

Synthesis of Functionalized Molecular Probes for Bioorthogonal Metabolic Glycoengineering



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To,

My parents, the memories of whom are most precious patrimony of my life!

My brothers and sister along with my wife Ansa and most beloved children,

Ateeb and Areesa with love and sincerity!

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Abbreviations

aaRSs	Aminoacyl-tRNA synthase
ABPP	Activity based protein profiling
AcO	Acetate
Ac ₂ O	Acetic anhydride
AcOH	Acetic acid
Ac ₄ ManNAz	Peracetylated- <i>N</i> -azidoacetylmannosamine
Anhyd	Anhydrous
Boc	<i>tert</i> -Butoxy carbonyl
Boc ₂ O	Boc anhydride
Bz	Benzoyl
BuOH	Butanol
BTES	2-[4-({Bis[(1- <i>tert</i> -butyl-1 <i>H</i> -1,2,3-triazole-4-yl)methyl]amino}methyl)-1 <i>H</i> -1,2,3-triazole-1-yl]ethyl hydrogen sulfate
Cbz	Benzyloxy carbamate
CC	Click chemistry
CHO	Chinese hamster ovary cells
CMP	Cytidine monophosphate
CMAH	Cytidine monophosphate- <i>N</i> -acetylneuraminic acid hydroxylase
CuAAC	Cu catalyzed azide alkyne cycloaddition
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	Dicyclohexyl urea
DEPT	Distortionless Enhancement by Polarization Transfer
DFT	Discrete Fourier Transformation
DIFO	Difluorocyclooctyne
DIPEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
Dns Cl	Dansyl chloride
DPA	Diisopropylamine
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EI	Electron ionization

ER	Endoplasmic Reticulum
ESI-MS	Electron spray ionization mass spectrometer
ETBE	Ethyl <i>tert</i> -butyl ether
Et ₂ O/DEE	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
EtONa	Sodium ethoxide
FAB	Fast atom bombardment
FDA	Food and drug administration
Fmoc	9-Fluorenylmethylcarbamate
G1/G2	Grubbs' catalyst 1 st /2 nd generation
Gal	Galactose
GalNAz	<i>N</i> -acetyl azide galactosamine
GlcNAc	<i>N</i> -Acetylglucosamine
GlcNAz	<i>N</i> -acetylazide glucosamine
HEp-2	Human laryngeal tumor cells
Hex	Hexane
HIV	Human immunodeficiency virus
HOMO	Highly occupied molecular orbitals
Hpg	Human plasminogen
IPA	Isopropyl alcohol
KDN	2-Keto-3-deoxy nononic acid
KHMDS	Potassium hexamethyl disilazide
LD ₅₀	Lethal dose causing death of lab animal up to 50%
LDA	Lithium diisopropylamide
LHMDS	Lithium hexamethyl disilazide
LUMO	Lowest unoccupied molecular orbitals
MALDI	Matrix-assisted laser desorption/ionization
Man	Mannose
ManNAc	<i>N</i> -Acetylmannosamine
ManNAz	<i>N</i> -acetylmannosazide
MeCN	Acetonitrile
Mito-GFP	Mitochondrial green fluorescent protein
MeONa	Sodium methoxide

MS	Molecular sieves
Neu5Ac	<i>N</i> -Acetylneuraminic acid
Neu5Gc	<i>N</i> -Glycolylneuraminic acid
Neu5Hept	<i>N</i> -Hept-6-ynoylneuraminic acid
Neu5Hex	<i>N</i> -Hex-5-ynoylneuraminic acid
Neu5pent	<i>N</i> -Pent-4-ynoylneuraminic acid
NHS	<i>N</i> -Hydroxysuccinimide
PCC	Pyridinium chlorochromate
Pd/C	Palladium on active carbon
PFP-TFA	Pentafluorophenyl trifluoroacetate
RCM	Ring closing metathesis
S1	Schrock's catalyst 1 st generation
Sia	Sialic acid
Sia5Az	5- <i>N</i> -Azidoacetylsialic acid
Siglec	Sialic-acid-binding immunoglobulin-like lectin
SPAAC	Strained-prompted azide alkyne cycloaddition
TAMRA	Tetramethylrhodamine
TBAF	Tetrabutylammonium fluoride
TBTA	Tris[(1-benzyl-1 <i>H</i> -1,2,3-triazole-4-yl)methyl]amine
TEA	Triethylamine
THTPA	Tris[(hydroxypropyltriazolyl)methyl]amine
Tf	Trifluoromethanesulfonyl (triflate)
Tf ₂ NPh	<i>N</i> -Phenyl-bis(trifluoromethanesulfonamide)
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl

1 Introduction

The cell surface of eukaryotes is decorated by carbohydrate conjugates of lipids and proteins known as glycolipids and glycoproteins, respectively. These structures, commonly known as glycoconjugates, are poised to mediate different activities of cell surface recognition, such as bacterial and viral binding to the host cell and leukocyte adhesion during an inflammatory response, signal transduction, as well as intracellular processes including trafficking of proteins to the lysosome, transcription and translation. Carbohydrates are commonly derived from periplasma or synthesized by metabolic cellular processes.^[1] Oligosaccharides covalently attached through the *N*-glycosidic or *O*-glycosidic linkage are responsible for the modification of proteins. On the other hand *N*-glycosylation occurs in Endoplasmic Reticulum (ER) and the Golgi.^[2]

The complexity of cellular systems is a big challenge for scientists to study biomolecules in natural environments. To understand and intervene the processes at DNA/RNA level, protein-protein interaction, enzymatic action, and cell surface modification, a detailed insight into the structural and chemical mode of action is a prerequisite.^[3] In recent years a number of technologies have been developed for selective modification in the living system of a selected species to understand cellular processes. One of the keys studying those processes is the bioorthogonal chemical reaction. A reaction, which is orthogonal with living conditions and is not disturbed by other moieties present in physiological environments and *vice versa*, is known as a bioorthogonal reaction.^[4] The ability to selectively tag a biomolecule in living cells or organisms enables researchers to:

- monitor its involvement in a given biological event as it progresses in its natural environment
- retrieve and analyze in detail the tagged molecule or its metabolites.

Metabolic glycoengineering has become a premier tactic for incorporation and glycoconjugation of chemical reporters, namely functional groups that possess unique reactivity orthogonal to natural biomolecules into cellular architecture.^[5-6] Theoretically, metabolic glycoengineering is a very straightforward technique. The reporter can simply be added to the cells culture from where it enters into the cell and the enzymatic machinery functions as a black box that performs the synthetically challenging task of creating a modified reporter.^[7] The first step out of two is the incubation of a synthetic monosaccharide in cell media or incorporation into living animals.^[4] This synthetic monosaccharide has a similar structure to a natural precursor but is adorned with a unique functionality- “the chemical reporter”. Once, the chemical reporter enters the system, it is

processed by biosynthetic enzymes in a manner similar to the natural precursor and is tagged with appropriate intra or extracellular target depending on the nature of the motif bearing it. The second step is the treatment of this modified metabolite of the reporter with a counter partner called chemical probe bearing complementary bioorthogonal functionality. The principle of metabolic glycoconjugation is illustrated in fig. 1.1.

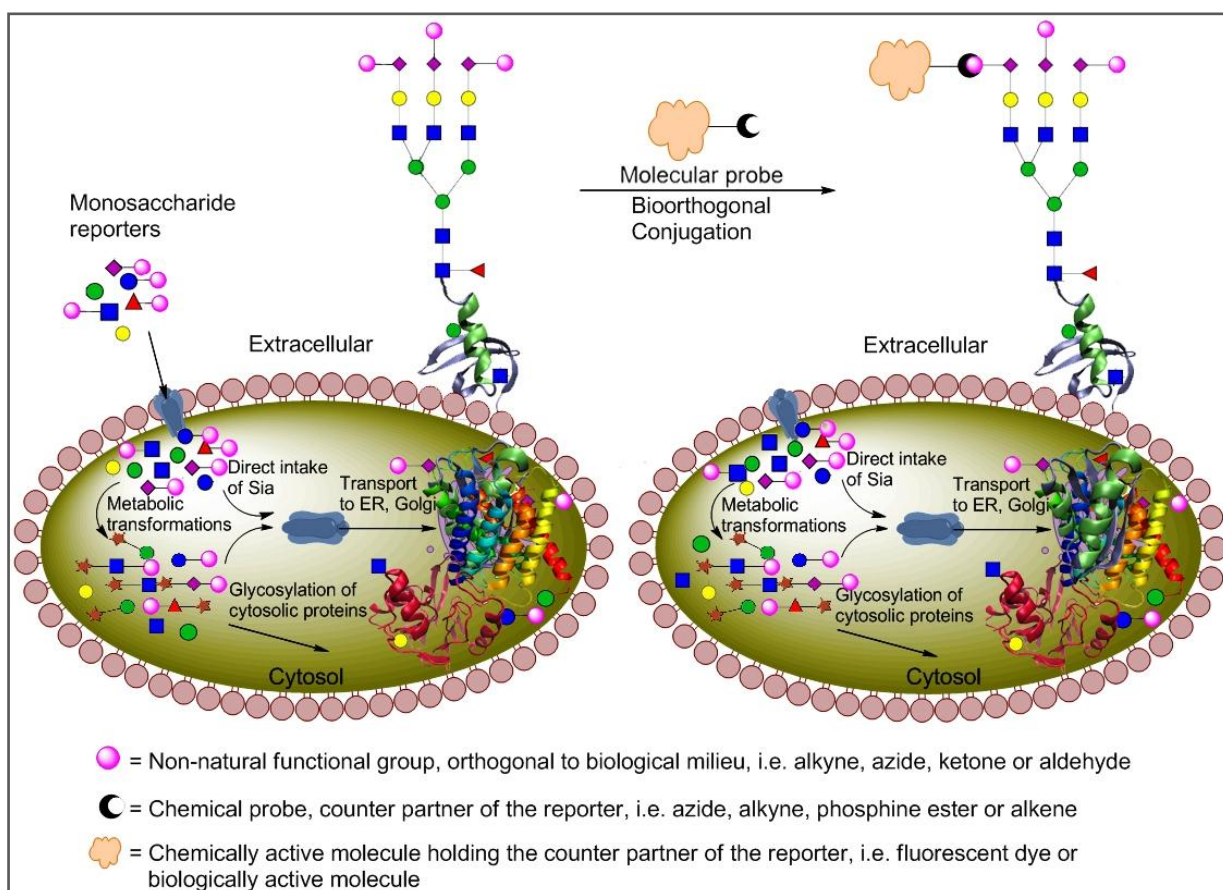


Figure 1.1: The bioorthogonal chemical strategy, showing a chemical reporter (light pink) linked to the substrate, is introduced into the target biomolecules (inside the cell and cell surface glycoconjugates) metabolically. In the second step the reporter is tagged with the probe through a bioorthogonal chemical reaction.

Bioorthogonal reporters are non-native, non-perturbing chemical motifs that can be modified in living systems through a highly selective reaction with exogenously delivered probes.^[8] Regardless of the route exploited, each enzyme involved in the installation must tolerate the unnatural motif. For this reason typical biophysical probes such as fluorescein cannot be used for direct modification to the metabolic substrate, as their relatively large size will interfere with enzymatic transformations. A small functional group is more likely tolerated by metabolic enzymes. The reporter and its partner must be mutually reactive in a physiological environment (37 °C, pH 6-8) and at the same time remain inert to the surrounding biological milieu. The metabolite could be a monosaccharide for glycan labeling, a nucleoside for DNA labeling, an

amino acid for protein labeling and a fatty acid for lipid labeling. The reporters with unnatural functionality which can be used for glycoconjugation may be glucose, mannose and sialic acid with appropriate metabolites. The most important of these are sialic acids and their modified derivatives due to their ability to decorate the cell surface glycoconjugates.^[9]

Different strategies for bioorthogonal glycoconjugation in practice are Staudinger ligation^[10], azide alkyne cycloaddition (click reaction)^[11] and condensation of a ketone/aldehyde with amine^[12] nucleophiles. However, azides have proven to be powerful reporters due to their small size and orthogonality to major biological functional groups; therefore, they minimally perturb a modified substrate.^[13] Despite the utility of Staudinger ligations, the phosphine reagents have certain liabilities. They may be susceptible to oxidation by molecular oxygen, limiting their shelf life and providing a pathway for rapid liver metabolism.^[14] Moreover, the reaction rates are relatively sluggish hindering the ability of Staudinger ligation to monitor rapid biological processes *in vivo*. Normal Cu(I) catalyzed [3+2] azide alkyne cycloaddition (CuAAC), described by Sharpless^[15] and Meldal^[16] is not favorable for *in vivo* applications due to the cytotoxicity of Copper (Cu). To overcome this limitation, Bertozzi et al. (2007) introduced strain-prompted azide alkyne cycloaddition (SPAAC) for Cu free click reactions which is currently considered the most efficient technique for metabolic labeling.

2 Bioorthogonal Chemistry

Bioorthogonal chemical reactions are paving paths for new innovations in biology and are becoming more popular due to their selectivity and biocompatibility. The reactants of these reactions remain inert under richly functionalized biological system and form chemoselective covalent bonds in cells and in some cases in living organisms.^[14, 17] This approach involves the incorporation of a unique chemical functionality known as “the chemical reporter” into a target biomolecule using the cell’s own biosynthetic machinery. These reporters are non-native, non-perturbing chemical handles that can be modified in living system through highly selective reactions with exogenously delivered molecular probes without disturbing the non-natural functionality. This two-step strategy can be used for detection or isolation, depending on the nature of the probe molecule.^[8] The bioorthogonality is difficult to achieve with reactions that are sensitive to water or utilize reagents sensitive to physiological conditions. Thus taking into account all the potential limitations of most chemical reactions, there are only few reactions that are suitable for tagging biomolecules. The most important of these reactions are the click reaction and Staudinger ligation.

2.1 Click Reaction

Click chemistry, a synthetic approach with vast potential, was first introduced by Meldal et al.^[16] and K. Barry Sharpless et al.^[18] in 2002. They independently described that the pre-existing triazole synthesis, discovered by Huisgen,^[19-20] could be effectively catalyzed by Cu(I), enabling the reaction to progress at rt. The reaction is governed by kinetic control and is a highly reliable and selective process. Sharpless has defined the criteria which a click reaction must follow, that is “*the reaction must be modular, wide in scope, high yielding, creates only inoffensive by-products (that can be removed without chromatography), stereospecific, simple to perform and that requires benign or easily removable solvents (ideally water)*”.^[15] Normal Huisgen reaction requires high temperature in the range of 80 °C to 90 °C and poor regioselectivity is attained (in the range of 1:1.6).^[18] The modified reaction conditions enabled, Huisgen 1,3-dipolar cycloaddition suitable for application in biological systems because of its capability to accomplish at rt. Moreover, other speciality of this reaction is its ability to occur at neutral pH, and possibility in water enabled it more favorable for bioorthogonal conjugations.^[3] Bertozzi et al. in 2007 described strain-prompted alkyne azide cycloaddition by the development of difluorinated cyclooctyne (DIFO).^[21] Both CuAAC and SPAAC have great potential for bioorthogonal labeling.^[4]

2.1.1 Cu catalyzed click reaction

The classical [3+2] cycloaddition of azides and unactivated alkynes was largely ignored since the time of its discovery in 1961 due to the severe reaction conditions.^[19] The discovery by Meldal and Sharpless that the click reaction can take place at rt in the presence of a Cu catalyst paved the way for its application in bioorthogonal glycoconjugation.^[15-16] The use of a Cu as catalyst also solved the problem of regioselectivity between 1,4 and 1,5 substitutions.^[18] Since the time of its discovery many attempts have been made to explain the mechanism and to optimize the reaction conditions. A generalized catalytic cycle is depicted in fig. 2.1.

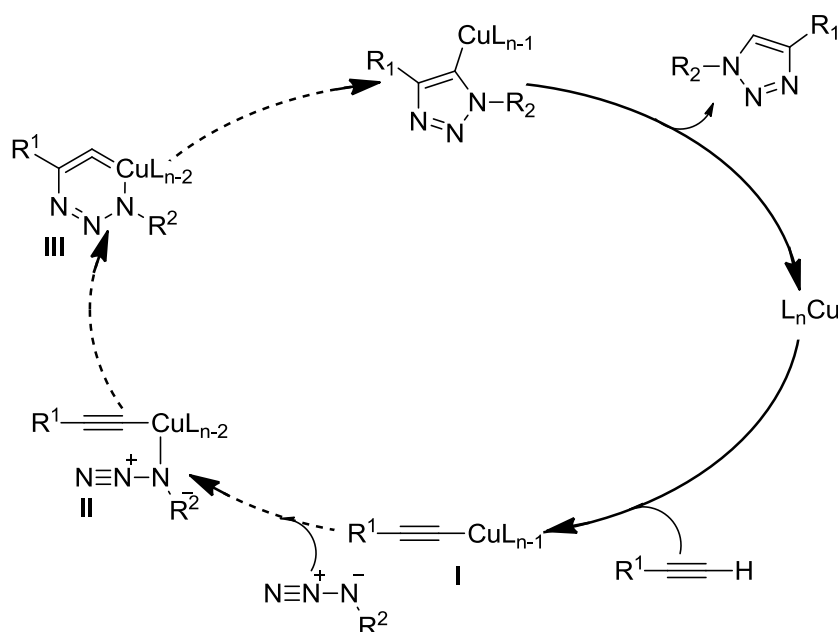


Figure 2.1: Mechanistic pathway proposed by Sharpless for Cu(I) catalyzed click reaction.^[18]

DFT studies have shown that the coordination of alkyne with Cu(I) is slightly endothermic in MeCN and exothermic in water, and favors an accelerated reaction rate in water. It also showed that the coordination of alkynes with Cu does not accelerate the rate of 1,3-dipolar cycloaddition. The resulting Cu-acetylide **I** coordinates with azide and the removal of another ligand takes place leading to the synthesis of an unusual six membered ring **III**. The closure of the catalytic cycle is demonstrated by the delivery of one H⁺ ion and leads to the contraction of the cycle finally yielding triazole.^[22] The first example of the applications of the click reaction catalyzed by Cu for bioorthogonal conjugation was reported by Meldal et al. This study coincidentally led to the peptidotriazoles by utilization of solid phase synthetic techniques and the click reaction in the presence of DIPEA and CuI as catalyst. These studies indicated that the reaction is mild and fully compatible with free amines, Fmoc and Boc-peptides, carboxylic acids and thioglycosides.^[16] Different efforts have been made to improve the efficiency, chemo and regioselectivity, scope

and compatibility of the reaction. It is known that the reaction rate is increased by polarizing the reactive groups, i.e. by installment of the electron withdrawing groups next to the alkyne triple bond. Another attempt to improve the efficiency of the reaction involved the use of Cu(I) stabilizing ligands to prevent oxidation to Cu(II).^[23-25] The ligands, recently developed for this purpose, are given in fig. 2.2. TBTA **1** (tris[(1-benzyl-1-*H*-1,2,3- triazole-4-yl)methyl]amine) is commercially available and commonly used to optimize click reactions in the presence of Cu(I) and DIPEA.

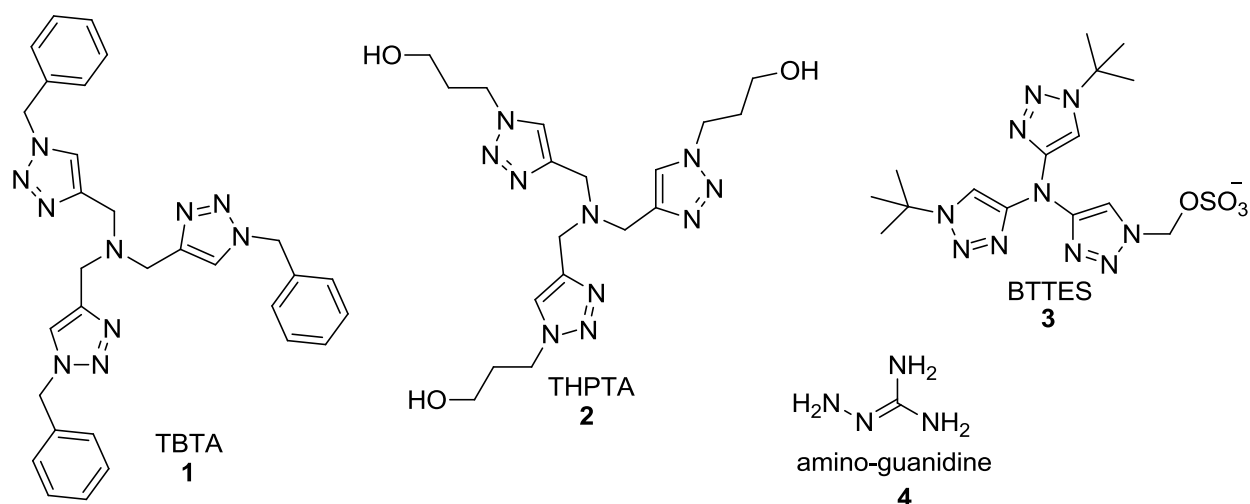


Figure 2.2: Different ligands stabilizing Cu(I) to enhance the click reaction rate.

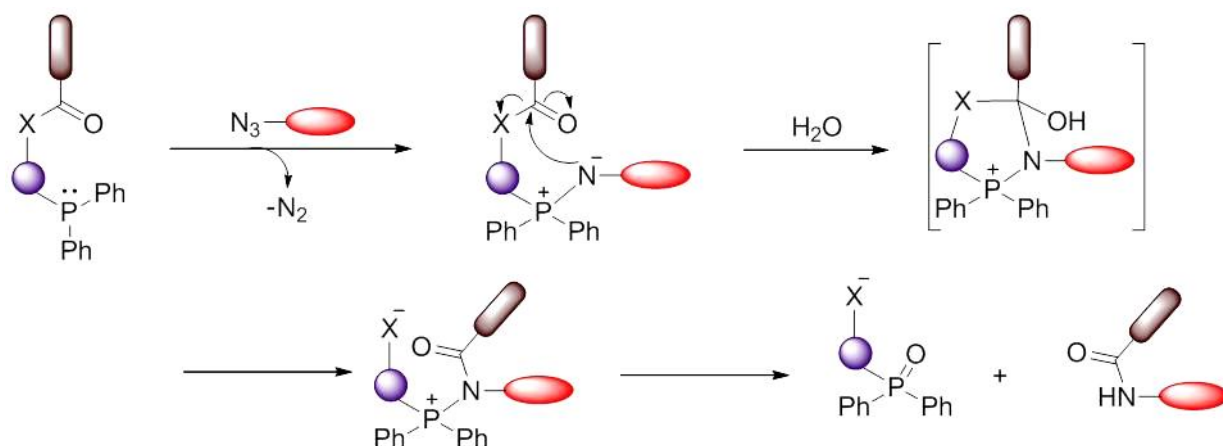
In 2004 K. B. Sharpless and V. V. Fokin described the application of TBTA along with other ligands and concluded that it was a powerful stabilizing ligand for Cu(I), protecting it from oxidation and disproportionation while enhancing its reactivity.^[23] They reported successful labeling of DNA by the use of TBTA without any fragmentation, which was intensely fragmented in the absence of ligand. However, insolubility of TBTA in water is a big barrier for its application in live cell media. M. G. Finn et al. recently described the synthesis of THPTA **2** (tris-[(hydroxypropyltriazolyl)methyl] amine) and achieved click reaction successfully on live mammalian cells by using $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and sodium ascorbate.^[25] Prior to their report, Peng Wu et al. reported the synthesis of BTTES **3** (2-[4-(bis[(1-*tert*-butyl]-1*H*-1,2,3-triazole-4-yl)methyl]amino) methyl)-1*H*-1,2,3-triazole-1-yl]ethyl hydrogen sulfate) and demonstrated that the introduction of one sulfate functionality to the ligand improved water solubility and enabled cell imaging of jurkat cells. They reported that the use of chelating agent BTTES **3** reduced cell death dramatically, which was observed significantly in the absence of it.^[24]

2.1.2 Cu free Click reaction

CuAAC accelerated by different Cu(I) stabilizing ligands has shown good efficiency, but the toxicity of Cu is still a big issue because it cannot be applied on live animals. The treatment of Zebrafish embryos in the presence of 1 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.5 mM sodium ascorbate and 0.1 mM TBTA led to the death of all embryos within 15 min.^[26] To avoid the drawbacks of toxicity and slow reaction rate, the only option is SPAAC, an alternate and efficient Cu free strategy. The story of strain prompted cycloaddition originates from study by Alder and Stein that described a higher reaction rate of dicyclopentadiene than pentadiene with azides.^[27-28] Investigation of cycloadditions with strained alkenes/alkynes and azides continued up to 1960, when Wittig and Krebs reported that “*the smallest cyclooctyne undergoes in cycloaddition like an explosion when treated with phenylazide*”.^[29] Bertozzi et al. synthesized cyclooctyne conjugated with biotin and found that it reacted with azide in good yield but the reaction rate was not faster than Staudinger ligation and considerably slower than CuAAC.^[30] However, it opened a route for investigations and a variety of cyclooctyne derivatives have been synthesized so far. Attempts to improve the reactivity of cyclooctyne involved introducing electron withdrawing groups next to the alkyne triple bond. Addition of one fluorine atom next to the alkyne triple bond increased the reaction rate up to four fold.^[31] However, double substitution of cyclooctyne with fluorine α to the alkyne triple bond dramatically increased the reaction rate up to 60 fold.^[21] The more efficient example of substituents-enhanced reaction rates is the application of difluorinated cyclooctyne, simply named DIFO **26** by Bertozzi et al. that exhibits a reaction rate constant of $10^{-1} \text{ M}^{-1}\text{s}^{-1}$, almost equiv. to CuAAC.

2.2 Staudinger Ligation

The classic Staudinger reaction, in which an azide undergoes reduction by reaction with triphenylphosphine to produce an aza-ylide intermediate, was developed by Hermann Staudinger in 1919.^[32] Staudinger ligation is a modified form of Staudinger reduction and was developed by Saxon and Bertozzi in 2000.^[33-34] In a modified form of Staudinger ligation the aza-ylide intermediate arranges itself in aqueous media to give an amide functionality incorporated with phosphine oxide, and named non-traceless Staudinger ligation. Soon after, Bertozzi^[33] and Raines^[35] reported traceless Staudinger ligation, in which the phosphine oxide is excised from the amide during hydrolysis in water as outlined in scheme 2.1. The presence of two aromatic groups hinders the oxidation of phosphine under ambient conditions.

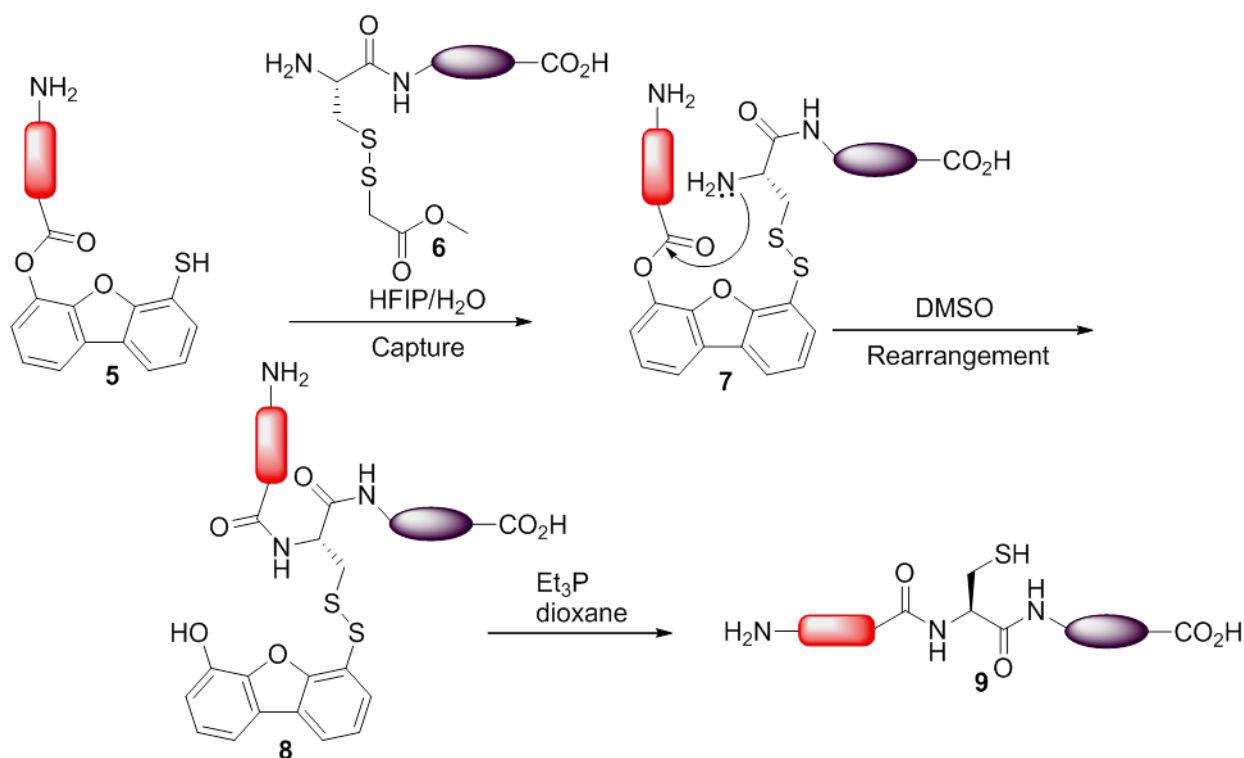


Scheme 2.1: The traceless Staudinger mechanism explained by Raines.^[33, 36]

Efficient chemo selectivity between the phosphine and the corresponding azide functionalities is a hallmark characteristic of traceless Staudinger ligation. The use of phosphinothioesters ($X = S$) instead of phosphinoesters leads to chemoselective ligation in the presence of unprotected functional groups in native proteins, thus avoiding unwanted side reactions.^[37] In addition to the chemoselectivity, the major advantage of traceless Staudinger ligation is its capability to develop peptide functionality between the chemical reporter and probe. It has been demonstrated that traceless Staudinger ligation is more advantageous for *in vivo* bioconjugation i.e. in mice which are the model laboratory animal for studying human diseases.^[38] It has a broad scope in biology for the labeling of DNA, peptide-peptide conjugation, protein modification and generation of microarrays and self assembling systems.^[39] An appealing feature of Staudinger ligation is its capacity to exhibit the invention of fluorogenic reagents for real-time imaging, as described by Lemieux et al. in 2003.^[40] However, in spite of all these milestones, it also has some drawbacks, the most important of which is slow reaction rate that hinders *in vitro* and *in vivo* applications.

