacology* **2012**, *224*, 231-240.

[70] V. Nawaratne, K. Leach, N. Suratman, R. E. Loiacono, C. C. Felder, B. N. Armbruster, B. L. Roth, P. M. Sexton, A. Christopoulos, *Mol. Pharmacol.* **2008**, *74*, 1119-1131.

[71] C. H. Croy, D. A. Schober, H. Xiao, A. Quets, A. Christopoulos, C. C. Felder, *Mol. Pharmacol.* **2014**, *86*, 106-115.

[72] E. Burgisser, A. De Lean, R. J. Lefkowitz, *Proc. Nat. Acad. Sci.* **1982**, *79*, 1732-1736.

[73] S. Friedman, M. Tauber, Y. Ben-Chaim, *Sci. Rep.* **2020**, *10*, 11177.

[74] R. O. Dror, H. F. Green, C. Valant, D. W. Borhani, J. R. Valcourt, A. C. Pan, D. H. Arlow, M. Canals, J. R. Lane, R. Rahmani, J. B. Baell, P. M. Sexton, A. Christopoulos, D. E. Shaw, *Nature* **2013**, *503*, 295-299.

[75] T. Kenakin, *Nat. Rev. Drug. Discov.* **2005**, *4*, 919-927.

[76] S. Suratman, K. Leach, P. Sexton, C. Felder, R. Loiacono, A. Christopoulos, *Br. J. Pharmacol.* **2011**, *162*, 1659-1670.

[77] C. Valant, C. C. Felder, P. M. Sexton, A. Christopoulos, *Mol. Pharmacol.* **2012**, *81*, 41-52.

[78] T. Kenakin, A. Christopoulos, *Nat. Rev. Drug Discov.* **2013**, *12*, 205-216.

[79] J. E. Marlo, C. M. Niswender, E. L. Days, T. M. Bridges, Y. Xiang, A. L. Rodriguez, J. K. Shirey, A. E. Brady, T. Nalywajko, Q. Luo, C. A. Austin, M. B. Williams, K. Kim, R. Williams, D. Orton, H. A. Brown, C. W. Lindsley, C. D. Weaver, P. J. Conn, *Mol. Pharmacol.* **2009**, *75*, 577-588.

[80] A. N. Pronin, Q. Wang, V. Z. Slepak, *Mol. Pharmacol.* **2017**, *92*, 601-612.

[81] J. R. Lane, P. M. Sexton, A. Christopoulos, *Trends. Pharmacol. Sci.* **2013**, *34*, 59-66.

[82] C. Valant, J. Robert Lane, P. M. Sexton, A. Christopoulos, *Annu. Rev. Pharmacol. Toxicol.* **2012**, *52*, 153-178.

[83] R. Schwyzer, *Ann. N. Y. Acad. Sci.* **1977**, *297*, 3-26.

[84] T. Disingrini, M. Muth, C. Dallanoce, E. Barocelli, S. Bertoni, K. Kellershohn, K. Mohr, M. De Amici, U. Holzgrabe, *J. Med. Chem.* **2006**, *49*, 366-372.

[85] J. Antony, K. Kellershohn, M. Mohr-Andra, A. Kebig, S. Prilla, M. Muth, E. Heller, T. Disingrini, C. Dallanoce, S. Bertoni, J. Schrobang, C. Tränkle, E. Kostenis, A. Christopoulos, H. D. Holtje, E. Barocelli, M. De Amici, U. Holzgrabe, K. Mohr, *Faseb J.* **2009**, *23*, 442-450.

[86] A. Bock, B. Chirinda, F. Krebs, R. Messerer, J. Bätz, M. Muth, C. Dallanoce, D. Klingenthal, C. Tränkle, C. Hoffmann, M. De Amici, U. Holzgrabe, E. Kostenis, K. Mohr, *Nat. Chem. Biol.* **2014**, *10*, 18-20.

[87] Y. Kofuku, T. Ueda, J. Okude, Y. Shiraishi, K. Kondo, M. Maeda, H. Tsujishita, I. Shimada, *Nat. Commun.* **2012**, *3*, 1045.