2.3 Native Chemical Ligation

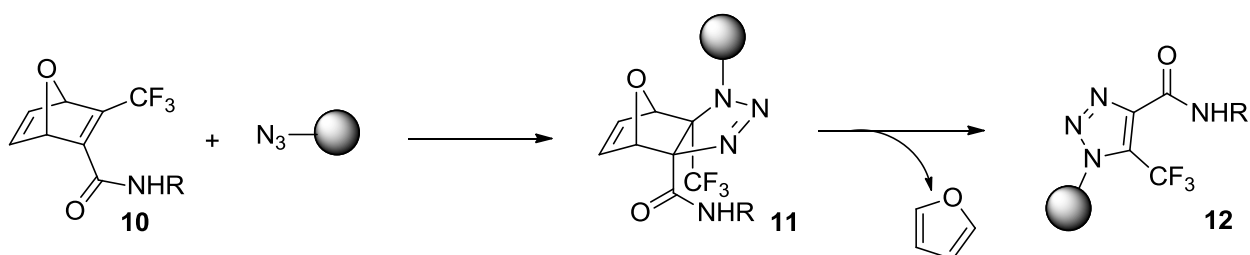
Native chemical ligation (NCL) was first introduced by Wieland et al. in 1953 and involves intramolecular aminolysis of thiophenol thioesters in the presence of amines to yield amides.^[41] The strategy was revolutionized in 1994 when Kent and co-workers reacted unprotected thioesters with N-terminal Cys peptides^[42] as shown in scheme 2.2. The mild reaction conditions, i.e. aqueous buffered solutions at neutral pH, make this strategy viable for peptide ligation. The nature of the amino acid at the C terminus of the thioester is an important factor for NCL, which was investigated by Dawson and co-workers.^[43] Still another factor that has a key influence on NCL is the nature of the thiol ester. The reaction requires rapid thiol-thioester exchange, which proceeds much more efficiently with aryl thioesters.^[44]



Scheme 2.2: Kemp's prior thiol capture strategy, HFIP = hexafluoroisopropanol.

2.4 Bioorthogonal Reaction with Alkenes

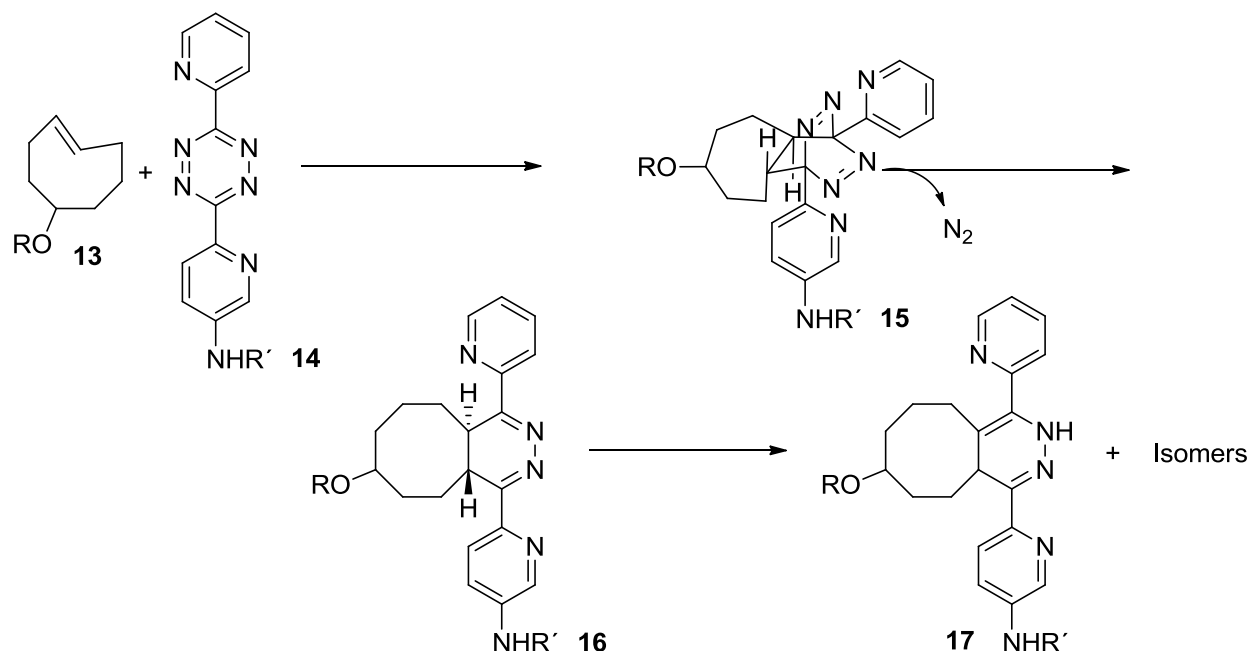
Alkenes undergo cycloaddition by reaction with 1,3-dipoles and dienes, prompted by ring strain or accelerated by light. The lower stability of triazoline, compared to the aromatic triazole, makes it less advantageous for bioorthogonal labeling.^[45] The first attempt to improve the reactivity was reported by Rutjes et al. by using an electron deficient olefin as shown in scheme 2.3. The reaction proceeds via a [3+2] cycloaddition with azide to give intermediate **11**, which then undergoes a retro-Diels-Alder reaction eliminating furan and finally yielding triazole **12**.^[46-47]



Scheme 2.3: Triazole synthesis by reaction between an alkene and azide.

Fox et al. illustrated that the reaction between tetrazine and strained alkenes proceeds very quickly using an inverse electron demand strategy with *trans*-cyclooctene and diperydyltetrazine as illustrated in scheme 2.4. Ruthenium catalyzed olefin cross metathesis also has potential for bioorthogonal reaction due to its tolerance of different functional groups.^[48-50] Work is being

done to modify the reactions conditions to operate in water, which represents a milestone for *in vivo* and *in vitro* applications.^[24, 51-52] Recently, Davis et al. developed modified proteins containing allyl sulfide groups through cross metathesis in water and *tert*-BuOH with a second generation of Hoveyda-Grubbs catalyst.^[53]



Scheme 2.4: Inverse demand Diels-Alder reaction of dipyrindyl tetrazine and trans-cyclooctene.^[54]

2.5 Applications of Bioorthogonal Labeling

Obtaining insight into the structure and functions of different cellular organs *in vitro* has been a hot topic for decades and covalently chemoselective modification of biomolecules has long been applied to the challenge by biochemists and biophysicists. The classic residue specific biomolecular alteration technique is inadequate for the parallel presence of the same residue in other biomolecules and makes it an unfavorable technique. A solution to this problem may be genetic tagging of fluorescent proteins to the target protein. However, this strategy is limited to protein modification only and is inadequate for other biomolecules like nucleic acids, glycans, lipids and certain protein regulatory processes like posttranslational modifications. The bioorthogonal modification has emerged as a unique strategy to covalently bind different biomolecules and to have insight into the dynamics and functions of biomolecule in living systems. The most common advantages of bioorthogonal labeling that make it unique to other techniques are:

- applicable to all classes of biomolecules, including proteins, nucleic acid, glycans and lipids

- extreme versatility, which enables the use of most probes desired by researchers
- scalable and suitable for functional annotation of individual biomolecules in living cells.

2.5.1 Protein labeling

Tirrel et al. pioneered a straightforward method for the introduction of unnatural molecules into proteins by subjecting cell to unnatural amino acids, particularly the aminoacyl-tRNA synthetases (aaRSs) that tolerated by the enzymatic machinery of the cell.^[55-56] This method opened the way to incorporate a number of bioorthogonal functional groups (alkynes, alkenes, azides) to the proteins of both *E. coli* and mammalian cells. Human plasminogen (Hpg) was employed to label newly synthesized proteins with an azidocoumarine dye by CuAAC in bacterial^[57] and mammalian^[58] systems as shown in fig. 2.3. Similarly lactic acid (Aha) has been used to interrogate newly synthesized protein by labeling with CuAAC, Staudinger ligation and more recently with cyclooctyne probes.^[59]

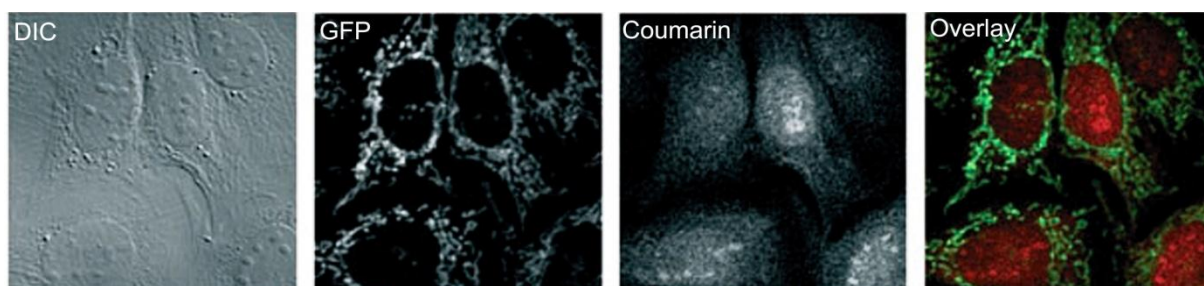


Figure 2.3: Adapted from Tirrell et al., describes the fluorescence labeling of protein in mouse embryonic fibroblasts with green fluorescent protein (MEF-GFP). Imaging of MEF-GFP cells pulse labeled in 1mM Human plasminogen (Hpg). First image on the left side comes from differential interference contrast (DIC). Each image is obtained under identical conditions to capture either GFP (second) or coumarin (third) image. The final overlay contains the superposition of GFP (green) and coumarin (red) image.^[58]

Azides and alkynes have also been installed in the capsids of viruses by replacing methionine residues with Aha and Hpg.^[60] Proteins can also be labeled by site specific modification (genetic encoding) of unnatural amino acids. This technique was first introduced by Schultz and co-workers^[61-62] as an *in vitro* strategy and since then has been in practice for *in vivo* applications.^[63] They introduced an *in vivo* system for site specific mutagenesis of unnatural amino acids by the selection of tRNA and aaRS that recognized the amber stop codon and unnatural amino acids respectively.

Activity based protein profiling (ABPP) is another strategy for protein labeling, which has enabled small molecular probes (>300 Da) target specific and eliminated the use of large molecular reporters (<300 Da). Its capability of using the small molecules enables it to focus on druggable enzymes, which show specific ligand binding. Initially ABPP uses an electrophile,

called the warhead, to covalently modify the target enzyme and finally conjugates with a probe.^[62]

2.5.2 Glycans labeling

Glycans cannot be genetically modified due to the lack of genetic templates and enzymatic activities that proteins possess. One solution to this problem is modification through metabolic labeling. This technique commonly known as metabolic oligosaccharide engineering was first applied to live cells and animals by Reuter et al. to alter the acyl side chains on the sialic acid residue by the use of *N*-acetylmannosamine (ManNAc) and its derivatives.^[64-65] The sialic acid residues were used to study host virus interaction,^[66] neuronal cell differentiation^[67] and poly sialic acid.^[68] Bertozzi et al. reported the incorporation of different functionalities like ketones^[69] and azides^[34] to the sialic acid residue on the cell surface glycoconjugates. This was a landmark study that introduced Staudinger ligation to bioorthogonal labeling as the Sia residues with azido functionality were detected by flow cytometry analysis. Later on they also reported that the azido functionality could also be introduced to live mice and zebrafish by the use of ManNAz or GalNAz as metabolic precursors and subsequently detected by either Staudinger ligation or a Cu free click reaction.^[70] The cell surface labeled by azido sialic acid was treated with DIFO-fluorophore conjugates by Baskin et al. through glycan transport experiments. They also applied the same technique to developing zebrafish embryos and for first time introduced the molecular imaging of glycans in a live organism^[70] as shown in fig. 2.4.

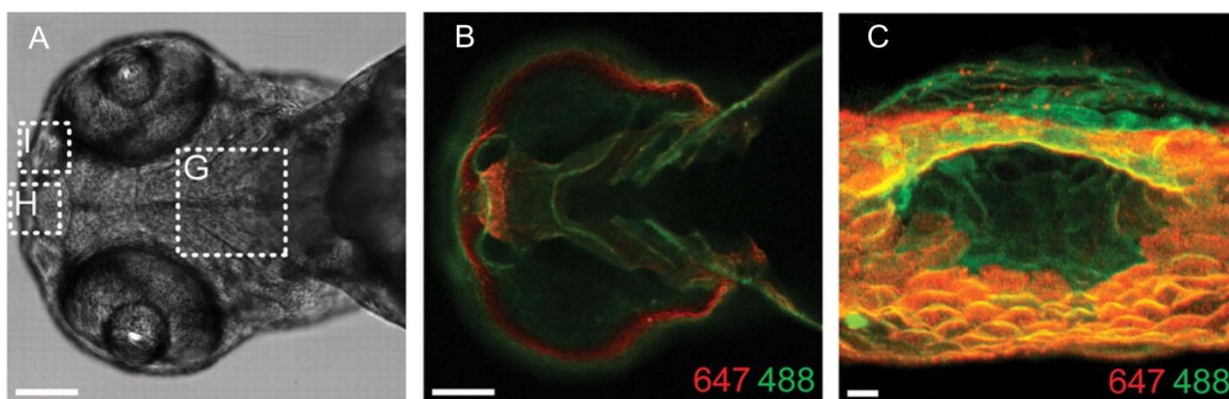


Figure 2.4: Adapted from Baskin et al. describes the identification of temporally distinct glycan populations during zebrafish development using two color labeling. Zebrafish embryo metabolically labeled with Ac₄GalNAz from 3 to 60 hpf were reacted with DIFO-647 between 60 and 61 and then treated with DIFO-488 either between 61 and 62 hpf or after an additional 1 h of the metabolic labeling with Ac₄GalNAz and reacted with same DIFO-fluorophore probe. (A) Bright field image of a ventral view of an embryo at 63 hpf. (B) Single plane fluorescence image of A displaying intense DIFO-488 (green) fluorescence and DIFO 647 (red) fluorescence (C) z-projection fluorescence images of the mouth region.^[70]

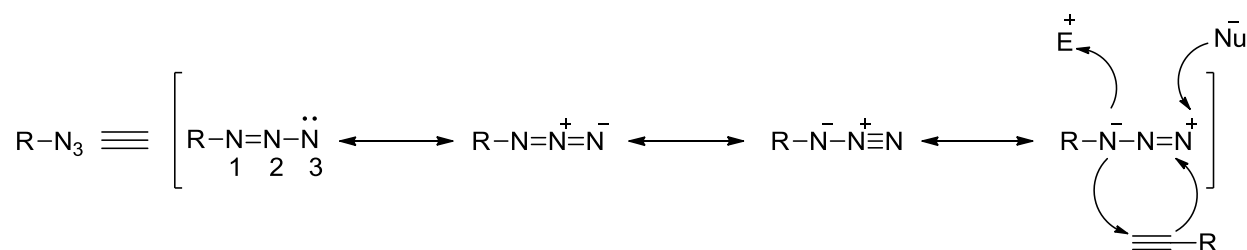
3 Bioorthogonal Reagents

A chemical motif incorporated into living environments for tagging and visualizing biomolecules without disturbing the biological environment is termed a bioorthogonal chemical reporters. Prescher and Bertozzi defined bioorthogonal reporters as “*non-native, non-perturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes*”.^[8] It could be a monosaccharide that expresses itself more likely on the cell surface glycoconjugates in the form of sialic acid derivatives and is useful for glycan labeling. In the case of DNA it could be a nucleoside, amino acid for protein or a fatty acid for lipid labeling.

A small molecule, quickly attached to a chemical reporter through a covalent bond in a bioorthogonal chemical reaction that tolerates complex biological environments, is termed as bioorthogonal chemical probe. The probes must have a complementary chemical moiety and capability to covalently bind with a partner (reporter) under physiological conditions. In addition to the counter functionality to the bioorthogonal chemical reporter, a probe molecule may carry different molecules with specific functionalities i.e. a fluorescent dye (fluorescent imaging) or a bioactive molecule (drug discovery).

3.1 Organic Azides

Organic azides, due to their unique functionality and nonexistence in nature are very popular in bioorthogonal reactions. The stability in physiological conditions and small size of azides have enabled biomolecular metabolites tagged with azide functionality to hijack biosynthetic pathways for numerous biomolecules, including glycans,^[71] proteins, lipids and nucleic acid derived factors.^[72-73] Organic azides undergo useful chemical reactions due to the mild positive charge on terminal nitrogen and thus behave as mild electrophile. In addition, a mild negative charge on N¹ enables them to behave as mild nucleophiles. This dipolar system is stabilized by resonance contributing structures as shown in scheme 3.1.



Scheme 3.1: Resonance contributing structures of an azide illustrating its reactivity for electrophilic and nucleophilic reactions.

Several approaches have been introduced for metabolic engineering, which make the use of azide functionality as a chemical reporter.^[70] Some of the commercially available chemical probes for this purpose are given in fig. 3.1. Recently, Best et al. reported the application of different azide probes for the labeling of proteins and lipids, and exploited different biological roles of phosphines.^[11]

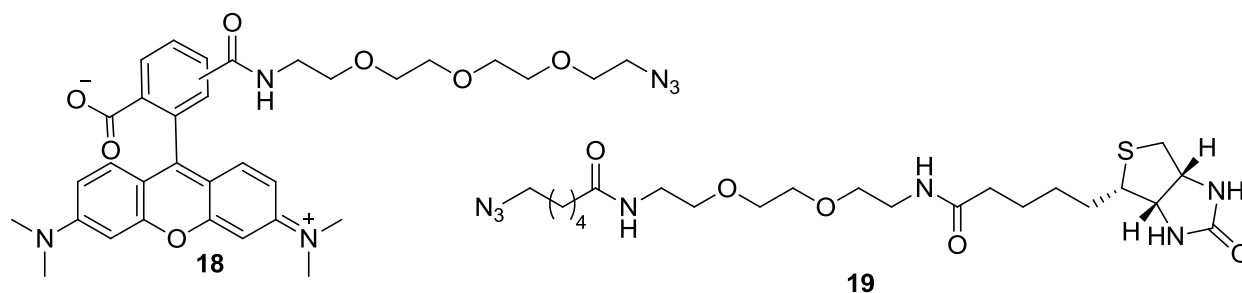
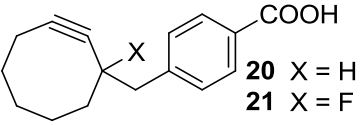
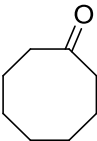
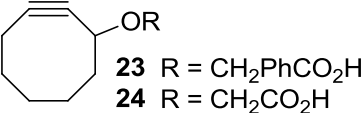

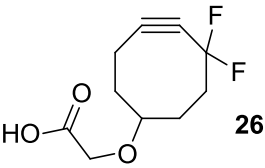
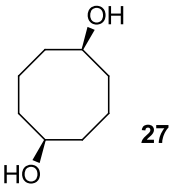
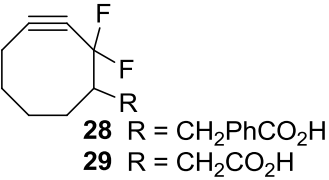
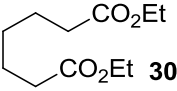
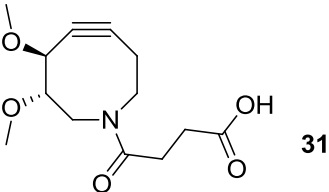
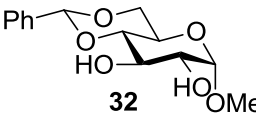
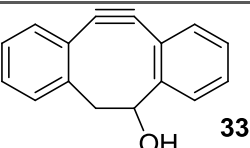
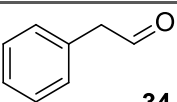
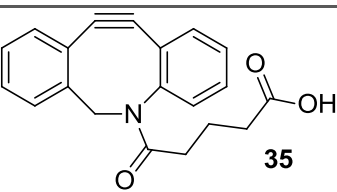
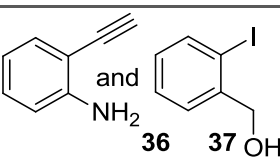


Figure 3.1: Chemical probes containing azide functionality.^[74]

3.2 Alkynes

Alkynes are important molecules involved in bioorthogonal labeling via the click reaction that are widely applied not only in biological science but in material science as well. Their stability and non-cytotoxicity make them ideal for use as bioorthogonal reagents and have complete compatibility with the biophysical environment, enabling alkynes to pass through metabolic pathways similarly to azides. Alkynes react almost exclusively with their counterpart azides without disturbing the surroundings due to the unavailability of alkyne functionality in cellular system. However, due to their specificity to the click reaction, they are not as versatile for bioorthogonal application compared to the azides. Terminal alkynes undergo Huisgen click reaction but only under intense conditions i.e. high temperature/pressure or in the presence of Cu(I) as a catalyst, preventing it from widespread application in bioorthogonal labeling. The concept that the cyclooctyne undergoes click reaction under less demanding conditions than terminal alkynes was first applied in bioorthogonal labeling when Bertozzi et al. introduced Cu free click reaction with difluorinated cyclooctyne. Lowering the LUMO level of the alkyne by the introduction of one fluorine atom adjacent to the triple bond increased the rate of the click reaction three fold.^[31] The introduction of second fluorine atom increased the reaction rate dramatically up to 60 times faster than cyclooctyne without any fluorine atom.^[21] This discovery opened new doors into the world of bioorthogonal glycoconjugation and since then a number of cyclooctyne derivatives with different functionalities have been developed, a list of which is shown in the table 3.1.

Table 3.1: Overview of the synthesis of important cyclooctynes and their reactivity for click reaction.

Sr.No.	Cyclooctyne based molecular Probes	Starting material	Steps	Yield	Reactivity $M^{-1}S^{-1}$	Ref.
1	 20 X = H 21 X = F	 22	4 5	12 15	1.2×10^{-3} 4.3×10^{-3}	[31]
2	 23 R = CH ₂ PhCO ₂ H 24 R = CH ₂ CO ₂ H	 25	4 5	52 36	1.3×10^{-3} 2.4×10^{-3}	[30] [31]
3	 26	 27	10	1.2	7.4×10^{-2}	[21]
4	 28 R = CH ₂ PhCO ₂ H 29 R = CH ₂ CO ₂ H	 30	8 10	27 21	4.2×10^{-2} 5.2×10^{-2}	[75]
5	 31	 32	11	5	3.0×10^{-3}	[76]
6	 33	 34	5	10	5.7×10^{-2}	[77]
7	 35	 36 and 37	9	41	3.1×10^{-1}	[78]

3.3 Ketones, Aldehydes and Alkenes

Ketones and aldehydes do not exist on the eukaryote cell surface and thus can be useful for bioorthogonal labeling. These motifs can bind not only proteins but also glycans and secondary metabolites. Rideout et al. for first time investigated the role of ketones and aldehydes in chemoselective drug assembly in the presence of living cells.^[79-80] Although suitable for chemical tagging in cell culture, aldehydes and ketones have a limited scope for application in

living organisms. The first hurdle is the pH requirement (5-6) which is unusual *in vitro*. In addition, aldehydes and ketones are not absolutely orthogonal to the living system as the metabolites of these functionalities are abundant in biological fluids in the form of sugars, pyruvates and various cofactors.^[8]

Alkenes due to their ability to undergo 1,3-dipolar cycloaddition through strain prompt or excitation by light are useful chemical reagents for bioorthogonal reactions. They react with azides and give cyclic products called triazolines. However, the instability of triazoline makes it unfavorable for bioorthogonal ligation. An important reaction of strain prompted alkenes with tetrazine was investigated by Fox et al. and exhibited high efficiency in water with a reaction rate of $10^3 \text{ M}^{-1} \text{ s}^{-1}$.^[54] In addition, olefin cross metathesis has also been investigated as a potential bioorthogonal chemical reaction. The approach has been applied by different people to achieve bioorthogonality^[48-50] using various metal catalysts. The 2nd generation Grubbs catalyst is currently approved to be most effective. Olefin cross metathesis also faces limitations due to the use of organic solvents that are incompatible with living system.

3.4 Aromatic Phosphines

Aromatic phosphines are important members of bioorthogonal chemistry and their reactivity with azide functional groups (Staudinger ligation) has become a topic of interest. The flexibility of phosphine has enhanced their value because a variety of different moieties with specific properties can be developed on them. Since the discovery of their application in Staudinger ligation, different phosphine reagents have been synthesized for studying various biological activities with bioorthogonal labeling. The most prominent phosphine derivatives carry fluorescent dyes (cell imaging), biotin (streptavidine adhesion) or some bioactive molecules (drug discovery). Biotin conjugated phosphine **38** (P-Biotin), shown in fig. 3.2, has been applied for protein tagging *in vivo* as reported by Ploegh et al.^[81] Background fluorescence in cell imaging is a big problem that can be overcome by the use of a nonfluorescent precursor like **39**, which becomes fluorescent after ligation with an azide.^[40]

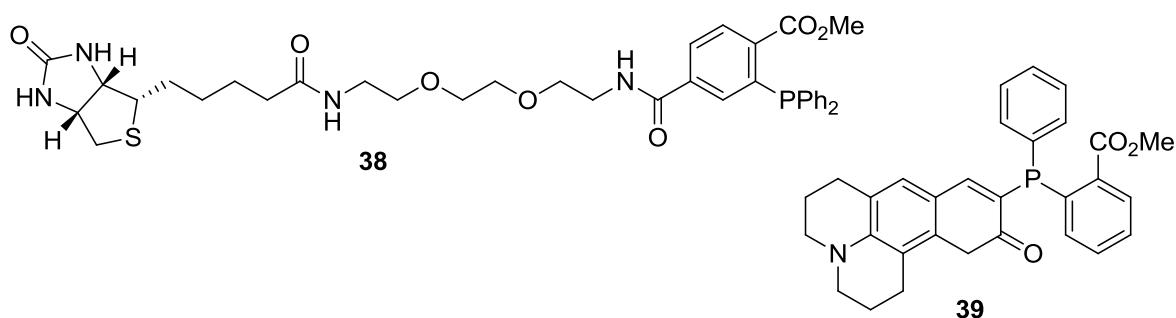


Figure 3.2: Phosphine probes containing biotin and fluorophore for Staudinger ligation.

3.5 Cytotoxicity of Bioorthogonal Reagents

Most of the organic azides being used in bioorthogonal reactions i.e. click reaction and Staudinger ligation are nontoxic. The ancestors of organic azides i.e. inorganic azides are more toxic than cyanide salts^[82] with a LD₅₀ in rats of 27 mg/kg in the case of NaN₃. Apart from its potential applications in the development of explosive and superconducting materials, the azide functionality has important pharmaceutical applications as enzyme inhibitor and antiviral treatments.^[83] The most common example of molecules containing azide functionality in antiviral treatment is azidothymidine (AZT) **40**, which was approved in March 1987 for HIV treatment.^[84] Some other medicines with an azide functional group are shown in fig. 3.3 including azidocillin **41**, zidometacin **42** and azidamfenicol **43**.

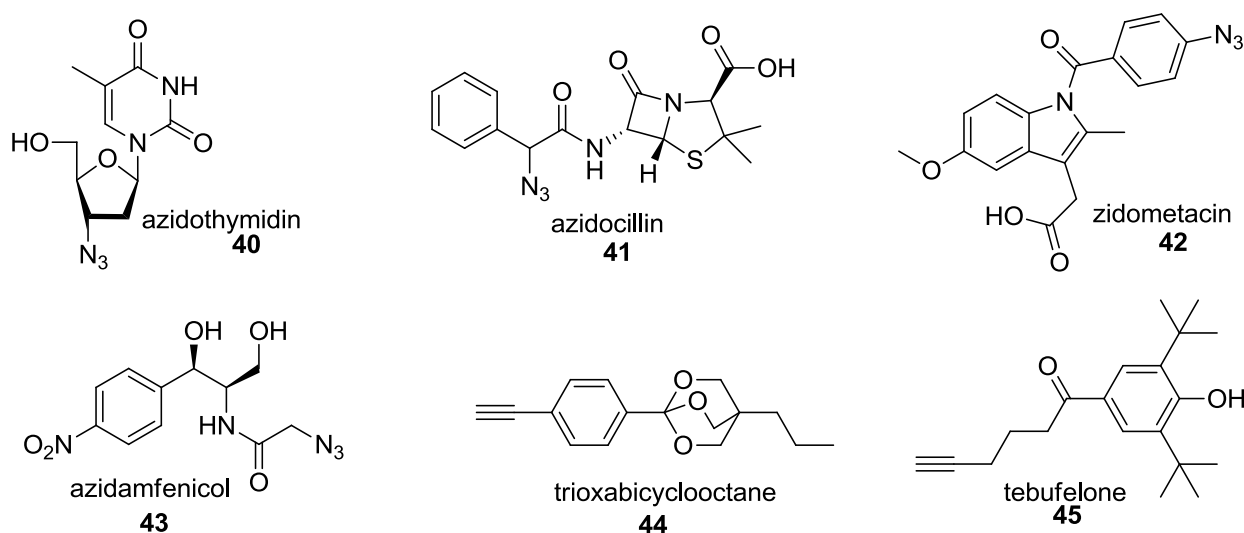


Figure 3.3: Different azide and alkyne molecules approved for medical applications.

Most of the alkyne derivatives are nontoxic but a few of them show cytotoxic effects. The trioxabicyclooctane **44**, a chloride channel blocker is toxic to both insects and mammals.^[85] Pyrethroids containing an alkyne functional group act on the central nervous system of insects by blocking their voltage dependent sodium channel. Several naturally occurring alkyne derivatives show herbicidal, fungicidal and insecticidal activities and are used as key intermediates in many agrochemicals. Tebufelone **45** is an anti-inflammatory agent and shows a lethal dose of 5 mg/kg observable adverse effect on rats, dogs and primates.^[86] CuAAC has cytotoxic effect due to the use of Cu, which has LD₅₀ in the range of 300 mg/kg in rats and 470 mg/kg for mammals.^[87] Tirrel et al. checked the toxicity of Cu when they treated *E. coli* that expressed proteins with associated azides with CuBr for 16 h. The *E. coli* subjected to CuBr survived the initial labeling but could not multiply any more.^[88]

4 Sialic Acids

Sialic acids (Sia) are prominent sugars, located on the terminal position of cell surface glycans can be an effective tool for cell surface modification through bioorthogonal reactions. *N*-acetylneuraminic acid (Neu5Ac) the most prominent member of the family is located on the terminal position of glycoconjugates in humans. They mediate cell-cell recognition and signal transduction processes involved in infection, inflammation or tumor formation.^[1] It has long been known that sialic acids are involved in myriads of interaction processes including viral infection such as emerging flu variants.^[89] Sia is a generic term for the *N*- or *O*- substituted derivatives of neuraminic acid, a nine carbon monosaccharide. They are widely distributed in animal tissues and bacteria, especially in glycoproteins and gangliosides.^[90] Neuraminic acid **46**, shown in fig. 4.1, does not occur naturally but many of its derivatives occur in animals and bacteria. The name neuraminic acid was introduced by a German scientist E. Klenk in 1941 in reference to the brain lipids from which it was derived as a cleavage product.^[91] The symbol commonly used for neuraminic acid is **Neu** also known as sialic acid with symbol **Sia**.

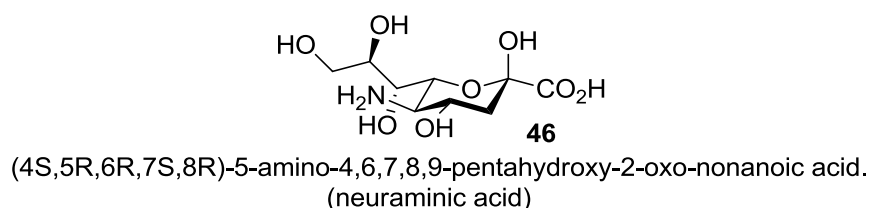


Figure 4.1: Neuraminic acid (Sialic acid or Sia), the primary member of the Sia class.

Sialic acids are a family of monosaccharides that contains more than 50 natural derivatives identified to date.^[92-93] Humans lack Neu5Gc **48** owing to a mutation in the CMAH (cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase) gene, which is responsible for the encoding of an enzyme required for the conversion of Neu5Ac **47** to Neu5Gc **48** as shown in fig. 4.2. Sia can occur freely in nature, but are generally found glycosidically linked to either the 3- or 6-hydroxyl group of galactose (Gal) residues or to the 6-hydroxyl group of *N*-acetylglucosamine (GlcNAc) residues. They can also exist as α -2,8-linked homopolymers commonly known as polysialic acids. Sialic acids are usually located at the exposed, non-reducing ends of oligosaccharide chains, and are transferred using α -2,3, α -2,6 or α -2,8 linkages to the sub-terminal sugars by a family of about 20 sialyltransferases. When sialylated oligosaccharide ligands are attached to protein and lipid carriers, the resulting glycoproteins and glycolipids have the potential to function as “counter-receptors” for Siglecs and other glycan binding proteins.^[94]

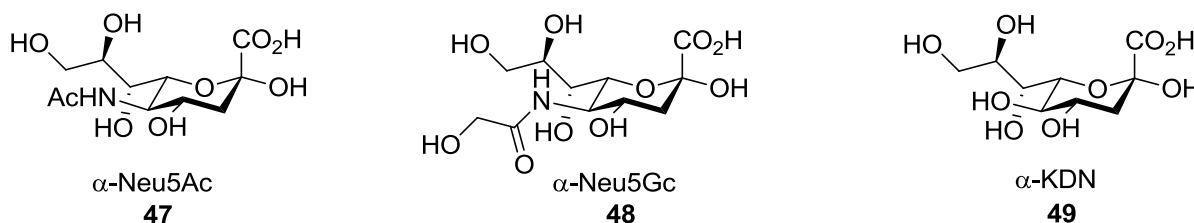


Figure 4.2: Sialic acids more commonly found in the nature.

4.1 Structural Diversity of Sialic Acid

With Over 50 known naturally occurring Sia derivatives the family shows remarkable structural diversity. Combinations of different glycosidic linkages with various substitutions as mentioned in fig. 4.3 give hundreds of ways in which sialic acids can present themselves on the surface of glycoconjugates. The largest structural variations of naturally occurring Sia are at C-5, which can be substituted with either an acetamido, hydroxyacetamido or hydroxyl moiety to form 5-*N*-Acetylneuraminic acid (Neu5Ac), 5-*N*-glycosylneuraminic acid (Neu5Gc) or deaminoneuraminic acids (KDN), respectively.

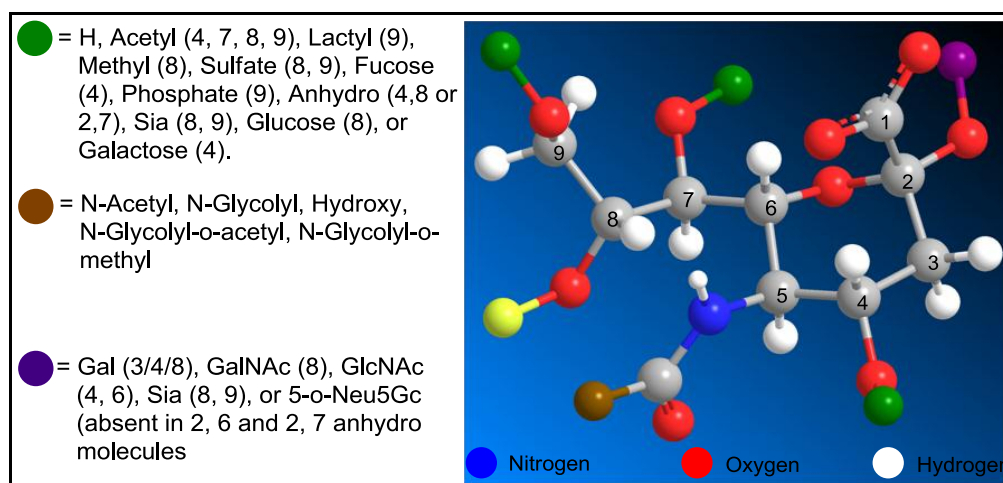


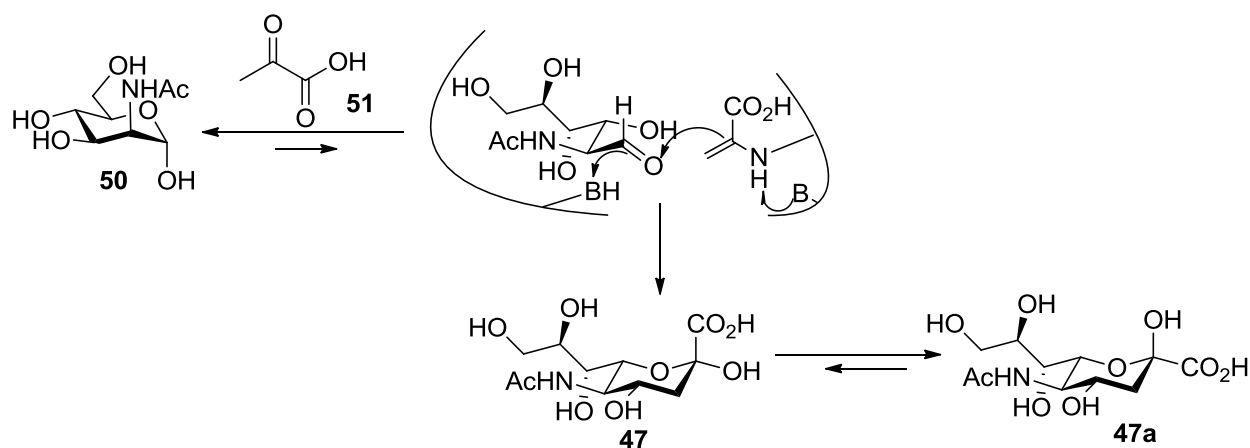
Figure 4.3: Diversity of different sialic acid derivatives. Natural substitutions that have been described to date (C-4, C-5, C-7, C-8 and C-9) are indicated.

Further structural diversity is generated primarily by a combination of the above mentioned variations at C-5, with modifications of any one of the hydroxyl groups located at C-4, C-7, C-8 and C-9. The structural diversity of sialic acids can determine and/or modify recognition by antibodies as well as by a variety of Sia binding lectins of endogenous and exogenous origin.^[95]

4.2 Synthesis of Sialic Acids

4.2.1 Enzyme catalyzed synthesis of sialic acids

Biosynthetically, Neu5Ac and KDN are believed to be the precursors of different sialic acid metabolites. The approach most commonly used for the synthesis of Neu5Ac involves condensation between *N*-acetylmannosamine (ManNAc) **50** and pyruvate **51**, catalyzed by the enzyme Neu5Ac synthase.^[96-99] The mechanism of the enzyme catalyzed reaction involves the formation of a Schiff base^[100] between a lysine moiety in the active site of the enzyme and pyruvate. The condensation between these Schiff bases and ManNAc **50** takes place in a general acid/base-catalyzed reaction as illustrated in scheme 4.1.^[101]



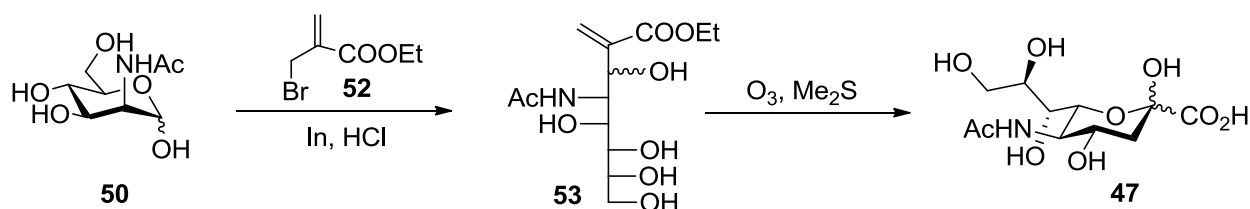
Scheme 4.1: Mechanism of Neu5Ac aldolase catalyzed reaction to access **47/47a**.

The biological function of Neu5Ac synthase is the degradation of Neu5Ac and as a consequence the reaction is shifted towards the retro-aldol side^[102] with an equilibrium constant of 12.7 M^{-1} . For synthetic purpose, however, the equilibrium can be shifted towards Neu5Ac by employing an excess of pyruvate.^[103]

4.2.2 Chemical synthesis of sialic acids

4.2.2.1 Indium catalyzed synthesis of sialic acids

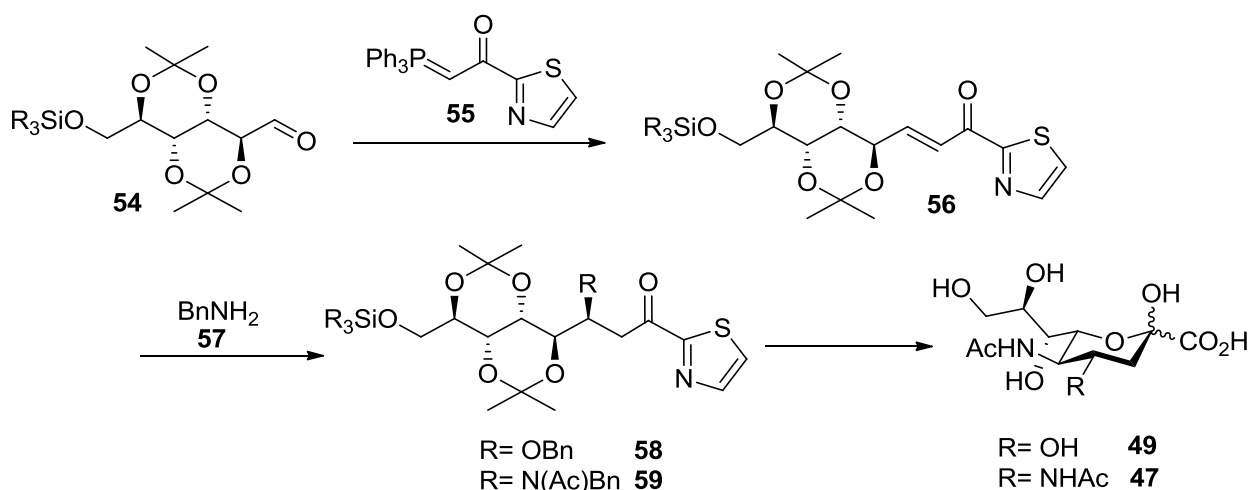
Several elegant approaches for the chemical synthesis of sialic acids have been reported.^[104] A short route, described in scheme 4.2, involves condensation between ManNAc **50** and ethyl α -bromomethyl acrylate **52** in the presence of indium in dilute aqueous HCl to give the corresponding enoate as a mixture of diastereoisomers (threo/erythro 4:1).^[105]



Scheme 4.2: Indium catalyzed synthesis of Neu5Ac.

4.2.2.2 Thiazol methodology for the synthesis of sialic acids

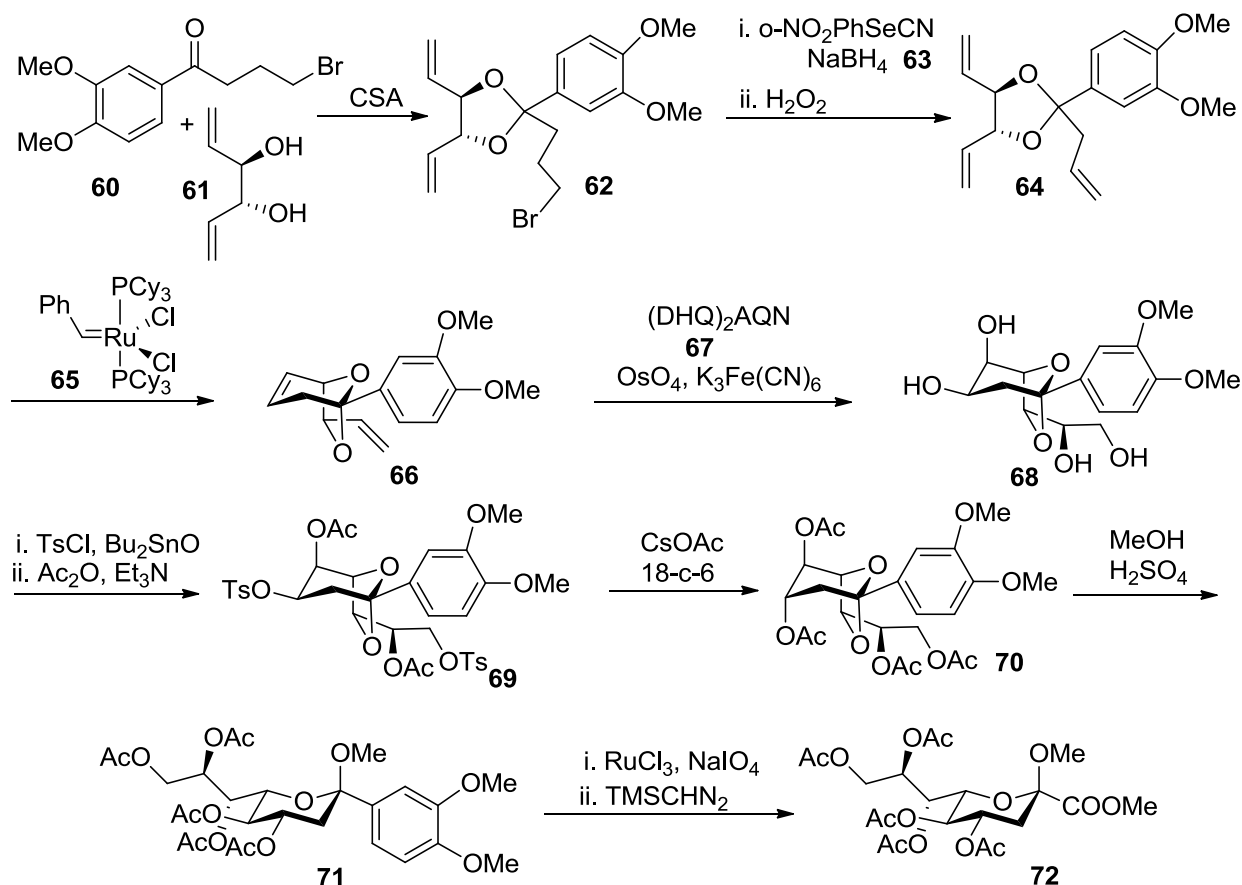
Dondoni and co-workers have employed thiazol methodology for the synthesis of sialic acid derivative possessing amine at C-4 as illustrated in scheme 4.3.^[106] The carbon frame work was obtained by means of a Wittig reaction between mannose derivative **54** and thiazolylyde **55**. Conjugative addition of benzylamine **57** to the resulting enone **56** predominantly gave the *syn* adducts **58** and **59**, which were then converted into 4-acetamido-4-deoxy-KDN derivative **49** and Neu5Ac **47** respectively.^[107]



Scheme 4.3: Thiazol methodology for the synthesis of KDN and Neu5Ac.

4.2.2.3 Ring closing metathesis reaction

A total synthetic approach of KDN was achieved by S. D. Burke et al., through a ketalization/ring closing metathesis reaction.^[108-109] As described in scheme 4.4, the ring closing metathesis of C-2 symmetric triene **64** in the presence of Grubb's catalyst **65** gave a substituted dioxabicyclo[3.2.1]oct-2-ene ring system **66**. Di-*O*-tosylate **69** was obtained as a result of double Sharpless hydroxylation followed by tosylation. Displacement of the tosyl group by treatment with 18-crown-6 after acetylation yielded peracetylated derivative **70** with the appropriate stereochemistry. Finally, the dimethoxy benzene moiety was unmasked by treatment with RuO_4 , an oxidizing reagent to yield the target KDN **72**.



Scheme 4.4: Total synthesis of KDN by ring closing metathesis.

4.3 Glycosylation of Sialic Acids

Advances in both chemical^[97] and enzymatic^[110] syntheses have provided reliable routes for the production of many complex sialosides.^[111] Synthetic compounds have proven to be of key importance for the determination of the biological roles of these glycoconjugates.^[112] Inter-glycosidic bond formation is generally achieved through condensation^[113] between a fully protected glycosyl donor^[114] bearing a potential leaving group at its anomeric center^[115] and a suitably protected glycosyl acceptor often containing only one free hydroxyl group.^[116]

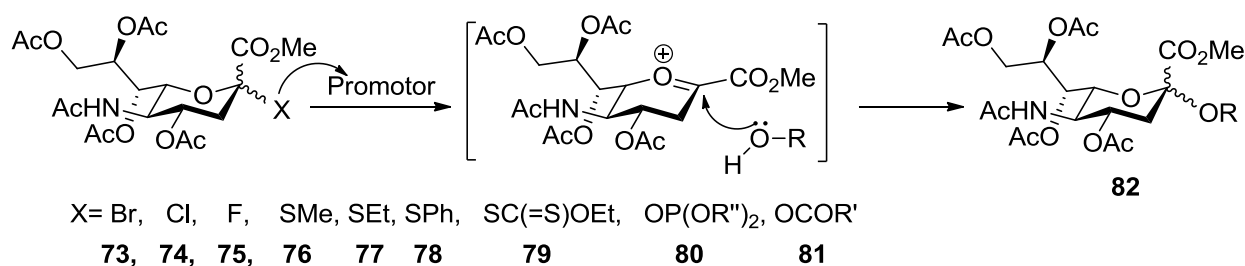
4.3.1 Direct chemical sialylation

Direct *O*-sialylation includes those methods that result in the formation of *O*-sialosides in one synthetic step, involving coupling between a glycosyl acceptor possessing a free hydroxyl group and a glycosyl donor with an appropriate leaving group at C-2. Some important methods for direct chemical sialylation are:^[117]

- 2-chloro derivatives as glycosyl donors
- 2-thio derivatives as glycosyl donors
- 2-xentates as glycosyl donors

- 2-phosphite as glycosyl donors.

In addition, there are some methods used for the direct chemical glycosylation of the sialic acids. Protic acid sialidation of 2-hydroxy derivatives of Neu5Ac have found some uses in the synthesis of alkyl sialosides.^[118] *N,N*-diacetyl derivatives of sialic acid have been shown to be highly reactive and efficient glycosyl donors.^[119-120] Kikkawa et al. introduced a new approach for the synthesis of glycosides^[121] through transglycosylation by reduction at C-1 of sialic acids as described in scheme 4.5.



Scheme 4.5: Direct chemical sialylation.

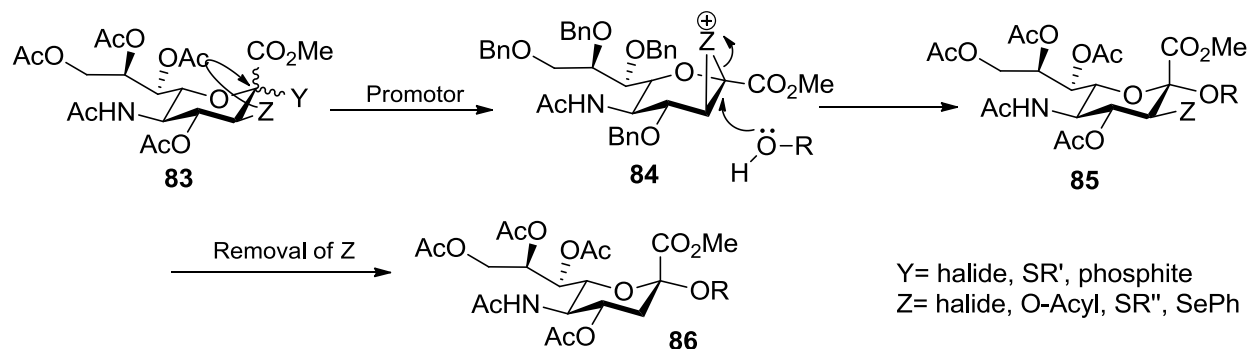
4.3.2 Indirect chemical glycosylation

Indirect methods afford *O*-sialosides in two or more synthetic steps, one of which may be a glycosylation. Most of these approaches involve modified sialyl donors possessing a participating functionality at C-3 in order to achieve better stereocontrol of sialylations.

The methods, which are more common, are:

- 3-Bromo-and other 3-*O*-auxiliaries
- 3-Thio and 3-seleno auxiliaries.

All available methods for glycosylation involve the use of a carboxyl group, as it is a more convenient way for glycosylation. The scheme 4.6 described an efficient route for the indirect glycosylation of methyl α -thioglycoside of Neu5Ac reported by L. O. Kononov et al.^[122] Both unprotected and protected carboxyl group involving saponification followed by acetylation of hydroxy groups showed good efficiency towards methyl α -thioglycoside of Neu5Ac and yielded 86% product over two steps.



Scheme 4.6: Indirect chemical sialylation.

4.4 Importance of Sialic Acids

Sialic acids being naked to the environment are responsible for different cellular activities, including interaction of the cell with its environment ranging from cell-cell recognition, adhesion and the signal transduction between the cell and different pathogens involved in infection, inflammation and tumor formation.^[123] Most probably, the earliest discovered function of Sia was to serve as receptor for influenza A and B viruses, which express themselves with Sia through α -2,3 linkage much like the expression of H5N1, the bird flu virus.^[124-125]

4.4.1 Sialic acids and human brain

Due to their importance in the development of the brain, Sialic acids may also be defined as “*the sugars that engineer the evolution, function and pathology of the brain*”.^[7] Unlike most primate brains that stop growing soon after the birth, the human brain continues to grow at the same rate as the rest of the body. In 2002 Varki et al. reported that the functional loss of CMP-*N*-acetylneuraminic acid monooxygenase (CMAH, an enzyme that converts Neu5Ac to Neu5Gc), played an important role in the evolution of the human brain.^[92] This concept is supported by the evidence of the mutation that abrogated the activity of CMAH transcripts found in present-day humans. This mutation occurred after the Homo-Pan divergence but prior to the brain expansion during human evolution. Gangliosides level varies among different mammals significantly and humans, the most intelligent species have almost the double amount as compared to other species. Moreover the cerebral grey matter contains $\sim 900 \mu\text{g/g}$ (wet weight) of GSL bound Neu5Ac, which is many times more than $\leq 30 \mu\text{g/g}$ (wet wt.) found in most tissues and organs.^[126]

The malfunctioning of sialic acid may cause different disorders ranging from inborn errors of metabolism (sialuria, Salla disease etc.) to the late onset degenerative disorders of the brain including schizophrenia, Parkinson and Alzheimer diseases. Many neurologic disorders are also

caused by congenital defects in ganglioside biosynthesis and GM3 alone is included in several diseases. In mice, lack of GM3 synthase activity results in complete loss of hearing due to degeneration of Corti organs.^[127]

4.4.2 Sialic acids and bioorthogonal labeling

Monosaccharides like *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl mannosamine (ManNAc) undergo metabolic pathway yielding sialic acids as illustrated in fig. 4.4. Rutter et al. established that the mannosamine bearing unnatural groups like azide, ketone, alkyne, thiol etc. undergo biosynthetic pathways for the synthesis of sialic acid.^[34, 128] It was established by Saxon et al. that the ManNAc undergoes the metabolic pathway more quickly compared to the GlcNAc. The existence of a modified Sia on the cell surface can easily be exploited by a functional group that has affinity for Sia and is inert to the rest of functionalities on the cell surface. The proof of concept came from the work of Bertozzi and Saxon when they labeled the cell surface through Staudinger ligation.^[34, 128] In addition to the keto and azide functionalities, the cell surface sialic acids can be functionalized with terminal alkynes. However, the necessity of Cu for click reaction of terminal alkynes, makes it unfavorable for *in vitro* application, which can be overcome to some extent by the use of Cu(I) stabilizing ligands. Cell surface labeling has recently been reported *in vitro* by the use of different Cu(I) stabilizing ligands.^[129]

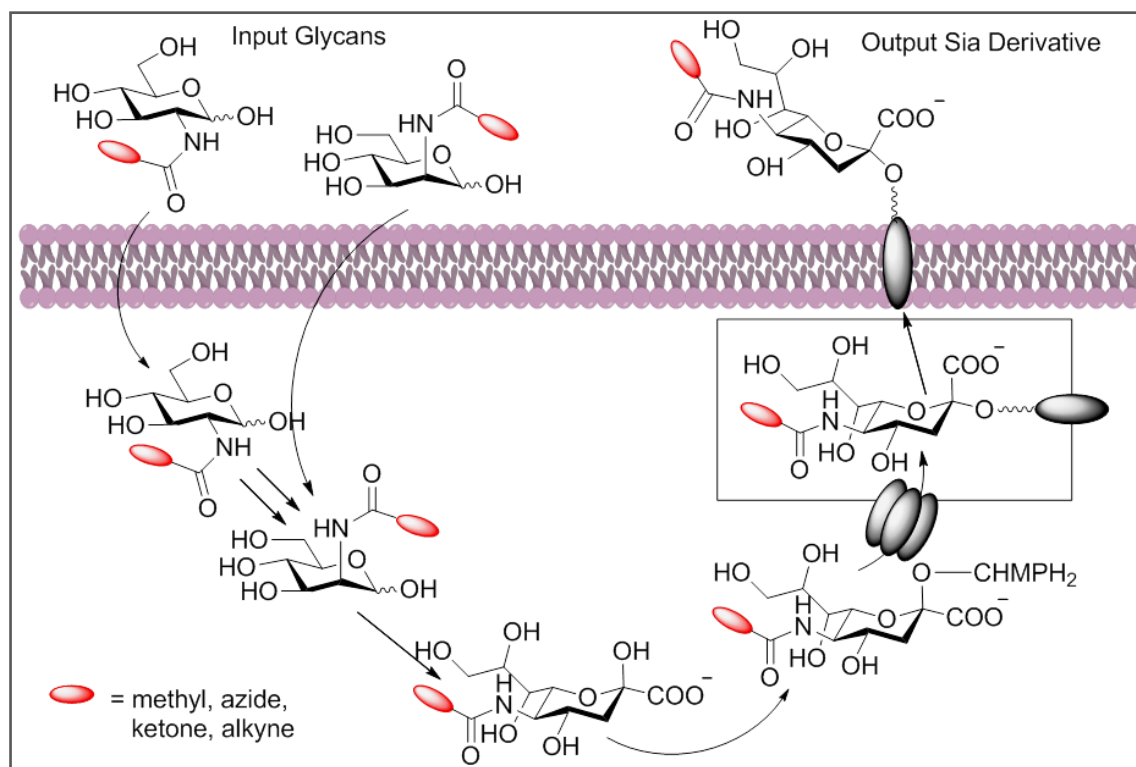


Figure 4.4: Sia biosynthetic pathway. Cell surface sialosides are synthesized by a series of enzymatic steps.

5 Glutarimide and Derivatives

Cyclic imides, such as succinimide, maleimide, phthalimide and glutarimide possess structural features, which confer potential biological activity and pharmaceutical use.^[130] These molecules contain an imide ring with general structure --CO--N(R)--CO-- and exhibit hydrophobic and neutral behavior, which enables them to cross biological membranes *in vivo*. The various classes of cyclic imides have received attention due to their antibacterial, antifungal, analgesic and antitumor activity.^[131-132] Glutarimides are the hydrolyzed cyclic imides formed as a result of cyclization of glutamine. These compounds are particularly important components of many extremely valuable drugs, some of which are shown in the fig. 5.1.