[88] V. O. Nikolaev, C. Hoffmann, M. Bünemann, M. J. Lohse, J.-P. Vilardaga, *J. Biol. Chem.* **2006**, *281*, 24506-24511.

[89] W. K. Seemann, D. Wenzel, R. Schrage, J. Etscheid, T. Bödefeld, A. Bartol, M. Warnken, P. Sasse, J. Klöckner, U. Holzgrabe, M. DeAmici, E. Schlicker, K. Racké, E. Kostenis, R. Meyer, B. K. Fleischmann, K. Mohr, *J. Pharmacol. Exp. Ther.* **2017**, *360*, 289-299.

[90] I. Cristofaro, Z. Spinello, C. Matera, M. Fiore, L. Conti, M. De Amici, C. Dallanoce, A. M. Tata, *Neurochem. Int.* **2018**, *118*, 52-60.

[91] C. Guerriero, C. Matera, D. Del Bufalo, M. De Amici, L. Conti, C. Dallanoce, A. M. Tata, *Cells* **2021**, *10*, 1877.

[92] X. She, A. Pegoli, C. G. Gruber, D. Wifling, J. Carpenter, H. Hübner, M. Chen, J. Wan, G. Bernhardt, P. Gmeiner, N. D. Holliday, M. Keller, *J. Med. Chem.* **2020**, *63*, 4133-4154.

[93] A. Pegoli, D. Wifling, C. G. Gruber, X. She, H. Hübner, G. Bernhardt, P. Gmeiner, M. Keller, *J. Med. Chem.* **2019**, *62*, 5358-5369.

[94] C. G. Weinhart, D. Wifling, M. F. Schmidt, E. Neu, C. Höring, T. Clark, P. Gmeiner, M. Keller, *Eur. J. Med. Chem.* **2021**, *213*, 113159.

[95] R. Messerer, M. Kauk, D. Volpato, M. C. Alonso Canizal, J. Klöckner, U. Zabel, S. Nuber, C. Hoffmann, U. Holzgrabe, *ACS Chem. Biol.* **2017**, *12*, 833-843.

[96] D. Volpato, M. Kauk, R. Messerer, M. Bermudez, G. Wolber, A. Bock, C. Hoffmann, U. Holzgrabe, *ACS Omega* **2020**, *5*, 31706-31715.

[97] L. Agnetta, M. Kauk, M. C. A. Canizal, R. Messerer, U. Holzgrabe, C. Hoffmann, M. Decker, *Angew. Chem. Int. Ed.* **2017**, *56*, 7282-7287.

[98] M. Jiménez-Rosés, M.-T. Matsoukas, G. Caltabiano, A. Cordomí, *J. Chem. Inf. Model.* **2018**, *58*, 1074-1082.

[99] R. A. Leppik, R. C. Miller, M. Eck, J. L. Paquet, *Mol. Pharmacol.* **1994**, *45*, 983-990.

[100] M. Bermudez, C. Rakers, G. Wolber, *Mol. Inf.* **2015**, *34*, 526-530.

[101] R. Capelli, A. Bochicchio, G. Piccini, R. Casasnovas, P. Carloni, M. Parrinello, *J. Chem. Theory Comput.* **2019**.

[102] K. Leach, A. E. Davey, C. C. Felder, P. M. Sexton, A. Christopoulos, *Mol. Pharmacol.* **2011**, *79*, 855-865.

[103] J. Klöckner, PhD thesis, *Design Subtyp-selektiver Agonisten und Antagonisten muskarinischer Rezeptoren*, Julius-Maximilians-Universität Würzburg **2013**.

[104] M. Szabo, T. Huynh, C. Valant, J. R. Lane, P. M. Sexton, A. Christopoulos, B. Capuano, *Med. Chem. Commun.* **2015**, *6*, 1998-2003.

[105] C. Matera, L. Flammini, M. Quadri, V. Vivo, V. Ballabeni, U. Holzgrabe, K. Mohr, M. De Amici, E. Barocelli, S. Bertoni, C. Dallanoce, *Eur. J. Med. Chem.* **2014**, *75*, 222-232.