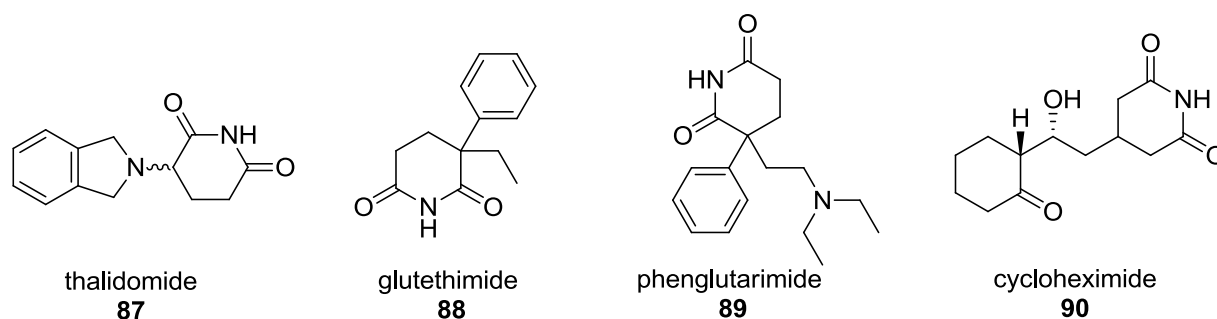
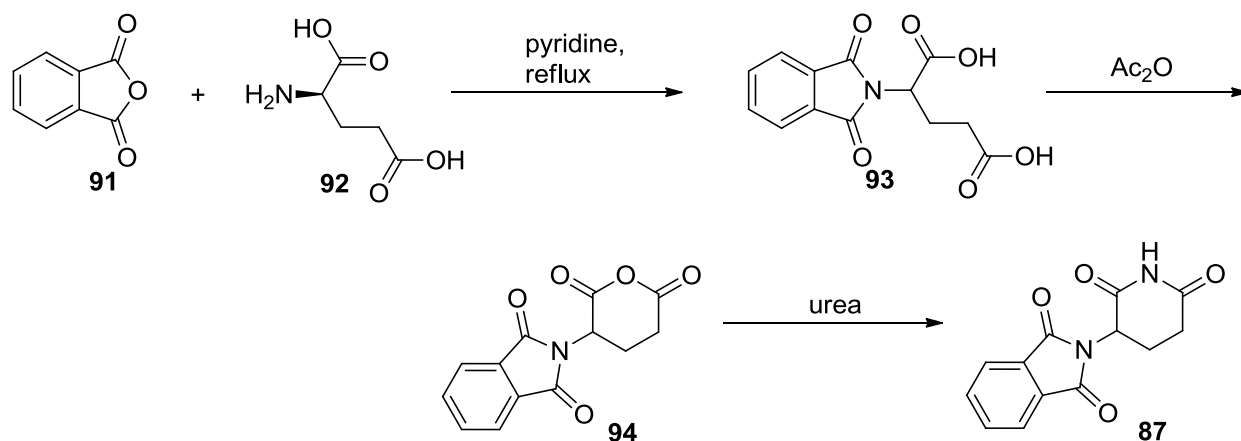


Figure 5.1: Glutarimide based biologically active molecules.

The glutarimide moiety is present in a great number of natural molecules and has a broad spectrum of pharmacological activities.^[133] The broad range of the applications of the glutarimide derivatives, both in medical and material sciences, has enabled them to attract the attention and a variety of the developments on these unique molecules have been made both in synthetic and application aspects.

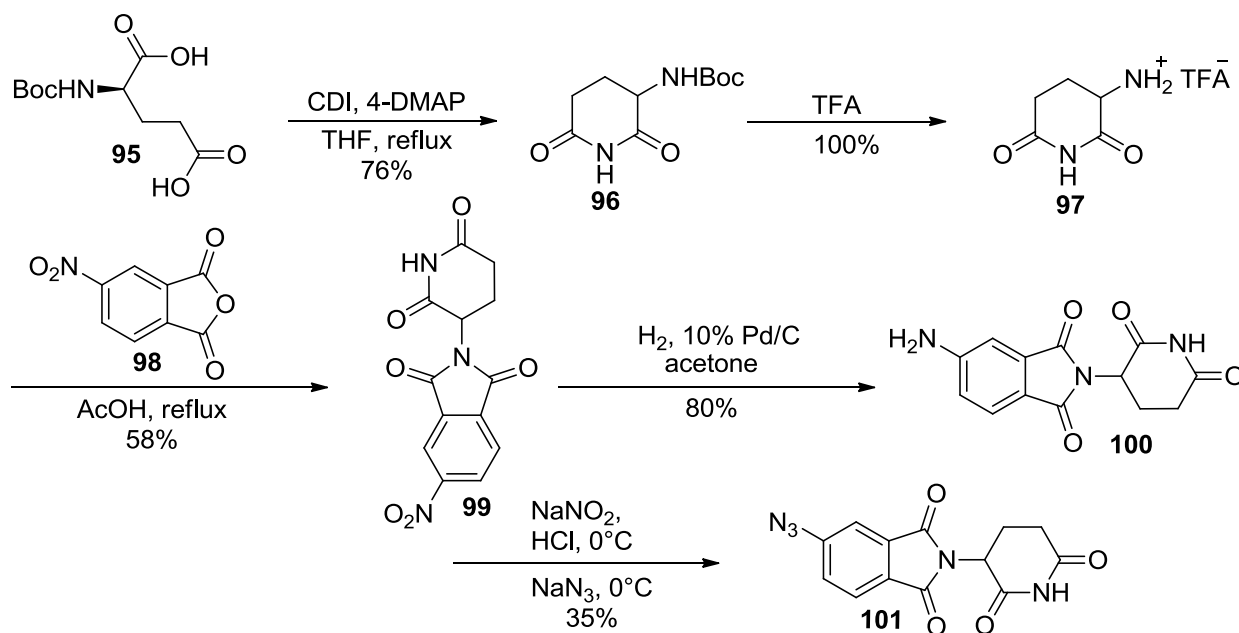
5.1 Synthesis of the Glutarimide Derivatives

The first synthesis of thalidomide **87**, an important member of glutarimide family is controversial due to different claimants i.e. CIBA (1954), Grunenthal GmbH (1950).^[134] Most methods reported so far for the synthesis of glutarimide derivatives start from glutamic acid.^[135-137] Commercially available L-glutamine is used as a precursor to achieve many target molecules. Adopting the same strategy, a concise two-step synthesis of thalidomide **87** was introduced by G. W. Muller et al. in 1999^[138] as described in scheme 5.1.



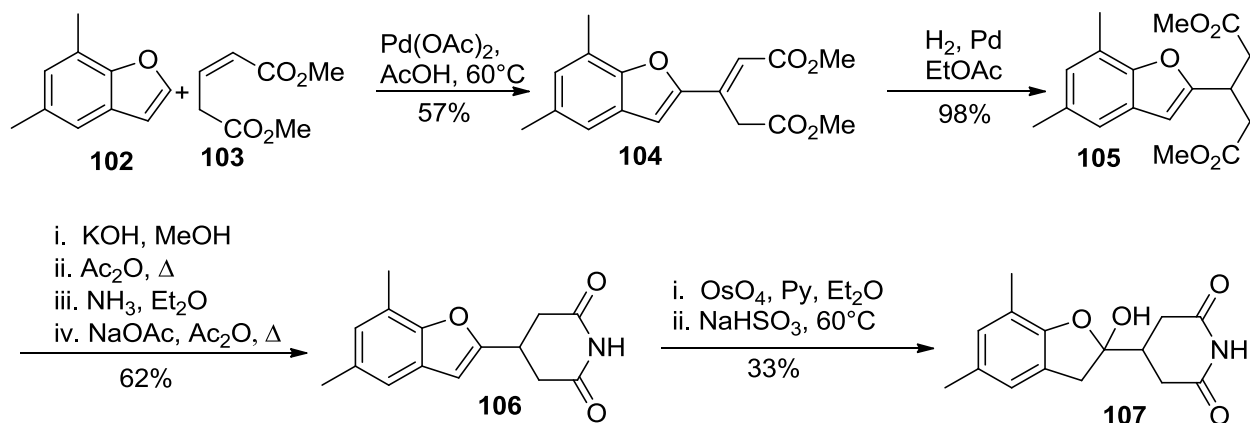
Scheme 5.1: Synthesis of thalidomide.

M. L. Brown and co-workers extended the work of Müller et al. and synthesized the azido derivatives of glutarimide **101** in two steps starting from isoglutamine **95** as described in scheme 5.2. In the first step, the glutarimide amine derivative was synthesized, which was then condensed with 4-nitrophthalic anhydride by refluxing in glacial AcOH in the second step.^[139]



Scheme 5.2: Synthesis of azido thalidomide.

H. Kiyota and co-workers described the synthesis of actiketal **107**, a glutarimide antibiotic in 2000.^[140] They got the desired actiketal by coupling of 5,7-dimethylbenzofuran and dimethyl glutaconate through a Pd-assisted reaction, described in scheme 5.3.



Scheme 5.3: Synthesis of actiketal.

5.2 Importance of Glutarimide Derivatives

Glutarimide derivatives have proven themselves as important moieties not only in medicinal science^[141-144] but also they have emerging potential in material sciences.^[145-147]

5.2.1 Medical importance of glutarimide derivatives

Glutarimide derivatives are a class of compounds possessing important bioactivities. It is suggested that the glutarimide moiety (2,6-piperidinedione) with the intact imide group (OC-NH-CO) and substituted at α or β position in the ring is acting as the carrier molecule, which transports biologically active substituents through cell membranes.^[148] Thalidomides have special clinical value in a number of pathological conditions such as erythema nodosum leprosum, rheumatoid arthritis, HIV-associated oral ulcers, and chronic graft versus host diseases.^[149] Both *in vitro* and *in vivo* studies have shown that thalidomides have the ability to inhibit tumor necrosis factor- α and its production.^[150] FDA has approved this drug for the treatment of erythema nodosum leprosum (ENL) and multiple myeloma.^[151] Racemic thalidomide **87** is the most well-known glutarimide derivative since the time it was introduced as a sedative drug in the late 1950s.^[152-154] It was first marketed in West Germany by Chemie Grunenthal GmbH as a clinically effective and extremely safe non-barbiturate sedative hypnotic in 1956. It became a popular drug in Europe, Canada, and Japan with a variety of names: Contergan®, Distaval®, and Isomin® due to low cost and high efficiency. Some of the important glutarimide derivatives are shown in fig. 5.2. Sesbanimide **108**, an alkaloid isolated from the seeds of *Sesbania drummondii* and *S. punices*, shows notable cytotoxicity against KB cells *in vitro* and potent inhibitory activity against P388 murine leukemia *in vivo*.^[155] Migrastatin **109** was isolated from a cultured broth of *Streptomyces sp.* as an inhibitor of tumor cell migration.^[156] The 14-membered lactone core in the structure of migrastatin is the essential part for its

inhibitory activity of tumor metastasis.^[157] Aminoglutathimide **110** being used for the treatment of metastatic breast cancer inhibits steroidogenesis at the aromatase sites.^[158] Antineoplaston A10 (*N*-[(3*S*)-2,6-dioxo-3-piperidyl]-2-phenyl-acetamide) is another glutarimide derivative, originally isolated from human urine and exhibits remarkable anticancer activity but lacks the toxicity of other common cancer drugs.^[133] It is assumed that A10 acts directly at the genomic level and alters the cellular responsiveness to steroidal hormones. Many glutarimide derivatives are partial agonists of the central nervous system (CNS) and their actions range from convulsive and analeptic to anticonvulsive and hypnotic. The importance of glutarimide moieties in antibiotics is well known and they act by inhibiting the synthesis of bacterial peptides at the initiation and extension steps.^[159] Cycloheximide is a powerful inhibitor of protein synthesis, which inhibits the transfer reaction (i.e. the release of deacylated t-RNA from P-site of ribosome) because of its specific binding interaction with a site on the 60 S ribosomal subunit.^[160] In spite of the anticancer behavior, glutarimide derivatives have been reported to show anti-inflammatory properties too, believed to be a critical component of tumor progression.^[161]

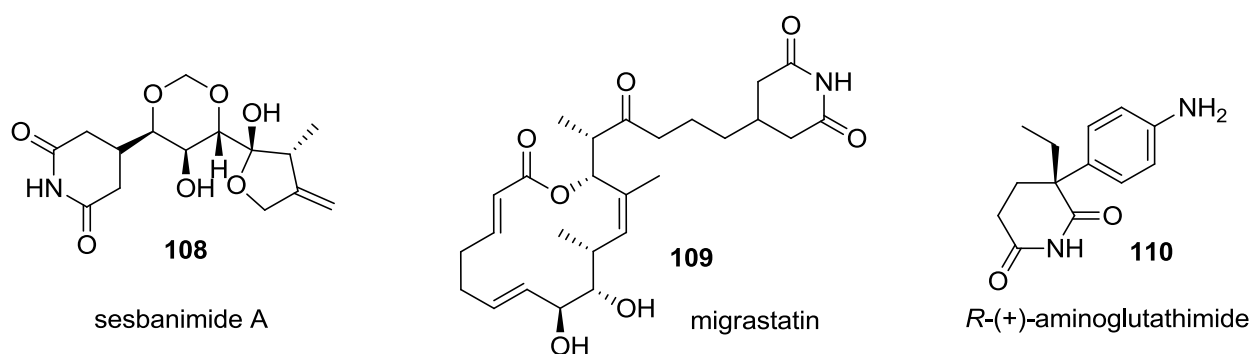


Figure 5.2: Some important Glutarimide derivatives with medicinal potential.

5.2.2 Potential of glutarimide derivatives in agriculture

In addition to the medicinal applications, the glutarimide derivatives have also exhibited the potential as antimicrobial and herbicides and fig. 5.3 illustrates some of them. E. G. Teach and co-workers have described a family of the glutarimide derivatives that show herbicidal behavior.^[162] CHX (Cycloheximide), a well-known inhibitor of protein synthesis, shows strong inhibitory activities against plant fungi, influenza virus, reovirus and arbovirus.^[163-164]

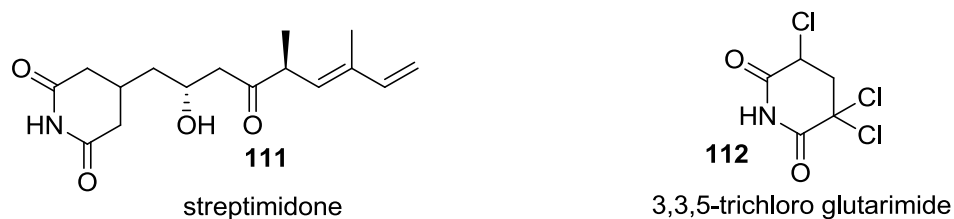


Figure 5.3: Glutarimide derivatives with potential in agriculture.

Streptimidone inhibits the development of plant diseases such as phytophthora blight on pepper, grey mould on cucumber leaves, and leaf blast on rice leaves.^[165] 9-Methylstreptimidone isolated from *Streptomyces sp.* is responsible for high antiyeast and antifungal activity of the *Streptomyces* fermentation and its antifungal activity is comparable with that of Streptimidone.^[166]

5.2.3 Glutarimide derivatives in material science

C. W. Struijk and co-workers have reported that the development of the glutarimide functionality in perylene dramatically increases its properties towards the conductivity to behave as semiconductor. They developed glutarimide ring on both sides of the perylene **114** with different side chains on the nitrogen atom as shown in fig. 5.4. Enhanced order in the liquid crystalline state resulted in a charge carrier mobility as high as $0.2 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, comparable to the highest values of already found for discotic materials.^[148] Glutarimide polymers have been reported to enhance the homogeneous distribution of iron nano particles on the surface by simple spin coating due to the complexation properties of glutarimide with iron.^[167] Polydimethylglutarimides **113** have been reported to exhibit resistance to deep UV (DUV) and have applications as the base layer in bilayer lift-off processes. These resists have glass transition temperature between 180-190 °C. However, these can be depatterende by the use of DUV and electron or proton beam exposure.^[146, 168-170]

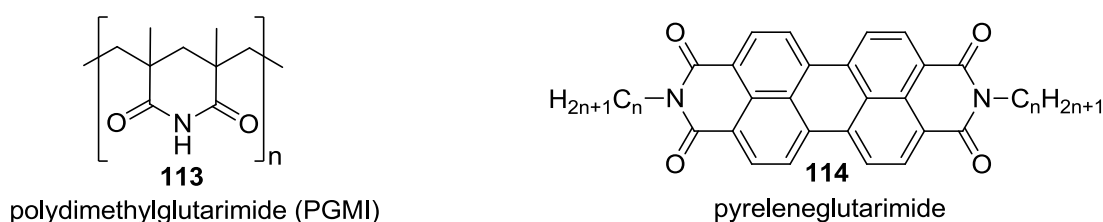


Figure 5.4: Glutarimide derivatives with potential in material science.

5.2.4 Toxicity of glutarimide derivatives

Aside from the variety of the useful applications of glutarimide derivatives in different fields as mentioned above, they also have a history of toxicity.^[171-173] One notorious tragedy in medicinal history is the congenital effect of thalidomide in pregnant women resulting birth defects in babies. Most prominent of these defects are amelia (absence of limbs), phocomelia (absence of most of the arms, extending like a flipper from shoulders), dysmelia (malformation, missing or extra limbs), bone hypoplasticity (incomplete development) and other like defects of ears, heart and internal organs.^[174] The small yet critical difference in molecular structure between

conformational form $R-(+)$ (safe, responsible for anti-inflammatory activity) and $S-(-)$ (dangerous) are responsible to the different behaviour of the molecule. In 2004 Frank et al. described the reactivity of thalidomide “*thalidomide derivative is a racemic glutamic acid analogue, consisting of $S-(-)$ and $R-(+)$ enantiomers that interconvert under physiological conditions. The $S-$ form potently inhibits release of tumor necrosis factor (TNF alpha) from peripheral mononuclear blood cells, whereas the $R-(+)$ form seems to act as a sedative, probably mediated by sleep receptors in the forebrain*”.^[134] Thalidomide may also cause some side effects like polyneuropathy, fatigue, skin rash and venous thromboembolism or blood clots, which could lead to stroke or myocardial infarction.^[175-176] Another objection on the use of glutarimide based drugs is memory loss due to the diverse effect on brain cells. Inhibition of ongoing constitutive protein-synthesis impairs replacement of the brain proteins undergoing degradation and this produces abnormal cerebral functioning, thus impairing memory storage.^[177]

6 Aim of Work

This work was designed to exploit the outermost position of the cell surface glycoconjugates through glycoengineering. The aim behind this work was the development of functionalized chemical probes for the labeling of cell surface glycoconjugates. Metabolic bioorthogonal cell surface labeling was pursued with-step strategy:

- bioorthogonal metabolic incorporation of the labeled glycans (chemical reporter) into the cell surface glycoconjugates
- direct chemical conjugation of the labeled cell surface glycans under physiological conditions.

In the first step the metabolic cell surface labeling was planned to achieve by tagging the cells glycoconjugates with modified glycans possessing non-native functional groups called chemical reporter. It was aimed to followe by conjugation of the modified cell surface glycans (reporters) with the chemical probes containing the counter partner as shown in fig. 6.1.

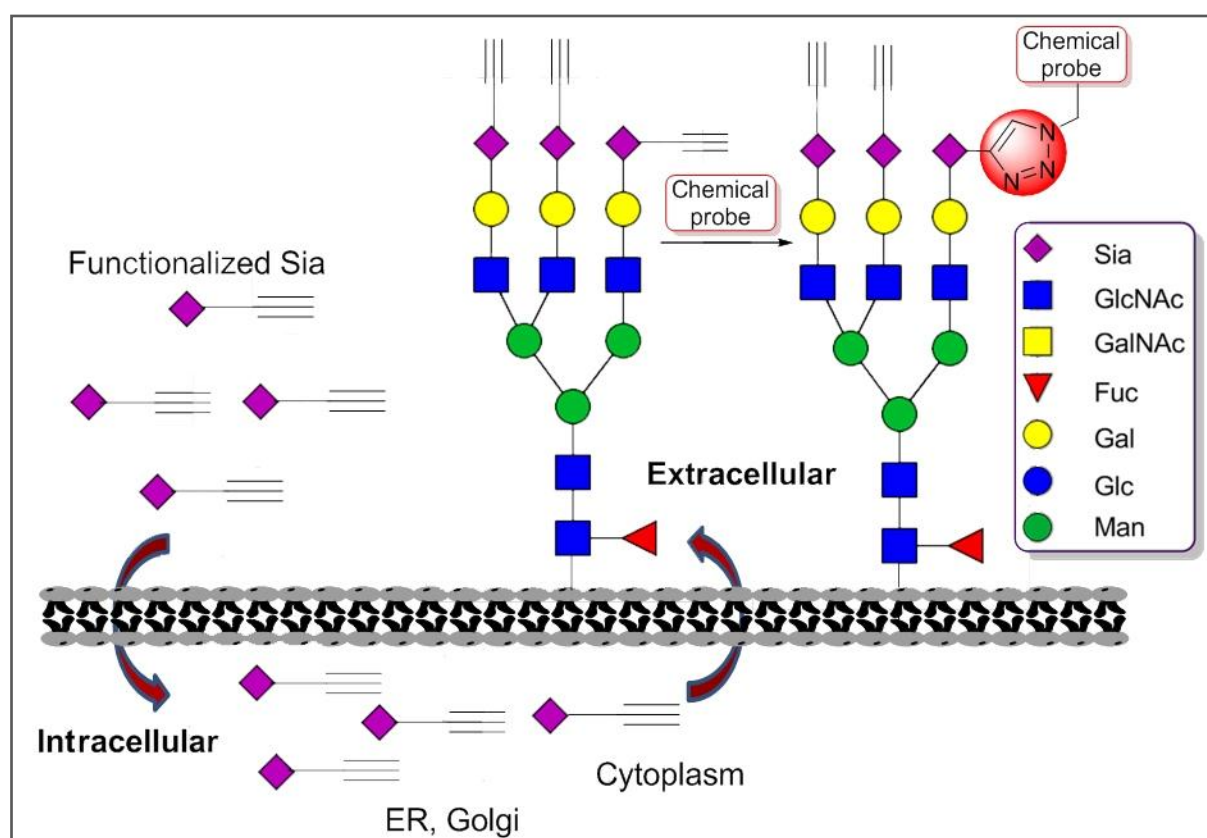
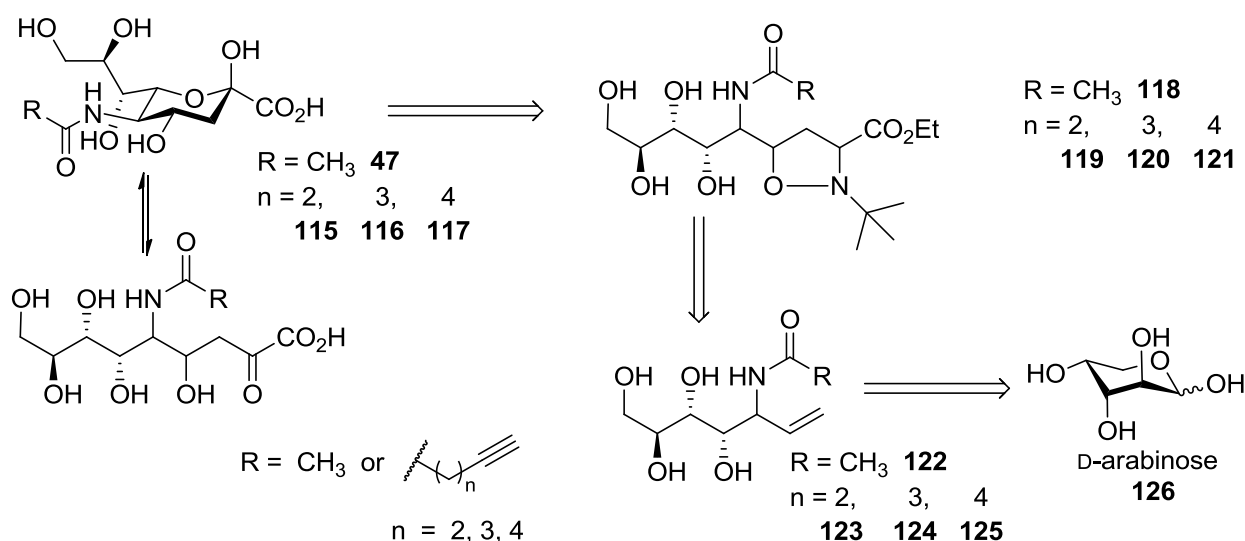


Figure 6.1: Schematic representation of the targeted glycoconjugation of cell surface modified sialic acids.

The aforementioned strategies include the incorporation of the modified *N*-acetyl mannosamine (ManNAc) and *N*-acetyl glucosamine (GlcNAc) for the introduction of the modified sialic acid derivatives on the cell surface. Here, we aimed to develop sialic acid derivatives containing

linkers with terminal alkyne functionality, and to study their intake by cell. To investigate the effect of the side chain towards the uptake of sialic acids by cells, and their expression on the surface, we envisaged their synthesis with different side chain length. The synthesis of Sia derivatives planned with an efficient three-step approach. The first step was a modified petasis reaction, followed by a 1,3-dipolar cycloaddition in second step. Finally, the Sia derivatives were obtained by a base catalyzed ring opening of isoxazolidines obtained in second step. A retrosynthetic route, for the proposed Sia derivatives, is shown in scheme 6.1.



Scheme 6.1: Retrosynthetic scheme for the targeted sialic acid derivatives.

To achieve the conjugation of the modified Sia through click reaction, we pursued the synthesis of different azides. As the aim of this work was cell surface labeling, the synthesis of fluorescent azides considered to be more beneficial for cell imaging, leading to the direct proof of the approach. Keeping in mind our goal, we synthesized azides of sugar moieties i.e. galactose azide **127**, glucosazide **128**, **129** along with fluorescent azides like fluorescein azide **130** and rhodamine azide **131** shown in fig. 6.2.

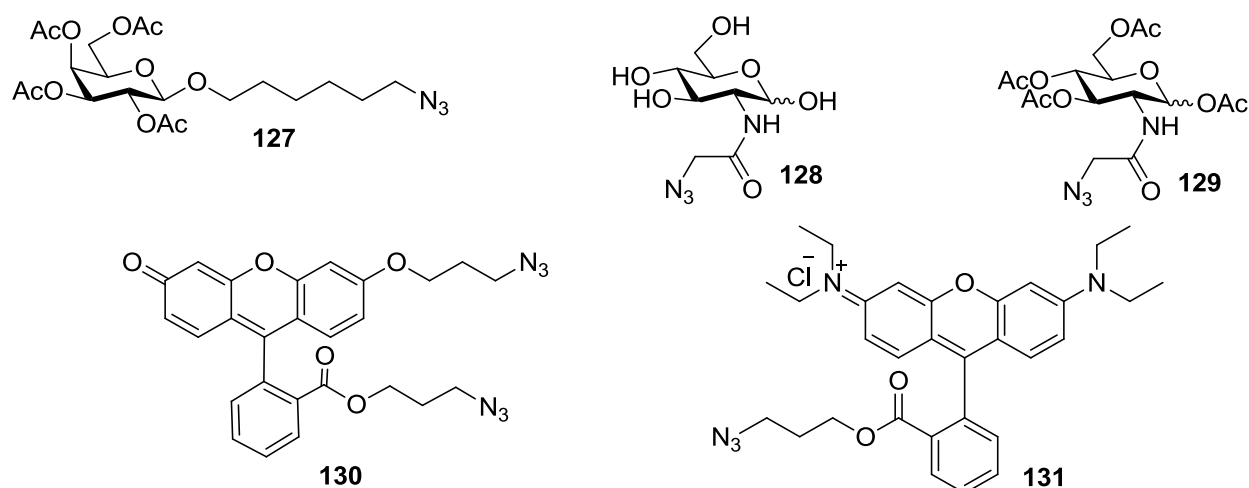


Figure 6.2: Different Azide molecules planned to be synthesized.

Our next goal was investigations on click reaction conditions and their optimization for the bioorthogonal reaction. Sharpless approach (CuAAC) cannot be applied *in vitro* due to the toxicity of Cu. To avoid cytotoxicity, we aimed to use Cu(I) stabilizing ligands, which cannot only enhance the reaction rate, but also reduce the toxicity by stabilizing Cu(I). After the investigation of Huisgen click reaction conditions, we focused on TBTA as Cu(I) stabilizing ligand and figured out its impact on the reaction rate. In order to achieve the cell imaging, the optimized reaction with TBTA was planned to apply on HEP-2 cells labeled by alkyne Sia derivatives, and cell imaging was envisaged by fluorescent microscopy after coupling the modified cells with fluorescein azide through click reaction.

The second part of this work was the investigation of click reaction under Cu free conditions by substituted alkynes with electron withdrawing groups and cyclic octynes. Aforementioned syntheses of cyclooctyne derivatives for Cu free click reaction are highly complicated. Our main focus was the introduction of simplified syntheses of cyclooctynes. First, we will investigate the synthetic pathway of already reported cyclooctynes (DIFO-I **26** and DIFO-II **28**) to develop a library for Cu free click reaction. The synthesis of the DIFOs was followed by the development of a simplified synthesis of the cyclooctyne **132**, a derivative with a cyclic amide functionality. A structural view of the synthesized octynes is given in fig. 6.3.

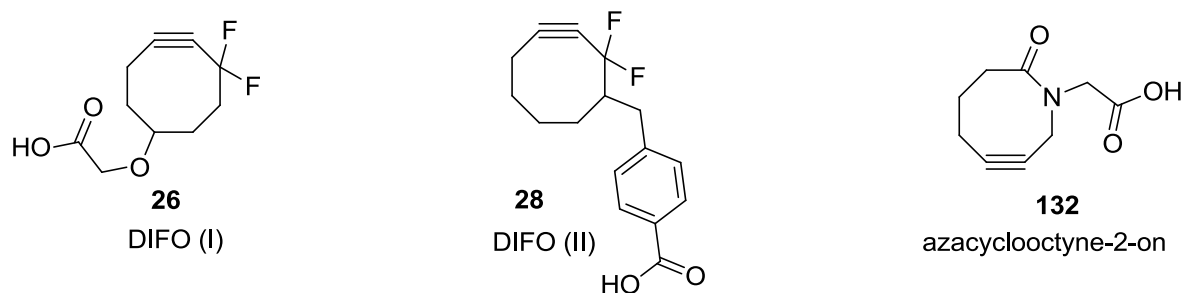
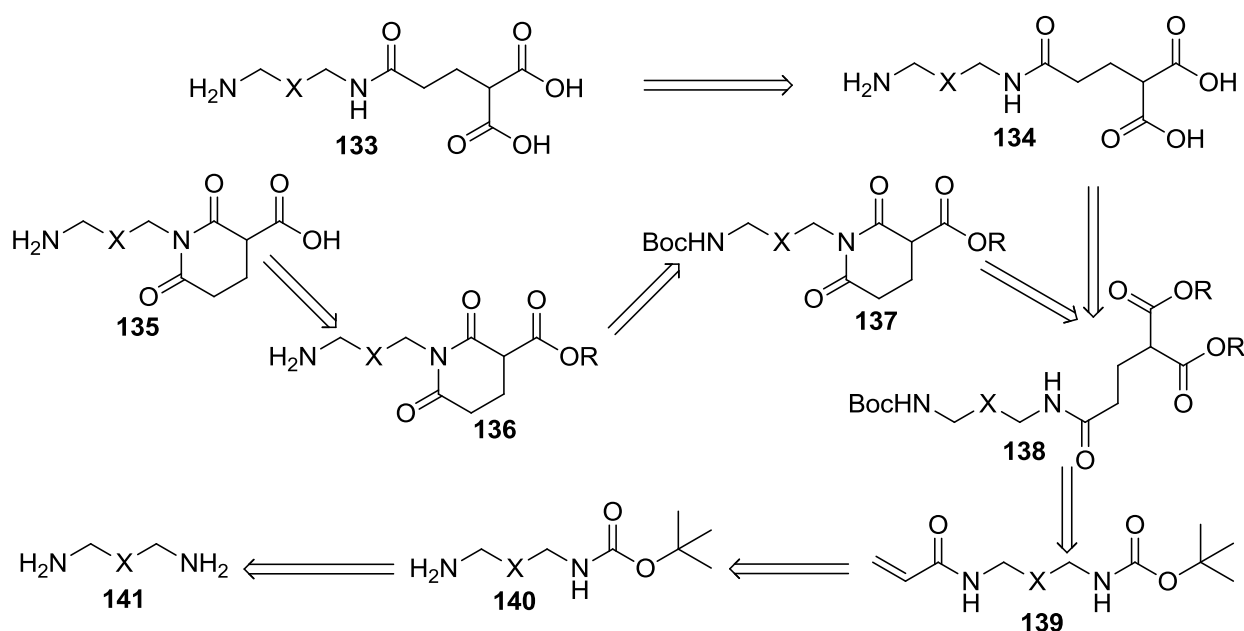


Figure 6.3: Structures of target cyclooctynes.

The third part of this work was designed to synthesize the reagents for the modification of cell surface polarity directly without involving the metabolic process. In this part, the synthesis of oxo-amino dicarboxylic acids and derivatives of glutarimide were envisaged. These molecules with highly polar nature, due to the presence of two carboxylic groups, in addition to the oxo-amino functionalities in the side chain were envisioned to have potential to change the cell surface charge. The synthesis of the target molecules was planned to achieve through a retrosynthetic pathway shown in scheme 6.2. The syntheses of the oxo-aza dicarboxylic acid moieties were further followed by their conjugation with different alkyne and azide moieties for the modification of cell surface.



Scheme 6.2: Retrosynthetic scheme for the targeted Glutarimide derivatives and amino dicarboxylic acids.

7.1 Sialic Acids

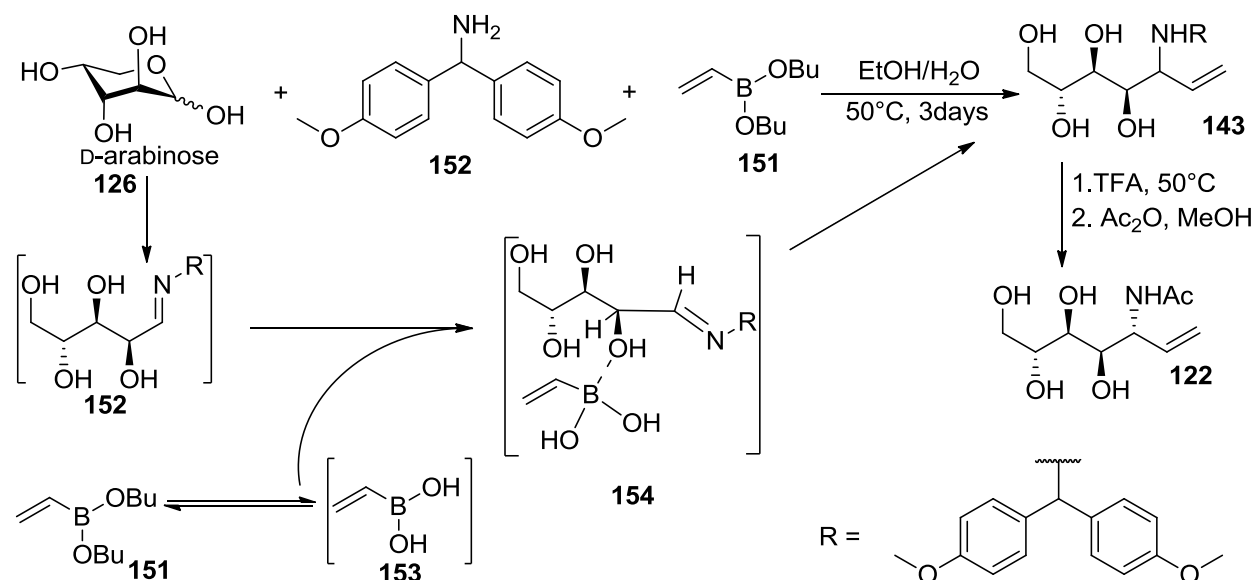
[illegible]

36

The first step was simultaneous introduction of an amino group containing a linker and a vinyl group to an aldose by means of a newly developed petasis reaction.^[179] This step makes the approach flexible for the synthesis of a variety of Sia, as a number of aldoses and amine derivatives carrying different functionalities can be introduced. The second step involves the conversion of the vinyl group into a γ -hydroxy- α -keto ester moiety through a diastereoselective 1,3-dipolar cycloaddition. The third and final step is a base catalyzed ring opening along with ester hydrolysis of the isoxazolidine obtained in second step. The potential efficiency of the reaction sequence is optimistic if there is not any involvement of extra steps i.e. protection and deprotection of functional groups and same thing is true here.

7.1.1 Modified petasis reaction

The work was started with the synthesis of naturally occurring D-N-acetylneuraminic acid **47** (D-Neu5Ac) to evaluate the route with the use of D-arabinose as a starting material. As shown in scheme 7.2, the first step was a condensation between α -hydroxy aldehyde (D-arabinose **126**), a secondary amine **152**, and a substituted vinyl boronic acid ester **151**. Vinyl boronic acid, a source of vinyl group in standard petasis reaction, is unstable and deteriorates easily in the presence of water.^[180] The commercially available dibutyl vinyl boronic acid ester was used in the petasis coupling, which proved a good vinyl source.



Scheme 7.2: One-pot synthesis and possible mechanism of intermediate **122** through boronic ester version of Petasis reaction.

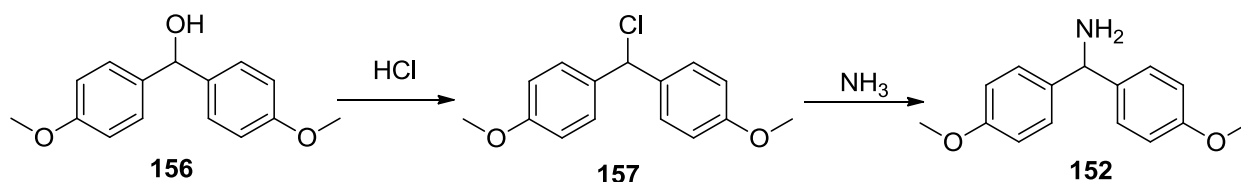
Water is considered to be necessary in the coupling of the boronic acid esters, which indicates that an *in situ* equilibrium between the boronic acid ester and boronic acid might occur under the

reaction conditions. Owing to this exchange process, the coupling of the dibutyl vinyl boronic acid had to be performed at a slightly elevated temperature (50 °C) and for a longer reaction time (3 days) in EtOH/H₂O (4:1). In contrast, the standard Petasis reaction takes place at 25 °C, in pure EtOH and the reaction time is 24 h. Rather than isolating compound **143**, the reaction mixture was treated with a catalytic amount of trifluoroacetic acid (TFA) and stirred for 24 h. After successful removal of bis(4-methoxyphenyl)methyl group from amine, selective conjugation of the amino group was accomplished by treatment with Ac₂O or activated esters **144**, **145**, **146** of alkyne acid/MeOH. The yield of the reaction was in the range of 55-60%, calculated on the basis of aldose sugar (D-arabinose).

As described above, we needed a secondary amine as a source of amine moiety in the synthesis of polyhydroxy olefinic amides. Thus, our initial target was the synthesis of amine **152** through the strategy described below.

7.1.2 Synthesis of 4,4'-dimethoxy benzhydrylamine **152**

Although amine **152** is commercially available, but due to its high cost and seldom availability, we decided to synthesize it in lab. Initially, the synthesis was attempted by treatment of 4,4'-dimethoxy benzhydrol with mesyl chloride at -50 °C in DCM.^[181] The mesylate was treated with ammonia, dissolved in THF at -50 °C, but the yield was not optimistic (25–30%). In a second approach, we decided to convert benzhydrol into the chloride **157**, which might be easier to convert into an amino functional group. Benzhydrol **156** dissolved in dry Et₂O was treated with HCl gas at -5 °C as described in scheme 7.3. The residue, obtained after the evaporation of Et₂O, was dissolved in DCM and treated with ammonia gas at 0 °C to afford the amine **152** as a white solid in a very good yield (>85%).

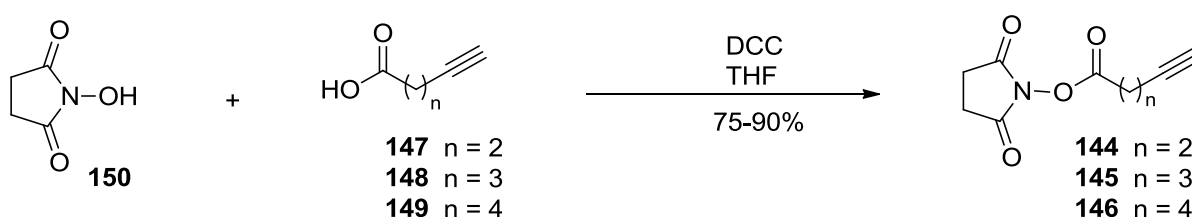


Scheme 7.3: Synthesis of 4,4'-dimethoxy benzhydrylamine **152**.

7.1.3 Esterification of carboxylic acids

The activation of the acids was achieved by treatment with *N*-hydroxysuccinimide **150** in the presence of *N,N*-dicyclohexylcarbodiimide (DCC). The first attempt was performed by the use of dioxane as the solvent at rt for 2.5 h.^[182] All the reactants were taken in the same ratio;

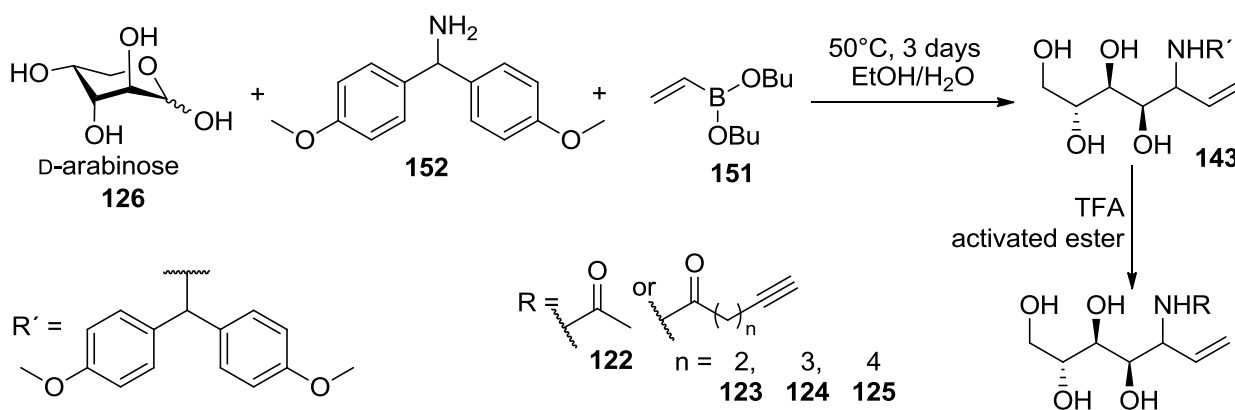
however, the reaction afforded very poor yields (10-20%) with acids **147**, **148** and **149**. To avoid these difficulties the reaction conditions were modified. It was assumed that rt is not suitable and the intermediate leading to the formation of the activated ester may decompose at ambient temperature. To avoid the complication, the reaction was carried out in THF at 0 °C for 1 h and then refrigerated overnight at 3-5 °C as described in scheme 7.4. Next day, the precipitated solid (DCU) was filtered and discarded. The solvent was evaporated and concentrate was purified by flash chromatography to afford the esters **144**, **145** and **146** in 85-90% yields.



Scheme 7.4: Activation of alkyne acids by esterification with *N*-hydroxysuccinimide and DCC.

7.1.4 Synthesis of polyhydroxy olefins **122**, **123**, **124** and **125**

The intermediates, polyhydroxy olefins **122**, **123**, **124**, **125** were synthesized through a modified petasis reaction shown in scheme 7.5. The reaction was initialized by treatment of the aldose sugar **126** with amine **152** and vinyl boronic acid ester **151** in a mixture of EtOH and water (4:1) for 72 h at 50 °C. The amino group was deprotected by the addition of trifluoroacetic acid (TFA) to the reaction mixture to get free amine by the removal of the 4,4'-dimethoxy benzmethyl group. After 24 h, the solvent was replaced by dry methanol and NaHCO₃ was added to deprotonate the amino group. Finally, acetic anhydride or either of the activated esters of acids (4-pentynoic acid **144**, 5-hexynoic acid **145** and 6-heptynoic acid **146**) was added. The reaction mixture was stirred at rt for 3 h followed by the removal of the solvent under reduced pressure without any workup.



Scheme 7.5: Schematic representation for the synthesis of olefinic intermediates.

After complete removal of the solvent, the crude product was subjected to flash column chromatography, eluting with DCM/MeOH (10:1). The isolation of the product was difficult to achieve due to the complication of impurities and bad elution. To obtain pure product, the crude mixture was chromatographed twice and in some cases three times. The product identities were justified by different analytical methods, i.e. NMR, mass spectrometry (ESI-MS) and optical rotation.

7.1.4.1 Discussion on the NMR spectra of olefins **122**, **123**, **124** and **125**

The ^1H -NMR study of the compounds **122**, **123**, **124** and **125** showed a broad singlet in the range of $\delta = 8.2$ ppm and such a far down field signal shows the presence of an amide group (fig. 7.1 for compound **125**). A ddd in the range of $\delta = 6.0$ ppm indicates the presence of a vinyl hydrogen at position 1'', and the presence of two triplets of doublet (td) at $\delta = 5.31$ ppm ($J = 17.3, 1.5$ Hz) and $\delta = 5.22$ ppm ($J = 10.5, 1.5$ Hz) indicate the presence of terminal hydrogen atoms of the vinyl group at position 2''. Furthermore, the downfield shift of a multiplet at $\delta = 4.56$ ppm of hydrogen at position 1 indicates the presence of the shielding effect of the amide nitrogen and confirms the presence of amide group at C-1.

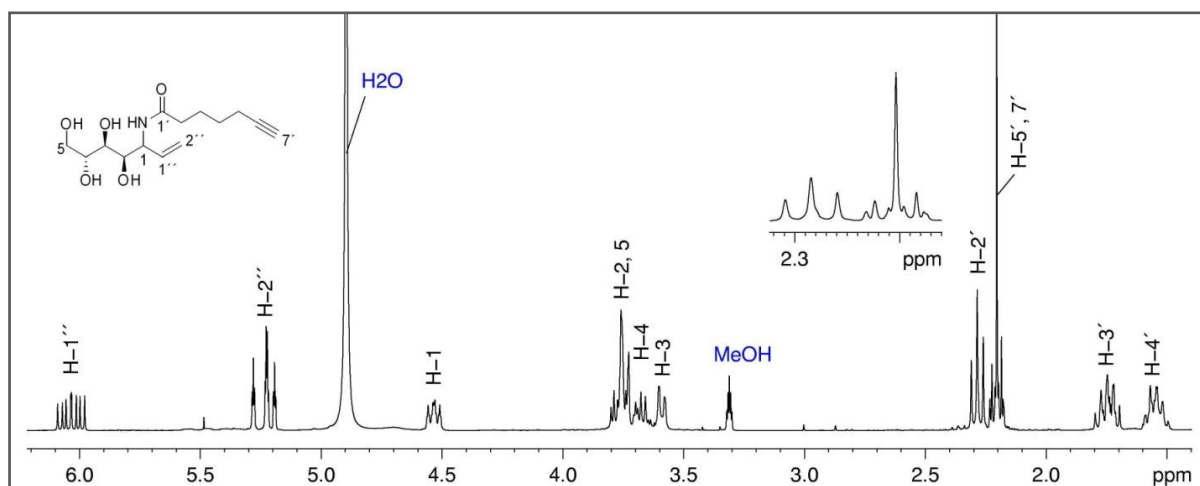


Figure 7.1: ^1H -NMR spectrum of polyhydroxy olefin intermediate **125** (MeOD, 300 MHz).

The carbonyl group is confirmed by ^{13}C -NMR shown in fig. 7.2, which shows a signal in the range of $\delta = 173.1$ - 174.5 ppm, typical for an amide group. ^{13}C -NMR further indicates the presence of a quaternary carbon of alkyne in the range of $\delta = 83.5$ ppm, which is further confirmed by ^{13}C -DEPT spectra. Furthermore, the proof for the presence of an olefinic double bond in ^{13}C -NMR spectra supported by two signals in the range of $\delta = 136.87$ ppm and $\delta = 116.95$ ppm, which confirm the presence of C-1'' and C-2''. The rest of the signals in the

range of $\delta = 69.7\text{--}72.7$ ppm also satisfy the presence of the sugar moiety. As a result of this discussion, it can be concluded that we have the right olefins, obtained as a result of one pot petasis reaction.

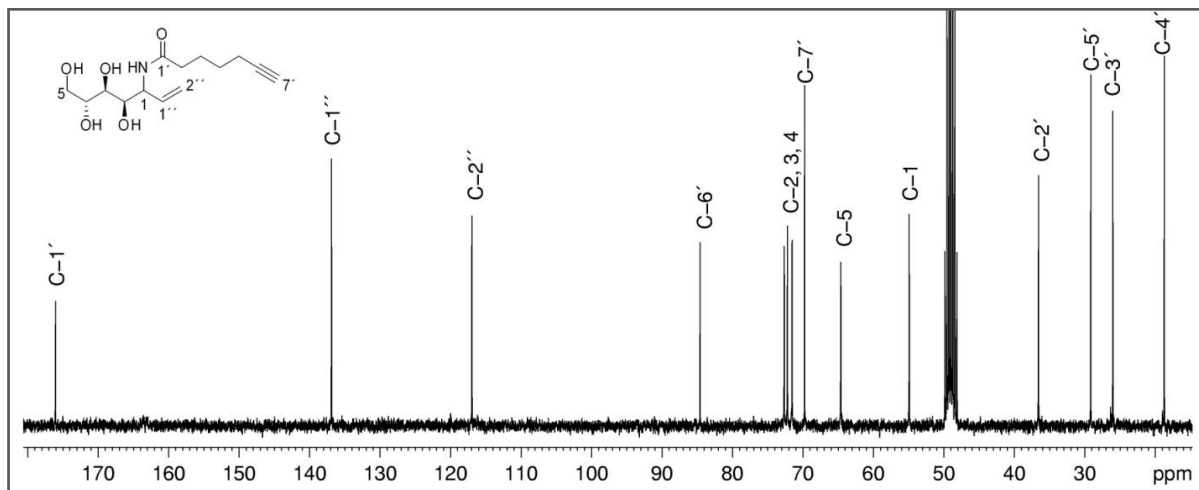
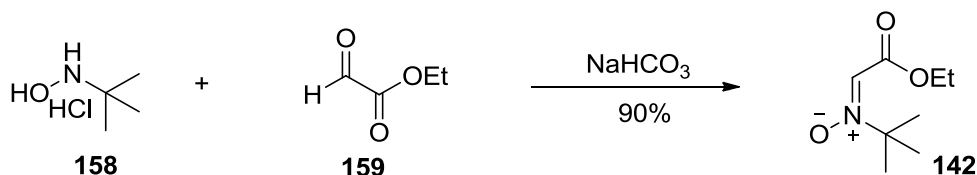


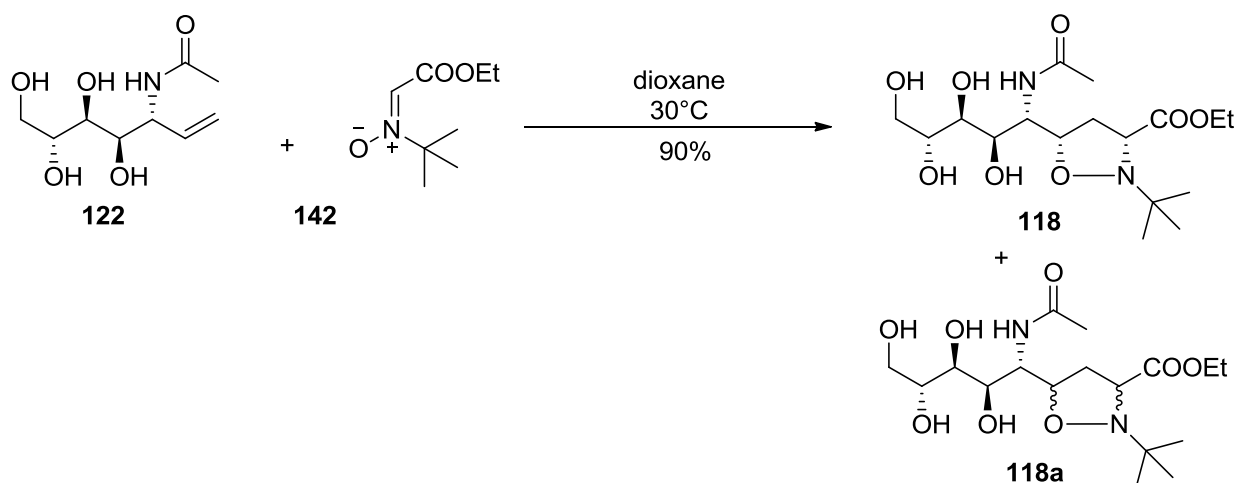
Figure 7.2: ^{13}C -NMR spectrum of polyhydroxy olefin intermediate **125** (MeOD, 75 MHz).

7.1.5 Synthesis of isoxazolidines **118**, **119**, **120** and **121**

The successful syntheses of olefinic intermediates were followed by their conversion into γ -hydroxy- α -keto acid moieties (isoxazolidines). One possibility could be the treatment of vinyl intermediate with nitrile oxide, but it suffers from the impossibility to achieve selective cleavage at N-O in the next step due to the presence of C=N bond.^[180] Another possibility to get the desired intermediate could be the use of nitron, but the possible problem might be the acidity of the carbon atom on α position to the ester functionality. However, it could be achieved by the use of a strong base like MeONa. It has been reported that the use of *N*-methyl nitron provides a good yield of the desired intermediate, however, a complex mixture of diastereoisomers that are difficult to isolate. The issue of diastereoselectivity was resolved by the use of *N*-*tert*-butyl nitron **142**, as a result of which 1,3-dipolar cycloaddition took place selectively. *N*-*tert*-butyl nitron not only gave a good yield of the isoxazolidine intermediate (>85 %), more importantly, the two isomers were found to have markedly different R_f values and were easy to separate by flash column chromatography. The ratio of the two isomers under optimal conditions (dioxane, 30 °C, reactant concentration 0.05 M) was 10:1 and the purification was performed by silica gel column chromatography. Such an advantageous nitron **142** was synthesized by the treatment of *N*-*tert*-Butyl hydroxylamine hydrochloride **158** with Ethyl glyoxylate **159** in the presence of a mild base like NaHCO_3 as shown in scheme 7.6.

Scheme 7.6: Synthesis of Nitron **142**.

Nitron **142**, containing a bulky group, i.e. *tert*-butyl, was used for 1,3-dipolar cycloaddition to the vinyl group as described in scheme 7.7. The reaction between nitron **142** and the vinyl moieties was carried out in the presence of anhyd. dioxane at a bit higher temperature (50 °C). The nitron was taken in excess (1.5 equiv. of the vinyl moiety) and the reaction was stirred for long time (14 days), until all of the reactants were consumed, monitored by TLC. Upon completion of the reaction, the solvent was evaporated and the crude mixture was purified by flash chromatography using DCM and MeOH (7:1). The reaction afforded a good yield of the products in the range of 85-90%. After purification, the product was confirmed by different analytical techniques, including ^1H -NMR, ^{13}C -NMR, ESI-MS and optical rotation.

Scheme 7.7: Synthesis of Isoxazolidine derivatives **118** and **118a**.

7.1.5.1 Discussion on the NMR spectra of isoxazolidines **118**, **119**, **120** and **121**

The ^1H -NMR spectra of the compounds **118**, **119**, **120** and **121** show the disappearance of the olefinic signals in the range of $\delta = 5.1$ -6.5 ppm, present in the educts **122**, **123**, **124** and **125**. As shown in fig. 7.3, the presence of an intense singlet in the range of $\delta = 1.0$ -1.2 ppm, possessing 9 hydrogen atoms indicates the *tert*-butyl group came from nitron and confirms the presence of nitron added to the olefinic double bond. Furthermore, it is also confirmed by the presence of a triplet at $\delta = 1.29$ ppm containing three hydrogen atoms along with a multiplet at $\delta = 4.21$ ppm containing two hydrogen atoms of CH_2CH_3 of the ester functionality of the nitron. The target

compounds were further confirmed by ^{13}C -NMR studies showing a signal in the range of $\delta = 175.0\text{--}175.2$ ppm of the carbonyl group from the ester linkage. As shown in fig. 7.4, an intense signal in the range of $\delta = 20.5\text{--}25.3$ ppm of three identical carbon atoms indicates three CH_3 groups of the *tert*-butyl group from nitrone, which is further confirmed by the presence of a signal in the range of $\delta = 61.2\text{--}63.8$ ppm assigned as secondary carbon further supported by ^{13}C -DEPT spectra.

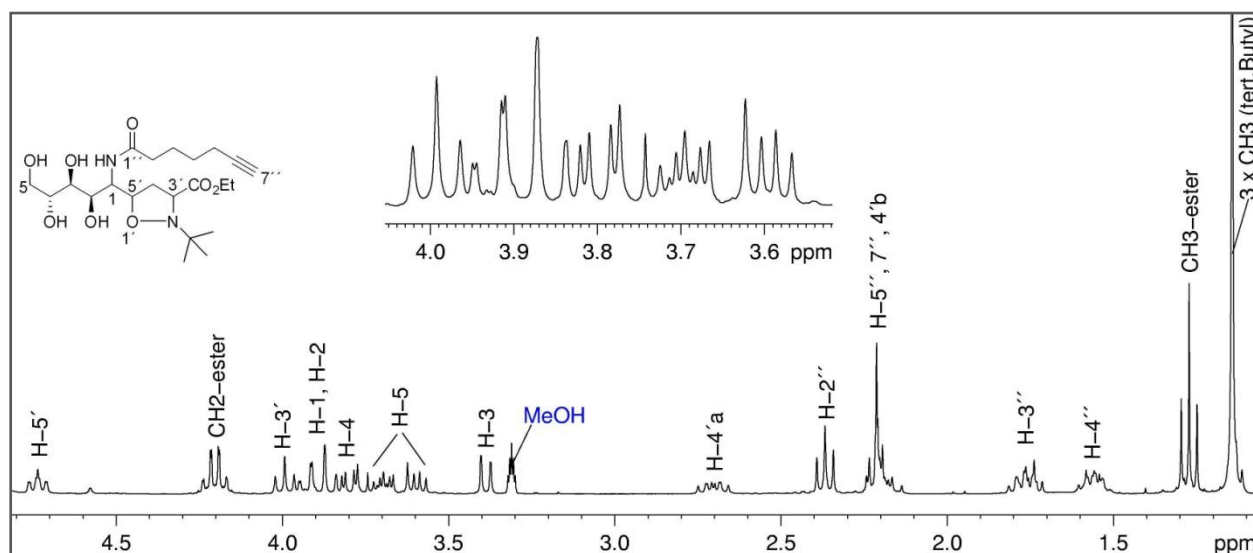


Figure 7.3: ^1H -NMR spectrum for isoxazolidine intermediate **121** (MeOD, 300 MHz).