[106] X. Chen, J. Klöckner, J. Holze, C. Zimmermann, W. K. Seemann, R. Schrage, A. Bock, K. Mohr, C. Tränkle, U. Holzgrabe, M. Decker, *J. Med. Chem.* **2015**, *58*, 560-576.

[107] L. Fang, S. Jumpertz, Y. Zhang, D. Appenroth, C. Fleck, K. Mohr, C. Tränkle, M. Decker, *J. Med. Chem.* **2010**, *53*, 2094-2103.

[108] M. Maspero, D. Volpato, D. Cirillo, N. Yuan Chen, R. Messerer, C. Sotriffer, M. De Amici, U. Holzgrabe, C. Dallanoce, *Bioorg. Chem.* **2020**, *96*, 103633.

[109] M. Szabo, C. Klein Herenbrink, A. Christopoulos, J. R. Lane, B. Capuano, *J. Med. Chem.* **2014**, *57*, 4924-4939.

[110] V. V. Dotsenko, S. G. Krivokolysko, V. V. Polovinko, V. P. Litvinov, *Chem. Het. Comp.* **2012**, *48*, 309-319.

[111] J. Kloeckner, J. Schmitz, U. Holzgrabe, *Tetrahedron Lett.* **2010**, *51*, 3470-3472.

[112] P. Sauerberg, P. H. Olesen, S. Nielsen, S. Treppendahl, M. J. Sheardown, T. Honore, C. H. Mitch, J. S. Ward, A. J. Pike, *J. Med. Chem.* **1992**, *35*, 2274-2283.

[113] X. She, A. Pegoli, J. Mayr, H. Hübner, G. Bernhardt, P. Gmeiner, M. Keller, *ACS Omega* **2017**, *2*, 6741-6754.

[114] B. E. Kane, M. K. O. Grant, E. E. El-Fakahany, D. M. Ferguson, *Bioorg. & Med. Chem.* **2008**, *16*, 1376-1392.

[115] J. Coste, D. Le-Nguyen, B. Castro, *Tetrahedron Lett.* **1990**, *31*, 205-208.

[116] G. Hiltensperger, N. Hecht, M. Kaiser, J.-C. Rybak, A. Hoerst, N. Dannenbauer, K. Müller-Buschbaum, H. Bruhn, H. Esch, L. Lehmann, L. Meinel, U. Holzgrabe, *Antimicrob. Agents Chemother.* **2016**, *60*, 4442-4452.

[117] D. Renck, P. Machado, A. A. Souto, L. A. Rosado, T. Erig, M. M. Campos, C. B. Farias, R. Roesler, L. F. S. M. Timmers, O. N. de Souza, D. S. Santos, L. A. Basso, *J. Med. Chem.* **2013**, *56*, 8892-8902.

[118] M. Lounasmaa, C.-J. Johansson, *Tetrahedron* **1977**, *33*, 113-117.

[119] L. V. Dyadyuchenko, V. D. Strelkov, S. N. Mikhailichenko, V. N. Zaplishny, *Chem. Heterocycl. Compd.* **2004**, *40*, 308-314.

[120] A. C. Castro, K. Chan, C. A. Evans, L. Li, Y. Liu, P. Ren, D. A. Snyder, T. E. Wilson, **2012**, *WO2012064973A2*, WIPO.

[121] R. Ding, Y. He, X. Wang, J. Xu, Y. Chen, M. Feng, C. Qi, *Molecules* **2011**, *16*, 5665-5673.

[122] C. Dallanoce, P. Conti, M. De Amici, C. De Micheli, E. Barocelli, M. Chiavarini, V. Ballabeni, S. Bertoni, M. Impicciatore, *Bioorg. Med. Chem.* **1999**, *7*, 1539-1547.

[123] M. De Amici, P. Conti, E. Fasoli, E. Barocelli, V. Ballabeni, S. Bertoni, M. Impicciatore, B. L. Roth, P. Ernsberger, C. De Micheli, *Il Farmaco* **2003**, *58*, 739-748.

[124] A. Bock, N. Merten, R. Schrage, C. Dallanoce, J. Bätz, J. Klöckner, J. Schmitz, C. Matera, K. Simon, A. Kebig, L. Peters, A. Müller, J. Schrobang-Ley, C. Tränkle, C. Hoffmann, M. De Amici, U. Holzgrabe, E. Kostenis, K. Mohr, *Nat. Com.* **2012**, *3*, 1044.