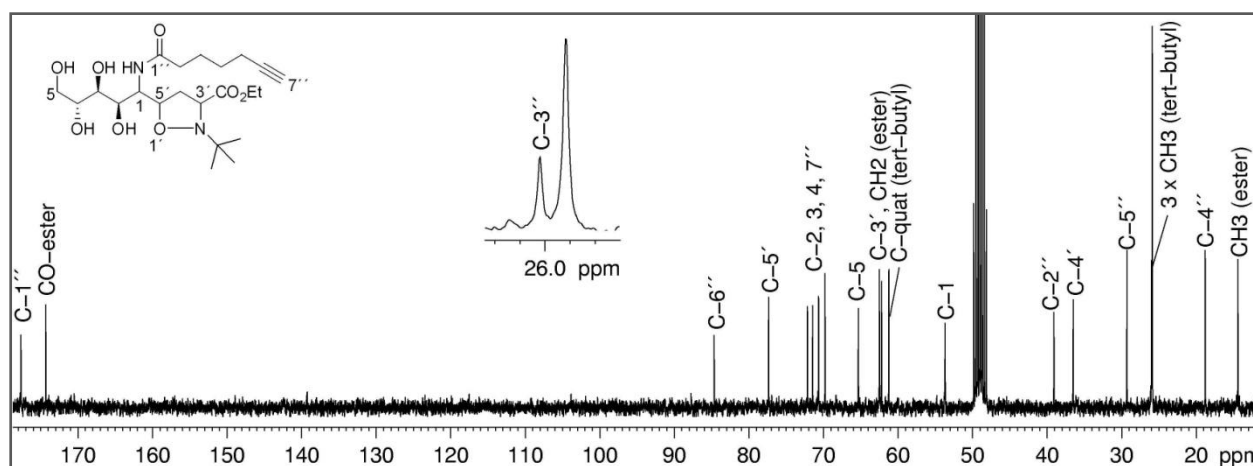
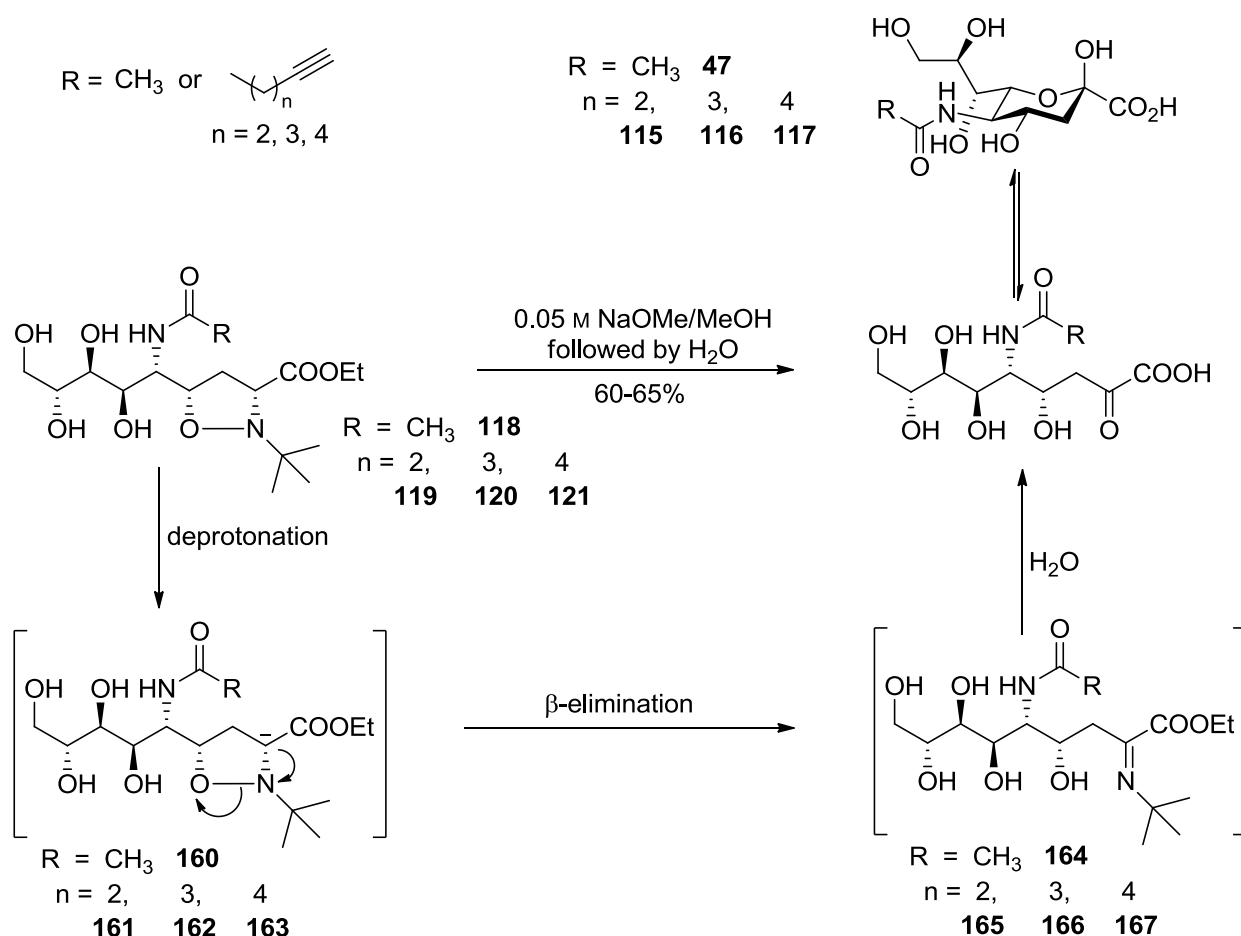


Figure 7.4: ^{13}C -NMR spectrum of isoxazolidine intermediate **121** (MeOD, 75 MHz).

All the remaining signals are in the same range indicated by the appropriate reactant olefins. The presence of the desired compounds was also confirmed by mass spectrometry (ESI-MS), which showed the masses of the intermediates in the same range as calculated theoretically.

7.1.6 β -Elimination towards sialic acid derivatives **47**, **115**, **116** and **117**

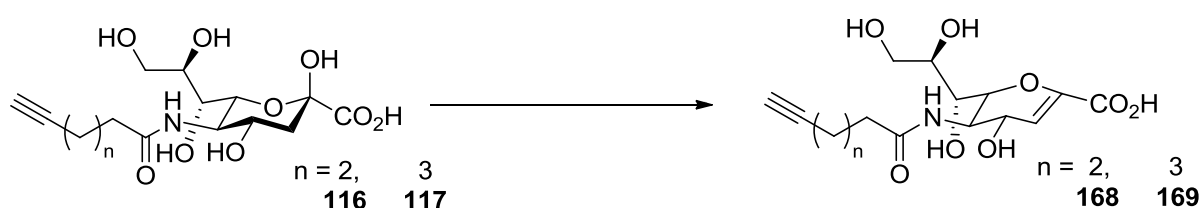
The isoxazolidines **118**, **119**, **120** and **121** were subjected to β -elimination by using 0.05 M MeONa in anhyd. MeOH as shown in scheme 7.8. After overnight stirring, the intermediate was hydrolyzed by the addition of water, stirred for further 24 h, and neutralized on an ion exchange resin. For the purification of the product, we tried different resins, the first of which was the Dowex-50. Initially, the acetate ions of the resin were exchanged with formate-ions by washing with 0.01 N HCOOH and then with MeOH to remove the excess of the acid. However, the product was decomposed during elution from resin column, approved by different analyses.



Scheme 7.8: Schematic representation of the β -elimination to afford sialic acid derivatives.

Then, we decided to use Amberlyst-15 just for the neutralization of the base moiety and purification with silica gel column chromatography. However, the elution of the Sia derivatives was not possible, even the use of different combinations of the eluting solvent could not solve the problem. We realized that the Sia derivatives were decomposed under even slightly acidic conditions and the same was true during column chromatography due to the slightly acidic nature of silica gel. Finally, we decided to make use of size exclusion chromatography by using

Biogel-2. It seemed difficult to achieve the purification with this technique as size exclusion chromatography is based on the difference of molecular size. However, there was a little difference in the masses of the target compounds and the impurities. In spite of little mass difference, the size exclusion chromatography worked excellently for the purification of Sia derivatives. In addition to the main reaction, we were also able to get the side products, which were assigned as the olefinic moieties by different analytical techniques. These were assumed to develop as a result of the acid prompted dehydration of the Sia derivatives ultimately affording a double bond between C-2 and C-3 in **168** and **169** as shown in scheme 7.9. This elimination was proposed to take place during neutralization of the basic media with acidic resin.



Scheme 7.9: Synthesis of olefinic Sia derivatives by dehydration.

7.1.6.1 Discussion on the NMR spectra of Sia derivatives **47**, **115**, **116** and **117**

The ^1H -NMR analysis of compounds **47**, **115**, **116** and **117** shows the absence of a triplet in the range of $\delta = 4.0$ ppm assigned as hydrogen at C-3' of isoxazolidine (reactant). Further confirmation of the quaternary nature of C-2 in the product comes from ^{13}C -NMR studies showing a signal in the range of $\delta = 97.5$ -99.0 ppm and the absence of the mentioned signal in ^{13}C -DEPT. Furthermore, the absence of an intense singlet in the products in the range of $\delta = 1.0$ -1.2 ppm assigned as three methyl groups of *tert*-Butyl in ^1H -NMR spectra of the isoxazolidines **118**, **119**, **120** and **121** indicates its removal in the products as shown in fig. 7.5. The presence of two downfield signals in the range of $\delta = 170.5$ -175.9 ppm in ^{13}C -NMR shown in fig. 7.6, represents two CO carbon atoms at C-1 and C-1'. The disappearance of a quartet in the range of $\delta = 4.5$ ppm and a triplet in the range of $\delta = 1.3$ ppm shows the absence of the ester functionality of isoxazolidines, which is hydrolyzed to the carboxylic acid in Sia derivatives. This is further confirmed by ^{13}C -NMR spectrum showing the absence of two signals in the range of $\delta = 14.2$ ppm and $\delta = 62.5$ ppm. The shift of CO signal from the range of $\delta = 174.3$ ppm to $\delta = 178.5$ ppm, the characteristic of an acid, also confirms the successful hydrolysis of the ester.

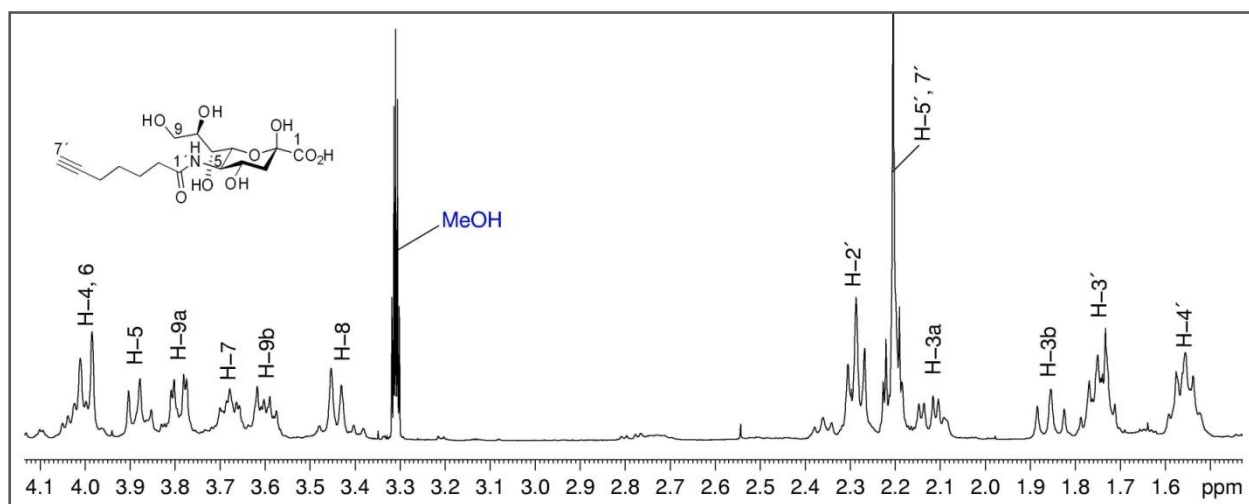


Figure 7.5: ^1H -NMR spectrum of Neu5Hept **117** (MeOD, 300 MHz).

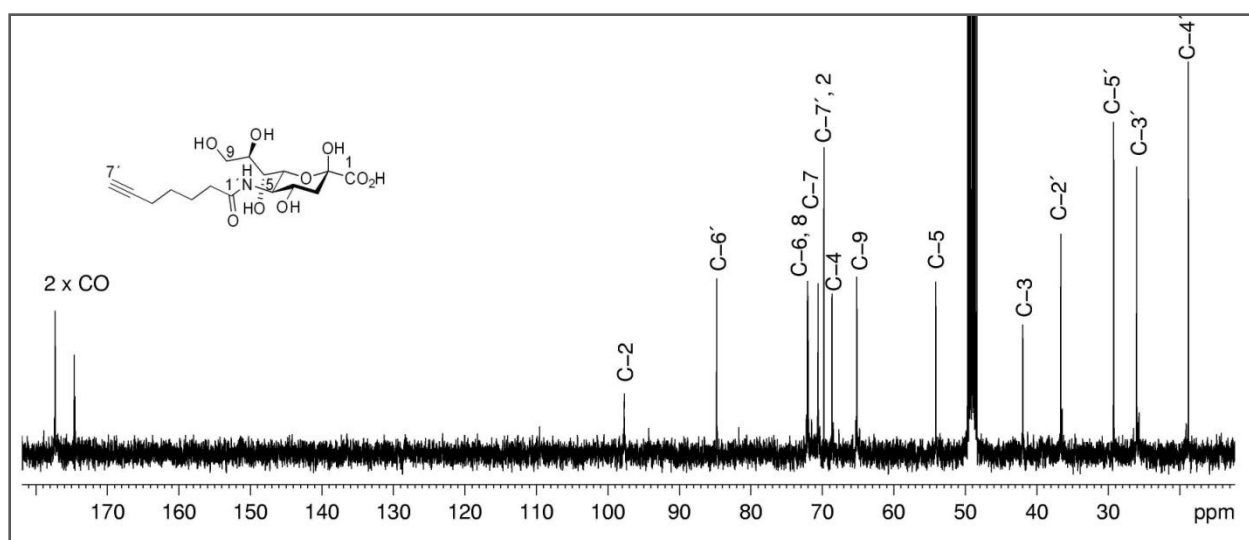


Figure 7.6: ^{13}C -NMR spectrum of Neu5Hept **117** (MeOD, 75 MHz).

The presence of the olefinic Sia derivatives **168** and **169** was confirmed by spectral data from NMR shown in fig. 7.7 and 7.8, further supported by mass analyses. Two new signals in ^{13}C -NMR spectra in the range of $\delta = 132.9$ ppm and $\delta = 110.2$ ppm indicate the presence of an olefinic moiety. Furthermore, the disappearance of a signal in the range of $\delta = 132.5$ ppm in ^{13}C -DEPT spectra confirms the quaternary nature of the carbon C-2 and illustrates the presence of the mentioned structure for Sia derivatives with a double bond. Mass analysis by ESI-MS of the olefinic Sia derivatives also confirmed the presence of mentioned structure for the compounds **168** and **169**.

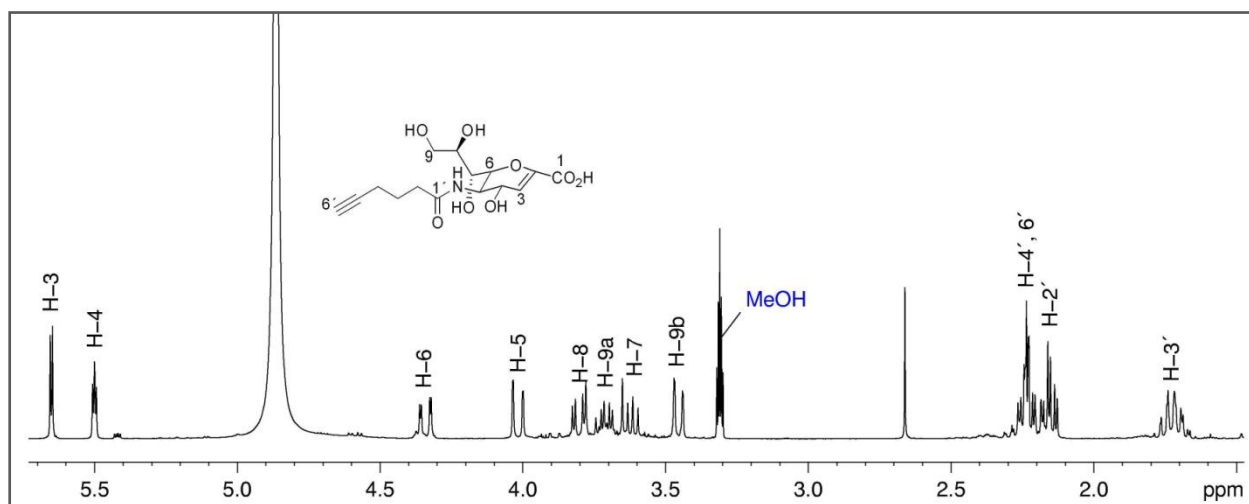


Figure 7.7: ^1H -NMR spectrum of the olefinic derivative **169** of sialic acid (MeOD, 300 MHz).

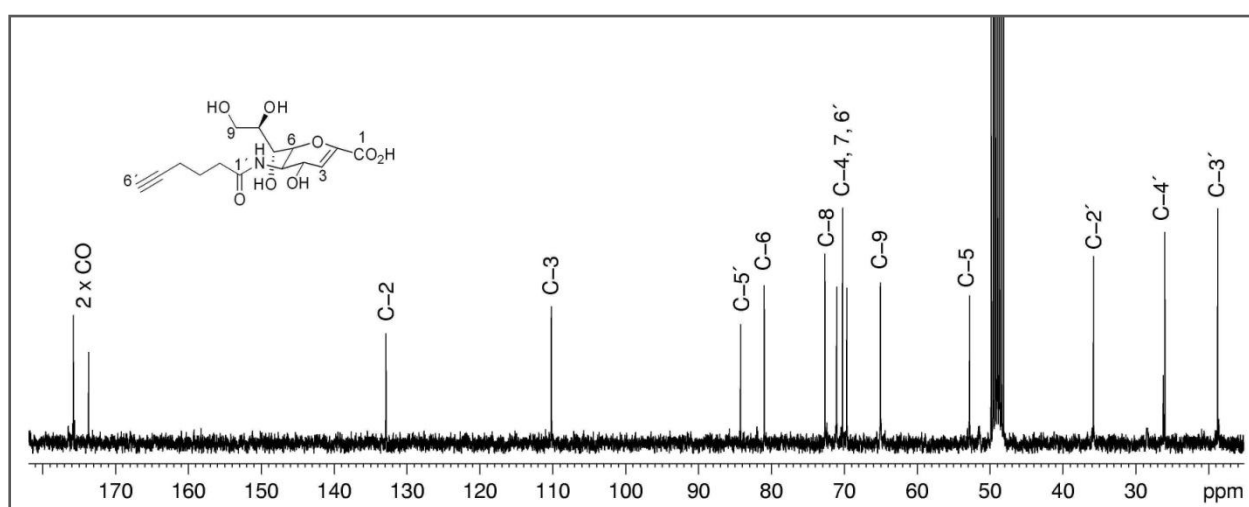


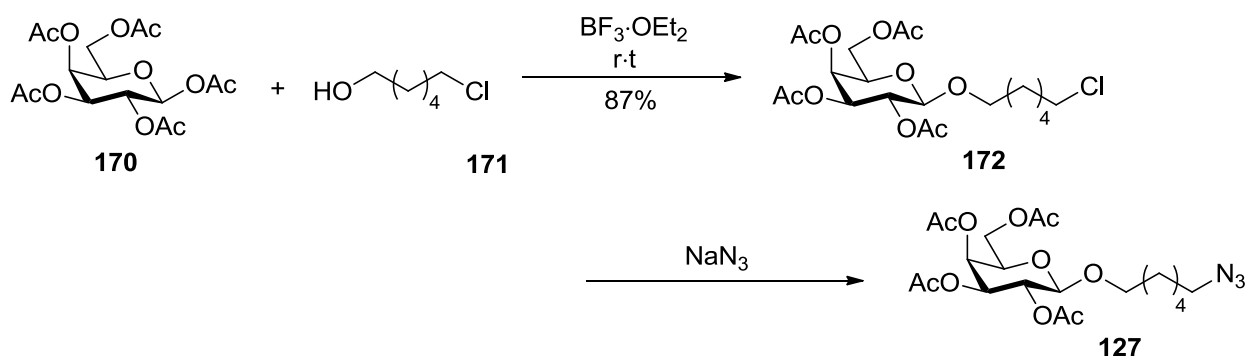
Figure 7.8: ^{13}C -NMR spectrum of the olefinic derivative **169** of sialic acid (MeOD, 75 MHz).

7.2 Synthesis of Azides

In our strategy we were interested in incorporating the synthesized sialic acid derivatives with their alkyne functionality into glycoproteins on human cells by metabolic glycoengineering. A successful incorporation should be demonstrated by a click reaction between the alkynes and their azide partners, most likely with a fluorescent tag. To extend our studies toward cells, we needed the azide reaction partner, to evaluate the reaction conditions. Keeping in mind the importance of azides for the click reaction, we synthesized a number of azide derivatives of different molecules like sugars and fluorescent dyes.

7.2.1 Synthesis of peracetylated galactose azide (**127**)

To envisage the optimization of click reaction, the first molecule synthesized was 6-azidoethyl 2,3,4,6-tetra-*O*-acetyl- β -galactose **127**. The target azide was synthesized in two steps as shown in scheme 7.10. The first step was the treatment of peracetylated galactose **170** with 6-chloro-1-hexanol **171** in the presence of boron trifluoride diethyl ether ($\text{BF}_3 \cdot \text{OEt}_2$) as a catalyst, which afforded the chloride **172** in 87% yield.



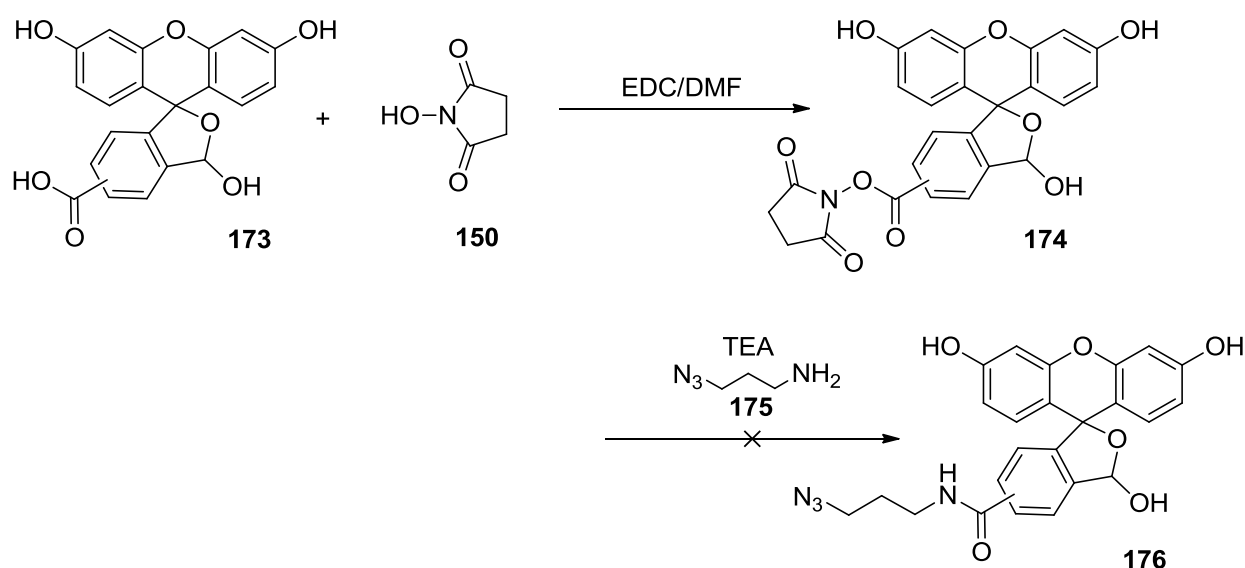
Scheme 7.10: Synthesis of 6-Azido hexyl-2,3,4,6-tetraacetyl β -galactopyranoside **127**.

The substitution on galactose ring was performed at 0 °C under highly dry conditions in anhyd. DCM because of the high sensitivity of the reaction towards moisture. To take further care, molecular sieves were used and all the additions were made under nitrogen atmosphere. The reaction was controlled by TLC and stirred until the reactants disappeared. As acetic acid is the side product of the reaction, NEt_3 was used for the neutralization of the reaction and deprotonation of the alcohol. Here, the retention of configuration was achieved at C-1 due to the anomeric effect of the neighboring acetyl group, which stabilizes the intermediate through a five membered ring and directs the nucleophile to attack from the same side; the leaving group has departed from. To achieve the synthesis of azide **127**, chloride **172** was treated with sodium azide in DMF at 70 °C for 4 h. After the complete conversion of reactants into the product, the solvent was evaporated *in vacuo* supported by the azeotropic mixture with toluene. The residue was dissolved in DCM, washed with brine and finally purified by flash chromatography to afford the azide **127** (97 %) as a thick colorless oil.

7.2.2 Synthesis of fluorescein azides **130** and **179**

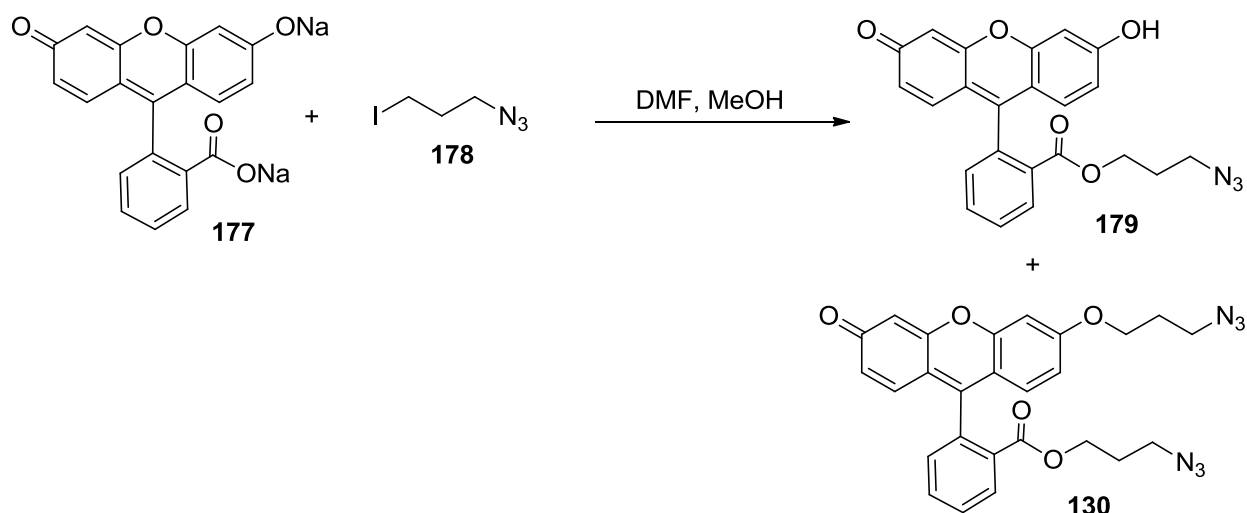
In pursuit of our ultimate goal, the next step was the synthesis of fluorescent azides as the counter partners of Sia alkynes for cell imaging by fluorescence microscopy. Fluorescent tags enable the analysis of both structural and functional information relating to cells and tissues and even more

complex system. Fluorescein exists in two tautomeric forms, the orange fully conjugated and colorless lactone, which is favored under acidic conditions.^[183] The excitation of fluorescein takes place at $\lambda = 494$ nm and the emission at $\lambda = 521$ nm. Different methods have been reported for bioorthogonal labeling via click reaction, affinity based protein profiling and cytosolic delivery of the probes by using fluorescence microscopy.^[14, 17, 75] In the first attempt, the synthesis of the desired azide was planned from fluorescein carboxylic acid **173** by esterification.^[184-186] As described in scheme 7.11, the target compound was attempted to achieve by activation of the acid **173** with *N*-hydroxysuccinimide (NHS) **150** in anhyd. DMF. The reaction was carried out in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a catalyst. However, neither NMR spectroscopy nor mass spectrometry supported the presence of the desired azide **176**.



Scheme 7.11: Synthesis of the azide derivatives of fluorescein acid **176**.

The approach was modified and instead of the fluorescein acid **173**, we decided to make the use of fluorescein. However, when the reaction of fluorescein was conducted with amino azide **175** directly in the presence of EDC and DMAP no reaction was observed. We assumed that the carboxyl acid of fluorescein is not active enough to react with amine in the presence of EDC and planned to activate it with NHS **150**. The reaction was carried out in the presence of EDC and DMF, however, the activation of the ester did not take place. Finally, we made use of the sodium salt of fluorescein **177** and an alkyl halide substituted azide.^[187] The reaction between disodium salt of fluorescein **177** and iodopropyl azide **178** (10 equiv.) in the presence of DMF and MeOH gave excellent results as illustrated in scheme 7.12.



Scheme 7.12: Synthesis of the azide derivatives of fluorescein sodium salt.

The product obtained after flash chromatography afforded two fractions. A fraction with $R_f = 0.34$ was double substituted fluorescein azide **130** in 65% yield and the second with $R_f = 0.31$ was mono substituted fluorescein azide **179** in 23% yield.

7.2.2.1 Discussion on the NMR spectra of fluorescein azide (**130**)

The ^1H -NMR spectrum of the product **130** as shown in fig. 7.9, displays a triplet at $\delta = 4.24$ ppm ($J = 5.9$ Hz) and a doublet of triplet (dt) at $\delta = 4.02$ ppm ($J = 5.9, 1.1$ Hz) showing the double substitution of propyl azide on fluorescein through an ester and an ether linkage respectively (3-H_2 and $1'''\text{-H}_2$). Two triplets at $\delta = 3.52$ ppm ($J = 6.5$ Hz) and $\delta = 3.03$ ppm ($J = 6.7$ Hz) each possessing two hydrogen atoms, indicate the presence of two azide groups (1-H_2 and $3'''\text{-H}_2$). The presence of two quintets at $\delta = 2.08$ ppm ($J = 6.3$ Hz) and $\delta = 1.52$ ppm ($J = 6.5$ Hz) are signature for H-2 and H-2'''. The remaining resonances are in the range of $\delta = 6.48\text{--}8.29$ ppm and show the presence of 10 aromatic hydrogen atoms of the fluorescein. All this information supports the double conjugation of fluorescein with propyl azide, which was further confirmed by ^{13}C -NMR spectrum shown in fig. 7.10. A downfield signal at $\delta = 187.21$ ppm assigned as carbonyl carbon (C-3'') of the fluorescein ring shows the open carboxylic form of fluorescein, which is more stable than the closed Spiro-Lactone. Another signal at $\delta = 166.69$ ppm indicates the presence of the ester carbonyl, with which propyl azide **130** is conjugated through the ester linkage. It is further supported by a signal at $\delta = 63.72$ ppm represents $-\text{CH}_2-$ attached to the fluorescein through ester bonding and assigned as C-3. The presence of the ether linkage, through which the second propyl azide is attached to the fluorescein molecule is supported by a signal at $\delta = 67.31$ ppm. Two signals at $\delta = 48.68$ ppm and $\delta = 48.65$ ppm are hidden behind the septet of methanol, but can be seen clearly in ^{13}C -DEPT spectrum, and reflect the presence of two carbon

atoms with azide groups. Furthermore, the presence of two $-\text{CH}_2-$ carbon atoms is supported by two signals at $\delta = 28.83$ ppm and $\delta = 29.51$ ppm. The rest of the signals come from the aromatic region of the fluorescein, in the range of $\delta = 110.0$ -168.5 ppm. All the spectral data of compound **130** shows the double substitution of the fluorescein, which is further supported by mass analysis (ESI-MS) showing m/z for $[\text{C}_{26}\text{H}_{22}\text{N}_6\text{O}_5 + \text{Na}]^+$ in the range of 521.21, in agreement with the theoretically calculated value 521.55.

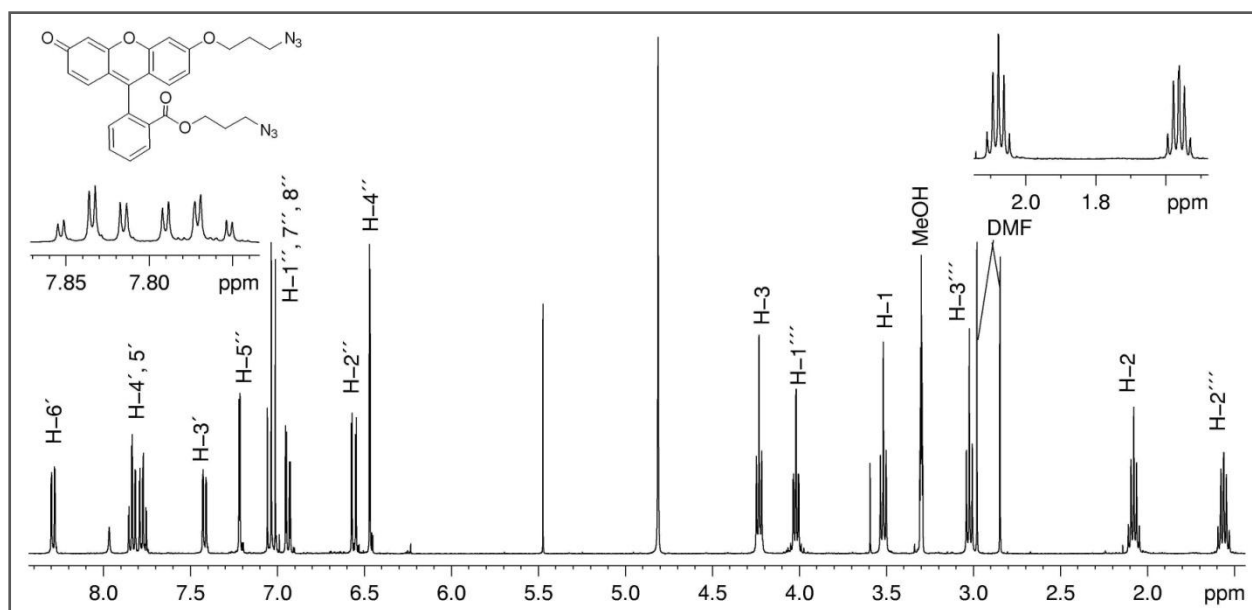


Figure 7.9: ^1H -NMR spectrum of doubly substituted fluorescein azide **130** (MeOD, 300 MHz).

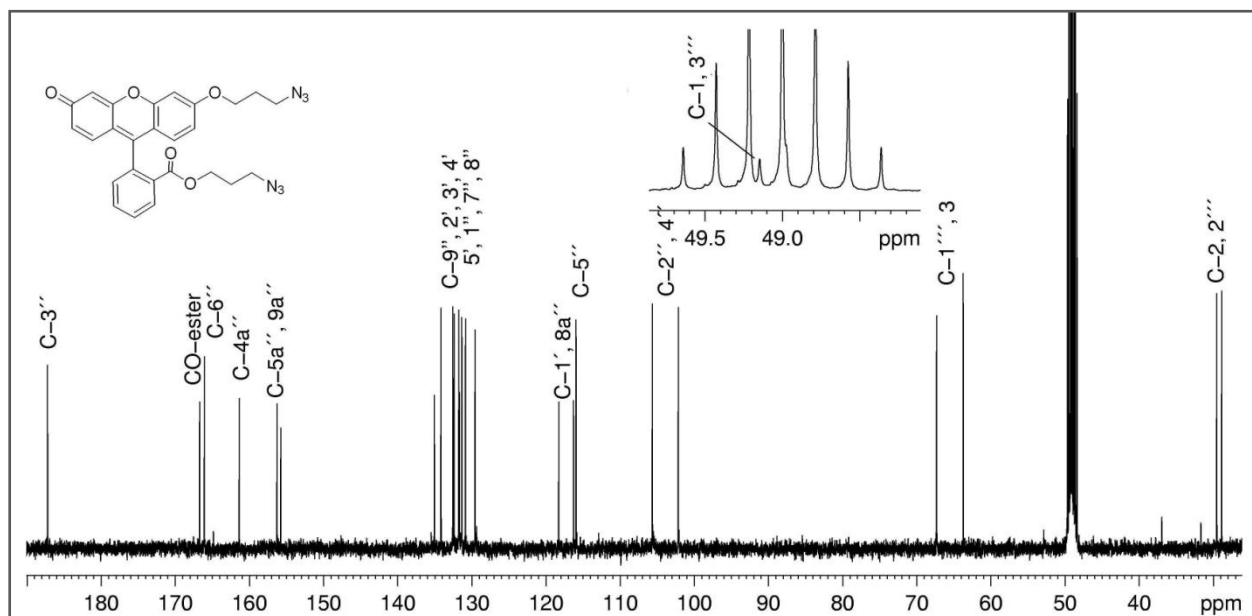
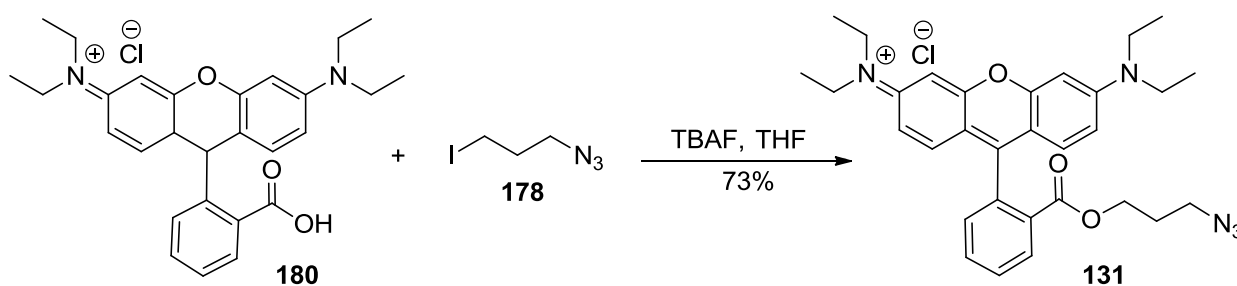


Figure 7.10: ^{13}C -NMR spectrum of doubly substituted fluorescein azide **130** (MeOD, 75 MHz).

7.2.3 Synthesis of rhodamine azide **131**

In our strategy for cell study, we were interested in developing a second fluorescent molecule to improve the versatility of our approach. For this reason, we selected rhodamine dye, which belongs to related chemical compounds called fluorone dyes, comprising rhodamine-B and rhodamine-6G as important members. Rhodamine-B **180** is an amphoteric dye, although it is usually considered basic in nature due to the overall partial positive charge. It has molar extinction coefficient of 1.06×10^5 at $\lambda = 542.7$ nm when dissolved in EtOH.^[188] The synthesis of rhodamine azide **131** was achieved by treatment of rhodamine-B **180** with azide **178** in the presence of tetrabutylammonium fluoride (TBAF) as a catalyst as shown in scheme 7.13. TBAF is a good phase transfer catalyst, but here it acts as a mild base to deprotonate the carboxylic acid. The reaction was carried out in THF and controlled by TLC. After the complete conversion of the reactants into the product, the solvent was evaporated and the crude product was subjected to flash chromatography, which afforded the desired azide **131** in good yield (73%).



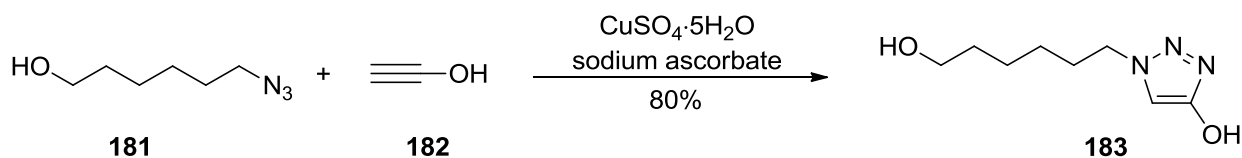
Scheme 7.13: Synthetic scheme for Rhodamine azide **131**.

7.3 Evaluation of Click Reaction Conditions

In order to chase our goal for studying the cell surface through click reaction, the first step was the investigation of the reaction conditions. Initially, we investigated the reaction conditions on simple azide and alkyne molecules, followed by its extension to complicated systems, i.e. fluorescent dyes and Sia derivatives.

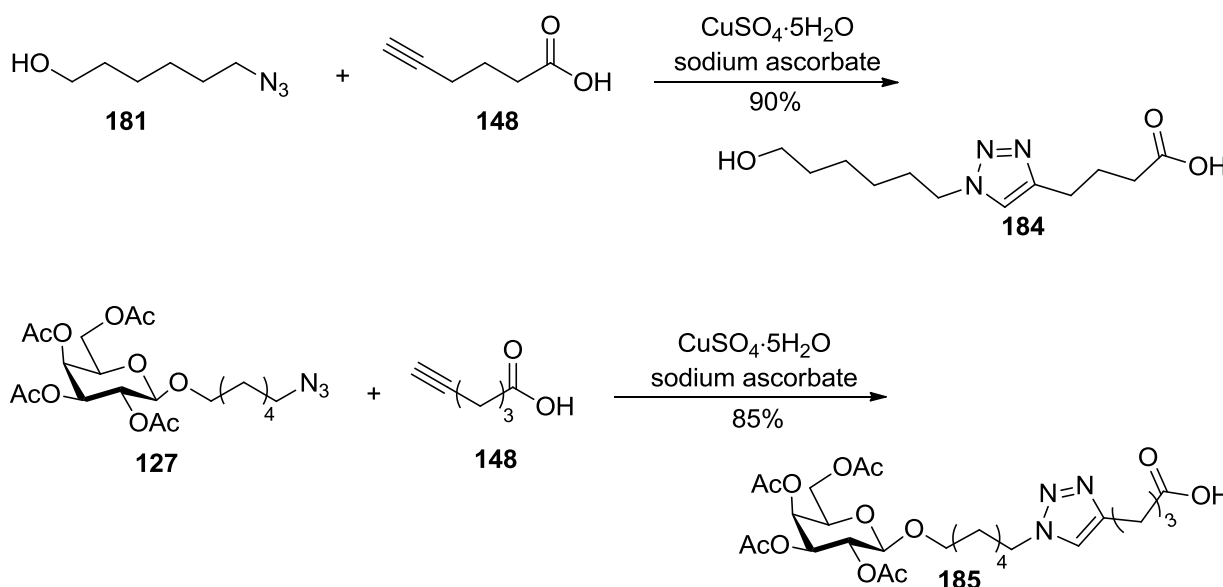
7.3.1 Synthesis of triazoles of sugar derivatives

To check the mode of the reaction, propargyl alcohol was treated with 6-azido-1-hexanol, using $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ as a source of Cu(I) and sodium ascorbate in 1:1 mixture of *tert*-BuOH and water as shown in scheme 7.14. The reaction proceeded smoothly and was recovered in very good yield, after isolation by flash chromatography.



Scheme 7.14: Copper catalyzed synthesis of triazole **183**.

After the successful synthesis of triazole **183**, the reaction was further extended to the conjugation of 5-hexynoic acid **148** with galactose azide **127** and 6-azido hexanol **181**, and afforded good yields of the triazole **184** and **185** (85-90%) as illustrated in scheme 7.15. The NMR studies as well as mass analysis by ESI-MS of the compounds showed the successful synthesis of triazoles **184** and **185**.



Scheme 7.15: Synthesis of different triazoles **184** and **185** through Cu catalyzed click reaction.

7.3.1.1 Discussion on the NMR spectra of the triazole **185**

The presence of the triazole **185** was confirmed by NMR analysis, which showed a singlet at $\delta = 7.78$ ppm in ^1H -NMR spectrum shown in fig. 7.11, indicating the presence of H-5' in triazole ring. Furthermore, a doublet of doublet at $\delta = 5.39$ ppm ($J = 3.2, 0.9$ Hz) indicates the presence of 5'''-H of the galactose ring. A doublet at $\delta = 4.63$ ppm ($J = 7.4$ Hz) represents 1_β '''-H, which shows the retention of configuration at C-2''' due to the anomeric effect of the neighbouring acetyl group during the substitution of side chain on the ring. Furthermore, two triplets at $\delta = 2.77$ ppm ($J = 7.5$ Hz) and $\delta = 2.36$ ppm ($J = 7.4$ Hz) indicate the presence of a $-\text{CH}_2-$ adjacent to the triazole double bond and a $-\text{CH}_2$ next to the carboxyl carbon of acid respectively. The rest of the signals also confirm the presence of the aliphatic chain of galactose azide **127**. The proof for the presence of four methyl groups was supported by four singlets in the range of

$\delta = 1.9\text{--}2.2$ ppm, each of which holds three hydrogen atoms. The ^{13}C -NMR spectrum displayed in fig. 7.12 shows five signals in the range of $\delta = 171.3\text{--}176.8$ ppm indicating the presence of five carbonyl carbons, out of which a signal at $\delta = 176.88$ ppm assigned as carbonyl carbon of hexynoic acid confirms the presence of the acid.

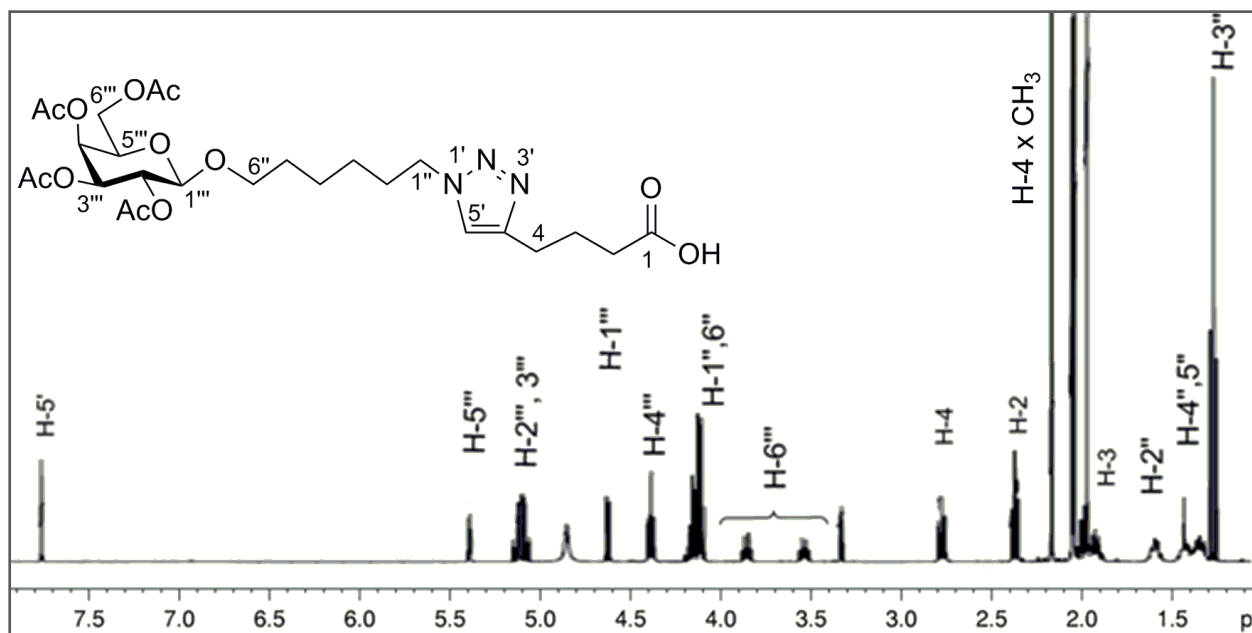


Figure 7.11: ^1H -NMR spectrum of galactose triazole **185** (MeOD, 300 MHz).

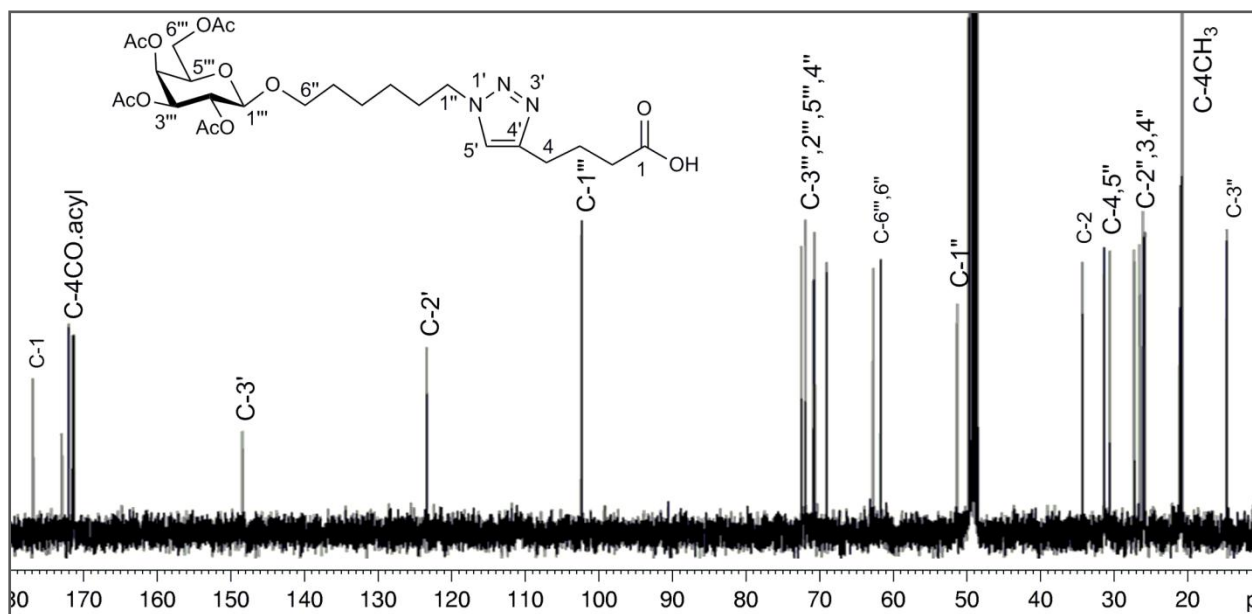


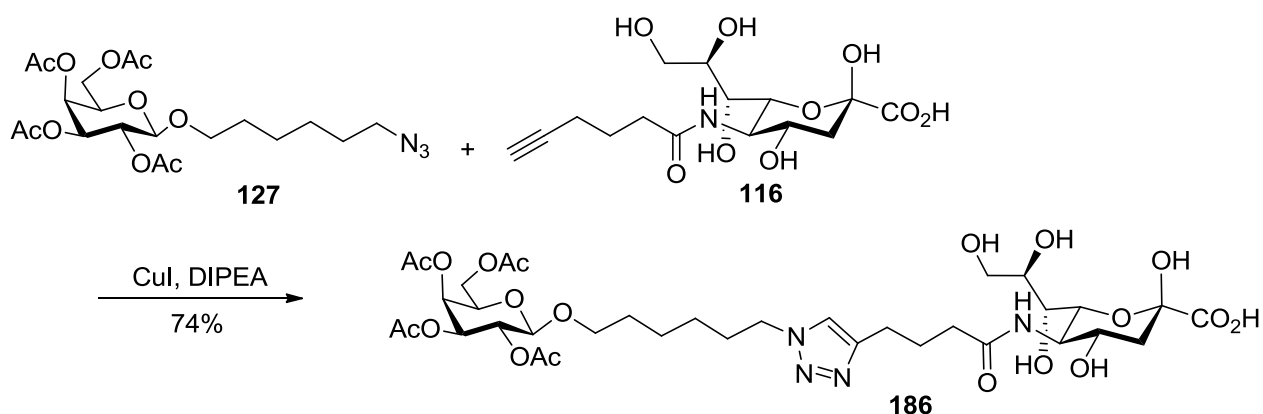
Figure 7.12: ^{13}C -NMR spectrum of the galactose triazole **185** (MeOD, 75 MHz).

Furthermore, two signals at $\delta = 148.37$ ppm and $\delta = 123.33$ ppm reflect the presence of C-4' and C-5' of the triazole ring, and the tertiary and quaternary nature of both carbons was confirmed by ^{13}C -DEPT spectrum. Another signal at $\delta = 102.25$ ppm assigned as C-1''' of the sugar ring confirms the β nature of it. The signals in the range of $\delta = 68.8\text{--}72.4$ ppm come from the carbons

of the sugar ring holding acetyl groups. Two signals at $\delta = 62.60$ ppm and $\delta = 63.72$ ppm reflect the presence of C-6'' of the aliphatic chain directly attached to the sugar molecule and C-6''' of galactose. Four signals in the range of $\delta = 20.5$ -20.9 ppm represent four methyl groups of acetylated galactose and confirm the complete protection of the sugar. The final evidence for the target molecule comes from mass analysis, which shows m/z for $[C_{25}H_{39}N_3O_{11}+Na]^+$ in the range of 608.23, in agreement with the calculated value 608.25.

7.3.2 Conjugation of the Sia with galactose azide

Successful synthesis of the triazoles **184** and **185** encouraged us to extend the approach for the conjugation of Sia derivatives with different azides. Initially, we selected Neu5Hex **116**, and subjected it to conjugation with galactose azide **127** under the Sharpless condition, i.e. by the use of $CuSO_4 \cdot 5H_2O$ and Na-ascorbate. After purification by flash chromatography, the product was analyzed by NMR, which gave some of the signals in the required range along with triazole. However, it was observe that some of the signal of sialic acid derivative were not in the appropriate position and reflected its decomposition. Moreover, the mass analysis of the product by ESI-MS gave a weak signal of the mass of the target triazole **186**. Further purification for two times could not improve the purity of the triazole. At this point we assumed that the Sia derivative was not tolerant of the basic Na-ascorbate. To check our hypothesis, we tried the reaction in the modified conditions under the presence of CuI and DIPEA as described in scheme 7.16.



Scheme 7.16: Synthesis of the triazole **186** catalyzed by CuI and DIPEA.

This time, the reaction proceeded excellently without any complications and the product **186** was purified by flash chromatography (75%) and its structure elucidated by NMR and mass analyses. The NMR analysis showed a singlet at $\delta = 7.82$ ppm in ¹H-NMR spectrum indicating the

presence of hydrogen from triazole ring. ^{13}C -NMR showed two signals at $\delta = 148.39$ ppm and $\delta = 123.51$ ppm assigned as triazole carbons C-4'' and C-5'' respectively, indicating the triazole functionality. All the rest of the signals satisfied the presence of both Neu5Hex and galactose azide moieties described above. The final proof for the target product came from mass spectrometry by ESI-MS, which showed m/z for $[\text{C}_{35}\text{H}_{54}\text{N}_4\text{O}_{19}-\text{H}]^-$ in the range of 833.09, and satisfied the calculated value 833.32 in the negative ion polarity mode. At the same time the mass was also confirmed by positive ion polarity, which showed m/z for $[\text{C}_{35}\text{H}_{54}\text{N}_4\text{O}_{19}+2\text{Na}]^+$ in the range of 880.12 in agreement with the calculated value 880.31.

7.3.3 Synthesis of fluorescein triazoles

The final step in our investigations for the optimization of the click reaction for bioorthogonal labeling was the conjugation of fluorescein azides with different alkynes especially the Sia derivatives. Keeping in mind the importance of fluorescein in bioorthogonal labeling, we coupled the fluorescein azides **130** and **179** with different molecules ranging from simple alkynes to glucose and Sia derivatives holding linkers with terminal alkyne functionality. In a first attempt, the fluorescein azide **179** was treated with 5-hexynoic acid **148** in the presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.001 equiv.) and sodium ascorbate (0.01 equiv.) and the synthesis of **187** was achieved successfully. The reaction was carried out in 4:1 mixture of *tert*-BuOH/ H_2O , and the mixture was stirred for 12 h, until the completion of the conversion, which was monitored by TLC.

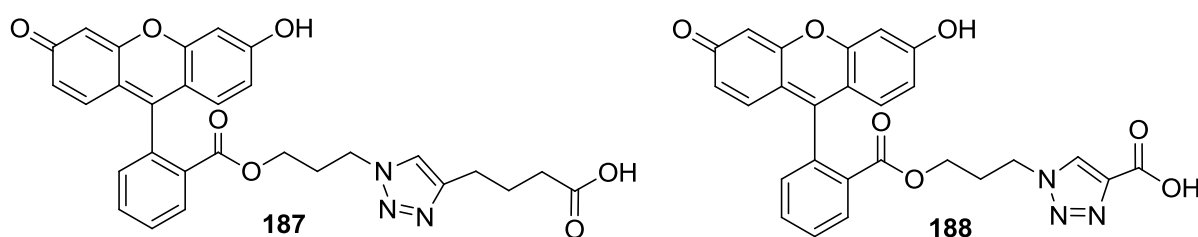


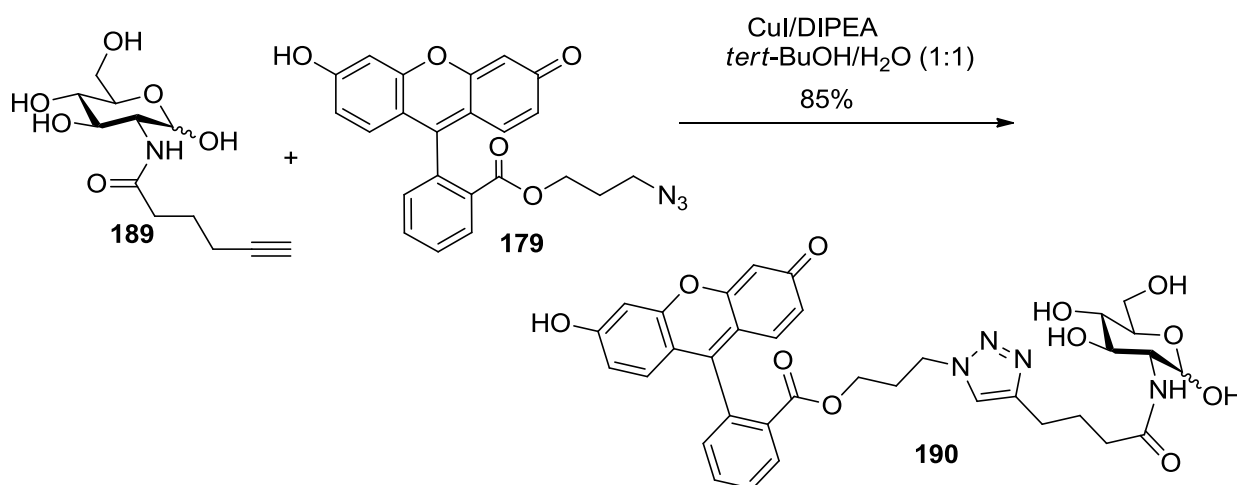
Figure 7.13: Fluorescein triazoles synthesized by Cu catalyzed click reaction.

Similarly, the synthesis of triazole **188** was achieved in 55% yield under the same conditions by using propargylic acid. Both triazoles i.e. **187** and **188** shown in fig. 7.13 were also synthesized in the presence of CuI (0.1 equiv.) and DIPEA (3.0 equiv.) in MeOH. It was observed that the reaction proceeded better as compare to the reaction with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and sodium ascorbate. After successful syntheses of the triazoles in the presence of both catalytic systems and appropriate solvents, we decided to change the solvent for the reaction catalyzed by CuI. To avoid the use of cytotoxic MeOH, the reaction was optimized using H_2O as solvent, but the reaction proceeded very slowly due to the lower solubility of the fluorescein azide in water.

However, the solubility problem was solved by the use of *tert*-BuOH/H₂O as solvent and afforded a good yield. The reaction was further investigated in the presence of water with 10% DMSO and that gave excellent yields. It was also observed that the triazole synthesis with propargylic acid proceeded better even with minute concentration (0.01 equiv.) of CuI as compare to the long chain alkynes. This behavior is due to the electron withdrawing effect of the carboxylic acid as the electron deficient alkynes undergo click reaction more efficiently than the normal terminal alkynes.

7.3.4 Conjugation of sugar derivatives with fluorescein

After successful conjugation of Sia with galactose azide **127** and the investigations on click reaction for fluorescein, attention was focused on the conjugation of Sia derivatives with fluorescein, so that the interaction of Sia can be examined in living organisms. In a first attempt, the reaction was carried out between 5-hexyne glucosamine **189** and fluorescein azide **179** in the presence of CuI and DIPEA, using *tert*-BuOH/H₂O (1:1) as solvent as illustrated in scheme 7.17. The reaction proceeded excellently and yielded triazole **190** (85%) as a dark orange solid.



Scheme 7.17: Synthesis of fluorescein triazole **190** with N-5-Hexyne Glc. amine derivative.

Successful application of the amended reaction conditions on the conjugation of fluorescein with glucose derivatives encouraged us for further proceedings, and the reaction was applied for the conjugation of fluorescein azide **130** and **179** with Neu6Hept **116** and olefinic Neu5Hex **168**. The conjugation was achieved successfully by using the reaction conditions mentioned above for the synthesis of the triazoles **191** and **192** in good yields shown in fig. 7.14.