[125] C. King, B. Barbiellini, D. Moser, V. Renugopalakrishnan, *Phys. Rev. B* **2012**, *85*, 125106.

[126] L. Stryer, *Annu. Rev. Biochem.* **1978**, *47*, 819-846.

[127] R. Yasuda, *Curr. Opin. Neurobiol.* **2006**, *16*, 551-561.

[128] C.-W. Chang, M. Wu, S. Merajver, M.-A. Mycek, *J. Biomed. Opt.* **2009**, *14*, 060502.

[129] J. P. Vilardaga, M. Bünemann, C. Krasel, M. Castro, M. J. Lohse, *Nat. Biotechnol.* **2003**, *21*, 807-812.

[130] C. Hoffmann, G. Gaietta, M. Bünemann, S. R. Adams, S. Oberdorff-Maass, B. Behr, J. P. Vilardaga, R. Y. Tsien, M. H. Ellisman, M. J. Lohse, *Nat. Methods* **2005**, *2*, 171-176.

[131] A. D. Stumpf, C. Hoffmann, *Br. J. Pharmacol.* **2016**, *173*, 255-266.

[132] J. Wess, *Pharmacol. & Therap.* **1998**, *80*, 231-264.

[133] M. J. Lohse, S. Nuber, C. Hoffmann, *Pharmacol. Rev.* **2012**, *64*, 299-336.

[134] B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269-272.

[135] N. Ziegler, J. Bätz, U. Zabel, M. J. Lohse, C. Hoffmann, *Bioorg. Med. Chem.* **2011**, *19*, 1048-1054.

[136] C. Galés, R. V. Rebois, M. Hogue, P. Trieu, A. Breit, T. E. Hébert, M. Bouvier, *Nat. Methods* **2005**, *2*, 177-184.

[137] P. Hein, M. Frank, C. Hoffmann, M. J. Lohse, M. Bünemann, *Embo J.* **2005**, *24*, 4106-4114.

[138] Q. Wan, N. Okashah, A. Inoue, R. Nehmé, B. Carpenter, C. G. Tate, N. A. Lambert, *J. Biol. Chem.* **2018**, *293*, 7466-7473.

[139] R. Irannejad, J. C. Tomshine, J. R. Tomshine, M. Chevalier, J. P. Mahoney, J. Steyaert, S. G. F. Rasmussen, R. K. Sunahara, H. El-Samad, B. Huang, M. von Zastrow, *Nature* **2013**, *495*, 534-538.

[140] C. J. Langmead, A. Christopoulos, *Br. J. Pharmacol.* **2013**, *169*, 353-356.

[141] A. Nayeem, A. Khatun, M. Rahman, *J. Pharmacogn. Phytotherapy* **2011**, *3*, 118-123.

[142] S. Budavari, *The Merck index : an encyclopedia of chemicals, drugs, and biologicals*, Merck, Rahway, N.J., U.S.A., **1989**.

[143] S. Hünig, P. Kreitmeier, G. Märkl, J. Sauer, *Arbeitsmethoden in der organischen Chemie*, Lehmanns Verlag, Berlin, **2006**.

[144] J. P. Kennedy, T. M. Bridges, P. R. Gentry, J. T. Brogan, A. S. Kane, C. K. Jones, A. E. Brady, J. K. Shirey, P. J. Conn, C. W. Lindsley, *ChemMedChem* **2009**, *4*, 1600-1607.

[145] R. L. Salvador, D. Simon, *Can. J. Chem.* **1966**, *44*, 2570-2575.

[146] K. Baum, D. Tzeng, *J. Org. Chem.* **1985**, *50*, 2736-2739.

[147] D. L. Musso, F. R. Cochran, J. L. Kelley, E. W. McLean, J. L. Selph, G. C. Rigdon, G. F. Orr, R. G. Davis, B. R. Cooper, V. L. Styles, J. B. Thompson, W. R. Hall, *J. Med. Chem.* **2003**, *46*, 399-408.