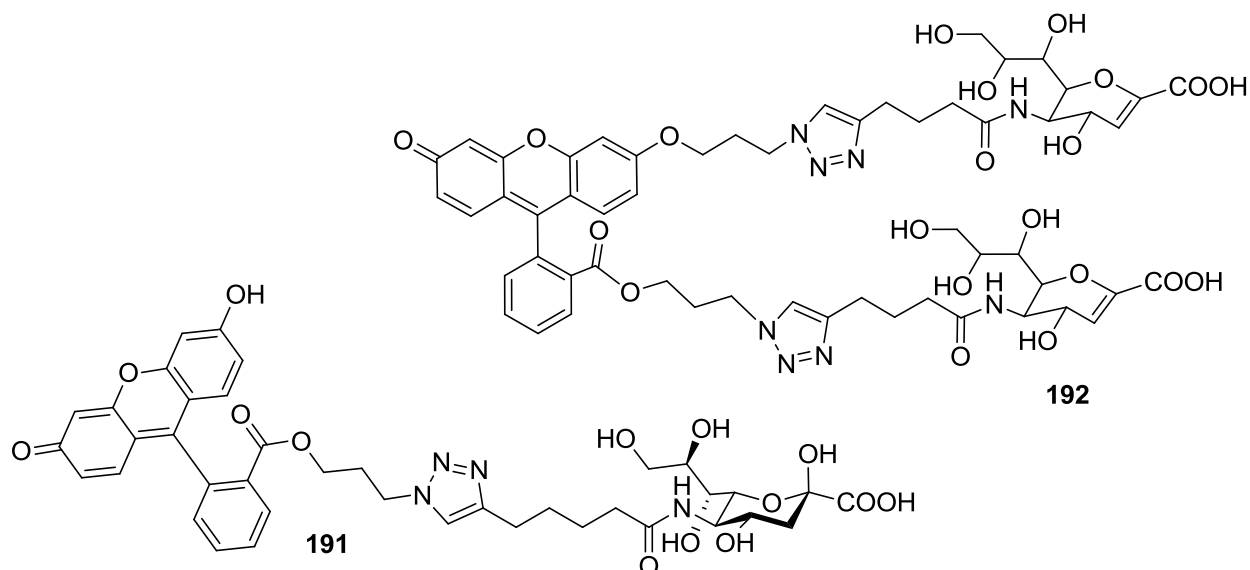


Figure 7.14: Fluorescein triazoles of Sia derivatives **191** and **192**.

7.4 Bioorthogonal Metabolic Labeling of HEp-2 and CHO-K1 Cells

The cell surface of eukaryotes is decorated by various types of glycans collectively called glycocalyx, out of which glycolipids and glycoproteins mediate the cell-cell recognition processes. The most important of these are the sialic acids due to their outermost position and their availability for different cellular activities with the environment. It has already been demonstrated that the acetylated sugars enter the cell by an active transport and a diffusion process depending upon the nature of the sugar. On the other hand, Sia derivatives with functional groups (azide/alkyne) enter the cell through a special process called pinocytosis and are incorporated into the cellular glycosylation machinery by an active transport system^[189] as described in fig. 7.15.

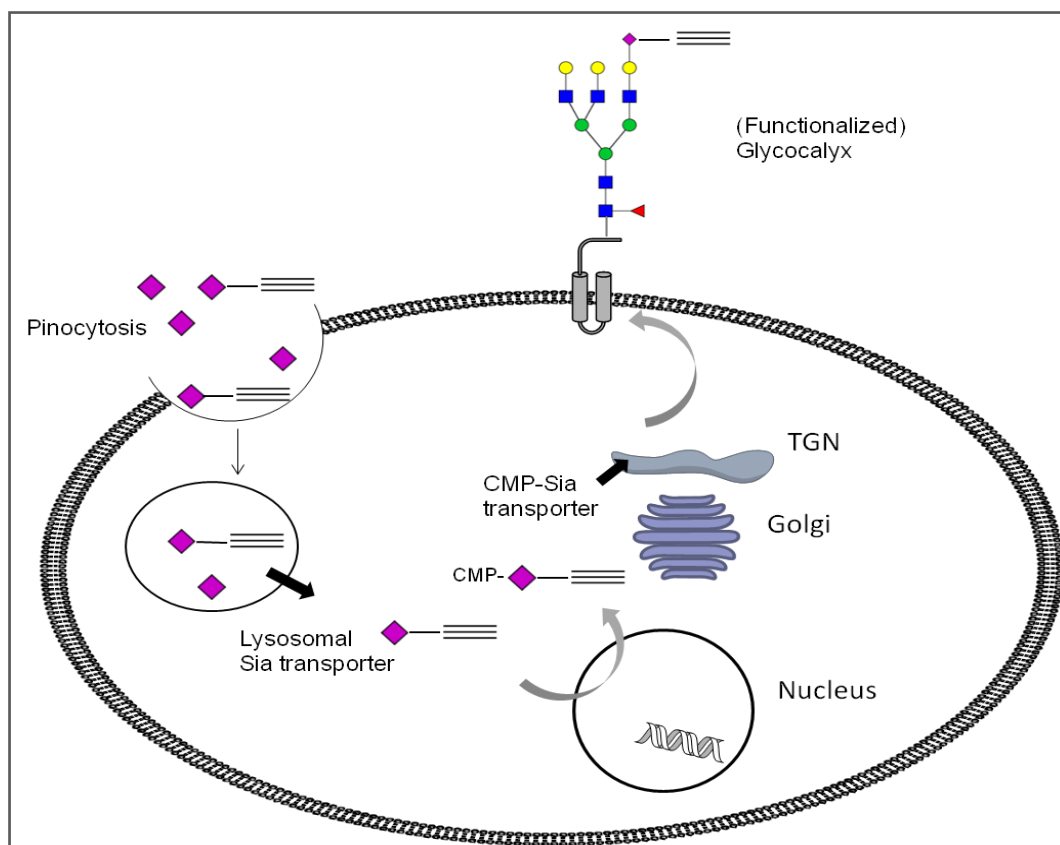


Figure 7.15: Proposed metabolic pathway of Neu5Hex based on unknown mechanism of Neu5Gc uptake.^[189] TNG: trans golgi network, CMP: Cytidine monophosphate, Sia sialic acid. (Adapted from Dr. Homann's PhD thesis)

The acetylated monosaccharides are processed by deacetylases, activated by nucleotide transferases and metabolized by isomerases and epimerases, and are ultimately incorporated into the natural carbohydrate pattern of the cell. Out of these, some modified sugars become part of the cell surface glycoconjugates and are accessible for chemical modification without any disturbance to the cell as shown in fig. 7.16. In order to achieve the cell surface glycoconjugation and ultimately their imaging by fluorescence microscopy, we selected the cell lines of human larynx carcinoma (HEp-2) and Chinese hamster ovary (CHO-K1). Metabolic labeling of the cell lines was performed by our colleague (Dr. Arne Homann) in order to study and characterize them.^[89] The Sia derivatives containing terminal alkyne functionality were introduced to the cell culture where they entered into the cells by endocytosis process and were incorporated into the posttranslational glycan patterns of proteins. After 24 h, cells were treated with fluorescein azide in the presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Na-ascorbate along with the addition of Cu(I) stabilizing ligand TBTA and further incubated for 1 h. The negative control of the cell labeling was also performed without the treatment of the cells with Sia derivatives.

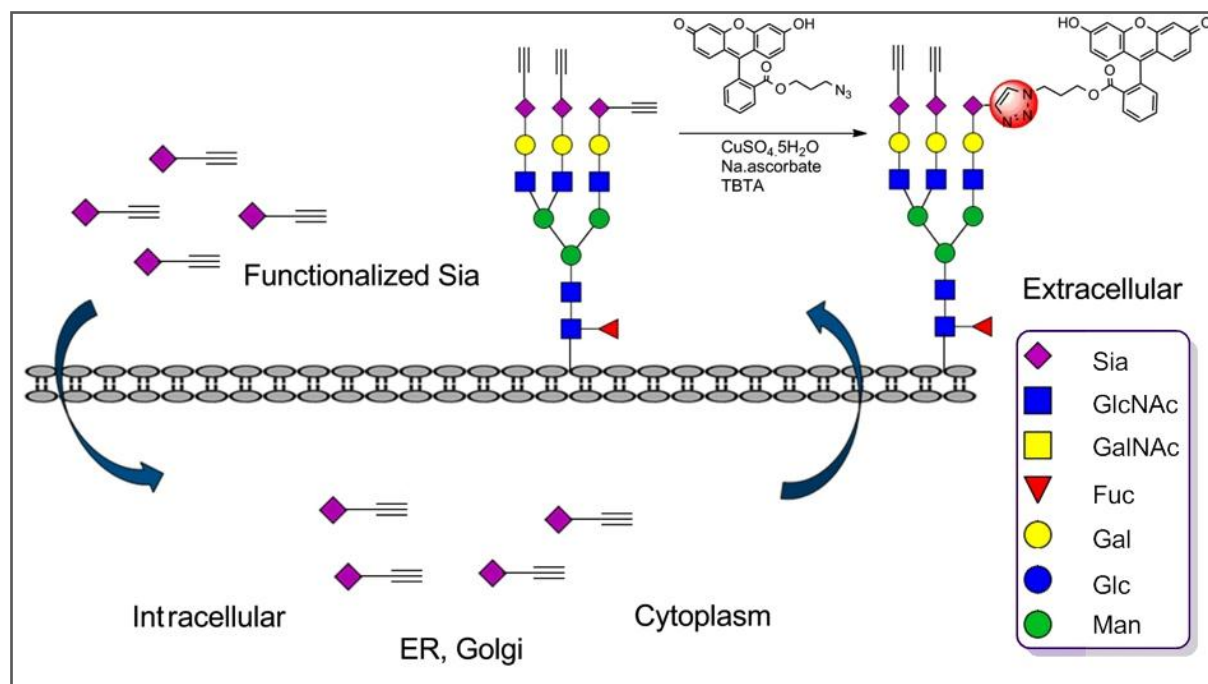


Figure 7.16: Schematic representation for two step bioorthogonal labeling of the cells.

After 1 h of incubation under click reaction conditions as mentioned above, the cells were washed and subjected to imaging by fluorescence microscopy at $\lambda_{\text{max}} = 525$ nm for fluorescein. In addition to the Neu5Hex **116**, GlcNAz **129** was also incorporated into the HEP-2 cells via metabolic labeling and the staining was performed by alkynylated TAMRA (Tetramethylrhodamine) and both of the results are shown in fig. 7.17.

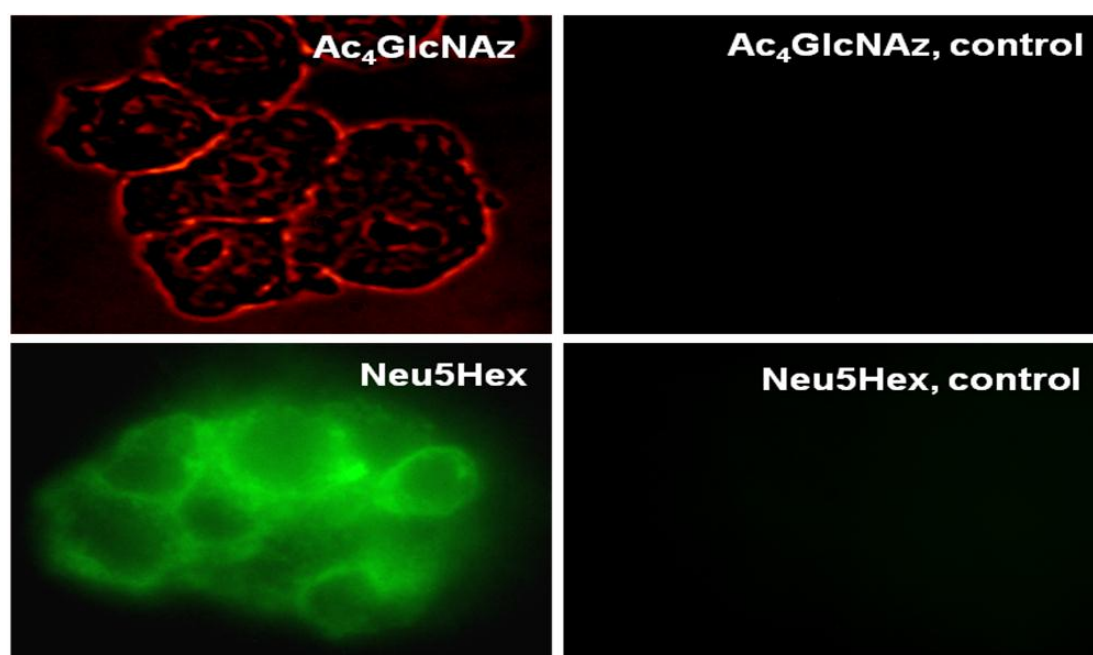


Figure 7.17: Top left: HEP-2 cells incorporated with Ac₄GlcNAz, labeled with alkynylated TAMRA at 580 nm. Bottom left: HEP-2 cells with incorporated Neu5Hex, labeled with fluorescein azide at 525 nm. On the right: background and unspecified staining controls at the same wavelength as corresponding pictures on left.

To check the versatility of the technique, the imaging of CHO-K1 cells was also performed by the injection of both GlcNAz and Neu5Hex into the cell culture and the labeling reaction was performed by alkynylated TAMRA and fluorescein azide respectively. However, a little background was observed in this case as shown in the fig. 7.18, which may be due to the overnight click reaction.

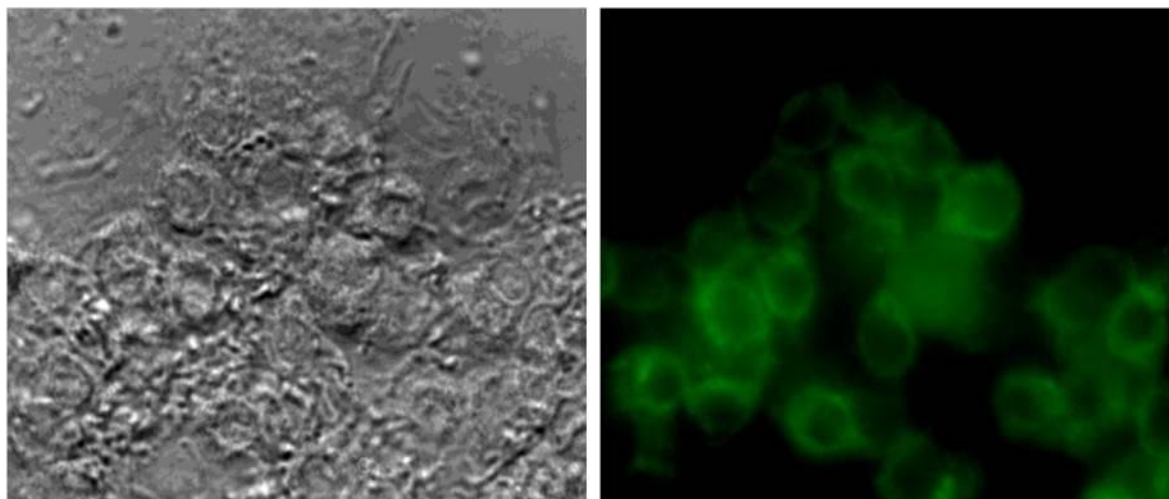


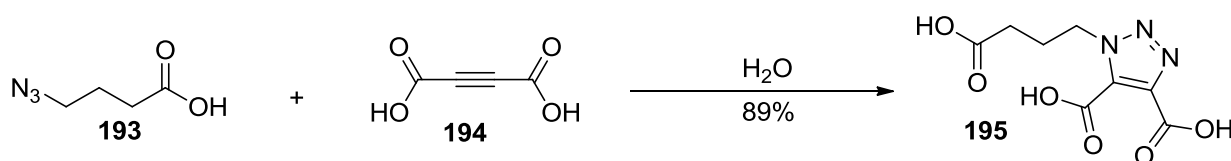
Figure 7.18: HEp-2 cells labeled with fluorescein azide. Bright field image (right) and fluorescein labeled cell surface at $\lambda = 525$ nm (left). Development of Cu free Click Reaction

In its classic form, the click reaction has limited application for biological systems due to the elevated temperature (Huisgen approach)^[20] or cytotoxicity of Cu (Sharpless approach).^[18] The Sharpless approach has a limited applicability, only for viruses, nucleic acids and proteins but not for bacterial and mammalian cells. The successful incorporation of the Sia on the cell surface leading to cell imaging under the mild conditions of the Huisgen click reaction, mentioned above, was followed by the development of the new possibilities for Cu free click reaction. Initially we focused on the use of the acyclic alkynes with electron withdrawing groups on adjacent positions to the alkyne triple bond. We decided to exploit the electron withdrawing effect of two carbonyl groups adjacent to the alkyne triple bond in acetylene dicarboxylic acid.

7.4.1 Cu free Click reaction with acetylene dicarboxylic acid

The reaction was conducted between acetylene dicarboxylic acid **194** and azidobutyric acid **193** in water without any catalyst as described in scheme 7.18. After 12 h, a solid was precipitated from the reaction mixture, the solvent was evaporated and the residue was subjected to purification by flash chromatography. The NMR and mass analysis of the compound indicated that triazole **195** was obtained in good yield (89%). The approach can be a useful tool for

bioorthogonal labeling, with the only drawback being the slow reactivity compare to the Cu catalyzed or strain prompted alkyne azide cycloaddition.



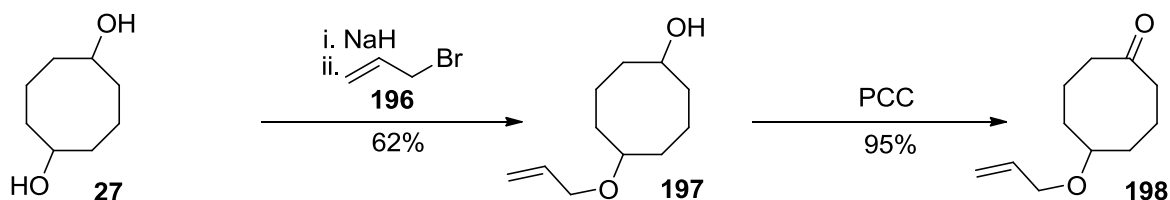
Scheme 7.18: Synthesis of triazole **195** through Cu free click reaction.

7.5 Synthesis of Cyclooctynes

To avoid the toxicity of Cu and long reaction time of Cu free click reaction of acetylene dicarboxylic acid, we decided to extend our studies towards the synthesis of cyclooctynes. A recent study by Bach and co-workers based on DFT calculations, showed a reduction in the activation energy for cycloaddition of phenyl azide to cyclooctyne of 8.2 Kcal/mol relative to that of the parent acetylene ($\Delta G = 16.2$ Kcal/mol).^[190-191] The introduction of two fluorides adjacent to the triple bond gives a further reduction of 2.1 Kcal/mol due to the inductive effect in DIFO (difluoro cyclooctyne), leading the reaction to take place at ambient temperature. To utilize the advantage of the reactivity of the cyclooctynes towards click reaction, we developed a library of pre-reported octynes in addition to the development of a new octyne with a simple synthetic approach.

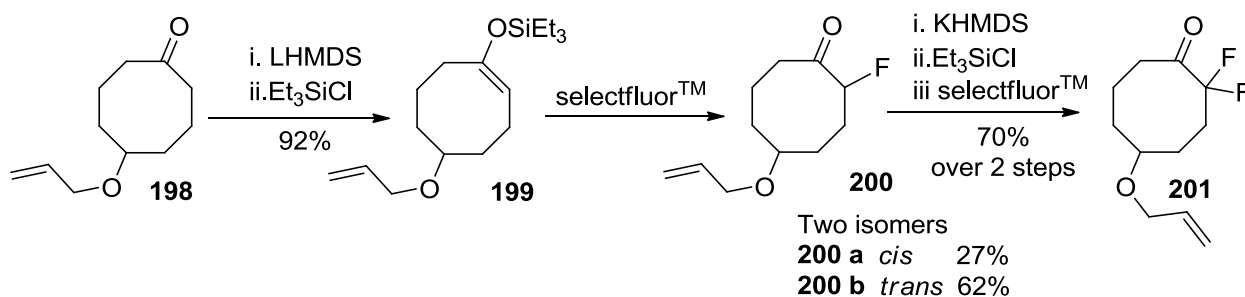
7.5.1 Synthesis of DIFO (I) **26**

To achieve the bioorthogonal reactivity with the click reaction, the alkyne was furnished with two powerful rate enhancing features, ring strain in the form of cyclooctyne and two electron withdrawing groups (fluorides). Synthesis of DIFO **26**, first reported by Bertozzi,^[21] was achieved by starting with cis-1,5-cyclooctanediol **27** in thirteen steps. In first step, described in scheme 7.19, the diol was selectively mono allylated by treatment with allylbromide **196** at 0 °C in the presence of NaH as base and DMF as solvent. After overnight stirring, the reaction was quenched by the addition of water followed by extraction of the product into Et₂O. The crude product was purified with flash chromatography, eluting with the gradient of Hex/EtOAc (8:1 to 2:1), *R_f* 0.28 in Hex/EtOAc (2:1), affording the desired allyl alcohol in 62%. In next step, the free hydroxyl group of allyl octanediol **197** was selectively oxidized to a ketone by stepwise addition of PCC (pyridinium chlorochromate) over 2 h. The mixture was further stirred for 1 h, concentrated and purified with flash chromatography, eluting with a gradient of Hex/EtOAc (8:1 to 3:1), *R_f* 0.29 in Hex/EtOAc (5:1), to afford the ketone **198** (93%) as a clear oil.



Scheme 7.19: Synthesis of allyloxy cyclooctanone intermediates **197** and **198**.

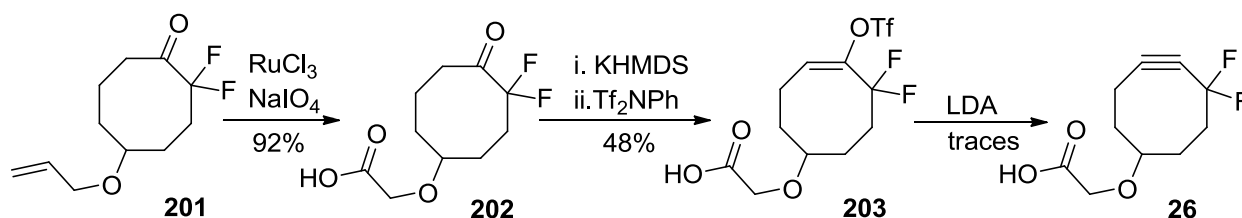
Cyclooctanone was further subjected to fluorination at α position to the carbonyl functionality, which was achieved in two steps. First step was the development of silyl enol ether, achieved by treatment of the ketone with LHMDS (lithium hexamethyldisilazide) in anhyd. THF at $-78\text{ }^{\circ}\text{C}$ as illustrated in scheme 7.20. The solution of ketone **198** in THF was slowly added via a syringe pump over 1 h to the solution of LHMDS in THF, followed by the addition of chlorotriethylsilane after 30 min of stirring at $-78\text{ }^{\circ}\text{C}$. After complete addition, the solution was allowed to warm to rt and stirred for another 1.5 h. The solvent was evaporated on the rotavap and the crude product was purified by flash chromatography, eluting with Hex/EtOAc (20:1) to afford the enol **199** in 90% yields, R_f 0.65 in Hex/EtOAc (10:1). Silyl enol ether **199** was then treated with selectfluor in the presence of DMF at $0\text{ }^{\circ}\text{C}$ to get the mono fluoro ketone. After 30 min the solution was warmed to rt, quenched by water, extracted with Et_2O and concentrated. TLC with a mixture of Hex/EtOAc (9:1) showed two spots of the product isomers with $R_f = 0.41$ (*cis*-isomer **200a**) and 0.27 (*trans*-isomer **200b**). The crude product was flashed through a silica gel column, eluting with a gradient of Hex/EtOAc (10:1 to 5:1) to get both isomers; *trans*-isomer (30%) and *cis*-isomer (58%). The *cis*-isomer **200a** was further treated with KHMDS in THF to convert into the *trans*-isomer **200b** (60%) along with *cis*-isomer (35%). The reaction was repeated until all of the *cis*-isomer was converted into the *trans*-product.



Scheme 7.20: Fluorination of cyclooctanone by selectfluor to afford **201**.

Monofluoro cyclooctane was then converted into the difluorinated derivative **201** by the repetition of last two steps i.e. synthesis of silyl enol ether and then its treatment with selectfluor. The only difference was the use of KHMDS instead of LHMDS for the synthesis of silyl enol ether **201** and purified by eluting in Hex/EtOAc (20:1) to afford the desired product (94%),

R_f 0.65 Hex/EtOAc (10:1). The monofluoro silyl enol ether was treated with selectfluor for 3 h at rt, the reaction was quenched with water, extracted with Et₂O, and the crude product was purified with flash chromatography, using Hex/EtOAc (10:1) as eluent, to afford the desired product **201** (68%) as a clear oil, R_f 0.58 in Hex/EtOAc (4:1). After successful introduction of two fluorine atoms on the ring, the next step was the conversion of the allyl functionality into carboxylic acid **202** as shown in scheme 7.21. Allyl ketone was treated with NaIO₄ and RuCl₃·H₂O in a mixture of CCl₄, CH₃CN and H₂O at 0 °C. After stirring for 15 min at 0 °C, the mixture was allowed to warm to rt and further stirred for 3 h followed by the concentration of the solvent. The concentrate was dissolved in 1 N HCl and brine and extracted with DCM. The crude product was subjected to flash chromatography, using a gradient of Hex/EtOAc (4:1 to 2:1) with 1% AcOH to afford the desired acid **202** (89%) as a colorless oil, R_f 0.3 in Hex/EtOAc (1:1) with 1% AcOH. In order to develop a triple bond, the ketone was converted into the vinyl triflate **203** by treatment with Tf₂NPh (*N*-phenyl-bis-(trifluoromethylsulfonamide)) at –78 °C, in the presence of KHMDS and THF as solvent. The reaction was quenched with water after the completion of the reaction, which was monitored by TLC. The solvent was evaporated and the concentrate was dissolved in 1 N HCl and brine. The crude product was purified with flash chromatography, eluting with a gradient of Hex/EtOAc (4:1 to 1:1) with 1% AcOH, R_f 0.33 in Hex/EtOAc (1:1) with 1% AcOH to afford the desired triflate **203** (62%) as a clear oil.



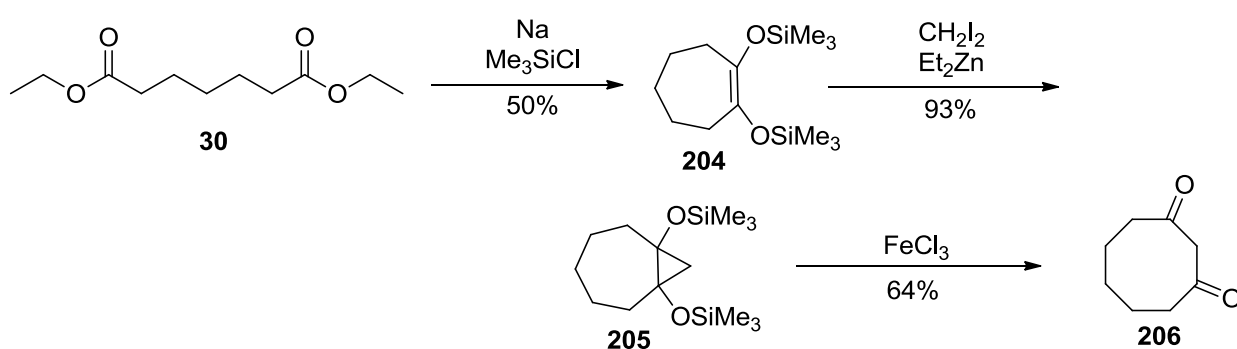
Scheme 7.21: Synthesis of difluorocyclooctyne (DIFO I).

The last step in the synthesis of DIFO (I) **26** was the treatment of vinyl triflate with LDA to afford the alkyne group. The reaction took place only in trace amounts due to the aggressive behavior of strong base towards free carboxylic acid. LDA was prepared by treatment of diisopropylamine with *n*-BuLi in THF at –78 °C, which was slowly added to the THF solution of triflate **203** via a syringe pump over 2 h at –78 °C and the mixture was stirred further for 1 h. The crude product obtained after evaporation of the solvent was subjected to the purification with flash chromatography, eluting with a gradient of Hex/EtOAc (4:1 to 1:1) in the presence of 1% AcOH. The reaction was tried a number of times but the staining on the TLC and isolation by column chromatography was always problematic. The first problem was the staining of the compound on TLC and for this purpose different staining reagents were applied, but all gave

poor results. The second problem was the same R_f value of both educt and product causing difficulties in isolation. After a detailed discussion and literature review, the TLC plates were pre-treated with silver chloride solution to bind the alkyne through its triple bond with silver so that it could not elute anymore. In this case only educt was able to elute, which was easy to detect on TLC plate. However, all these attempts used for the purification and isolation of the target compound were of minor success. NMR spectra of the compound showed small traces of the desired octyne molecule **26**. The ESI-MS analysis of the compound was also hardly able to verify the presence of the target alkyne. Finally, it was concluded in the light of our observation and literature review that the target compound DIFO **26** was not stable under the reaction conditions and deteriorated quickly.

7.5.2 Synthesis of DIFO (II) **28**

The synthesis of DIFO 1st generation **26** is not only a long process but also complicated and its intermediates suffer from decomposition, especially in the last step. The acid moiety in the final product is not show tolerance to the highly basic conditions of the elimination step. So there was need to simplify the reaction conditions and to have a better approach for the synthesis of cyclooctyne derivatives. The synthesis of the target DIFO (II) **28** was initiated by the development of cyclooctane 1,3-dione **206**, which was obtained through a synthetic approach introduced by Seagussa^[192] as shown in scheme 7.22. The first step was the cyclization of diethyl pimelate **30**, achieved through Bouveault-Blanc condensation proceeding through a free radical mechanism, also known as Acyloin condensation.^[193] The most useful application of the reaction is the synthesis of cyclic rings varying in size from 4 to 34 carbon atoms.^[194]

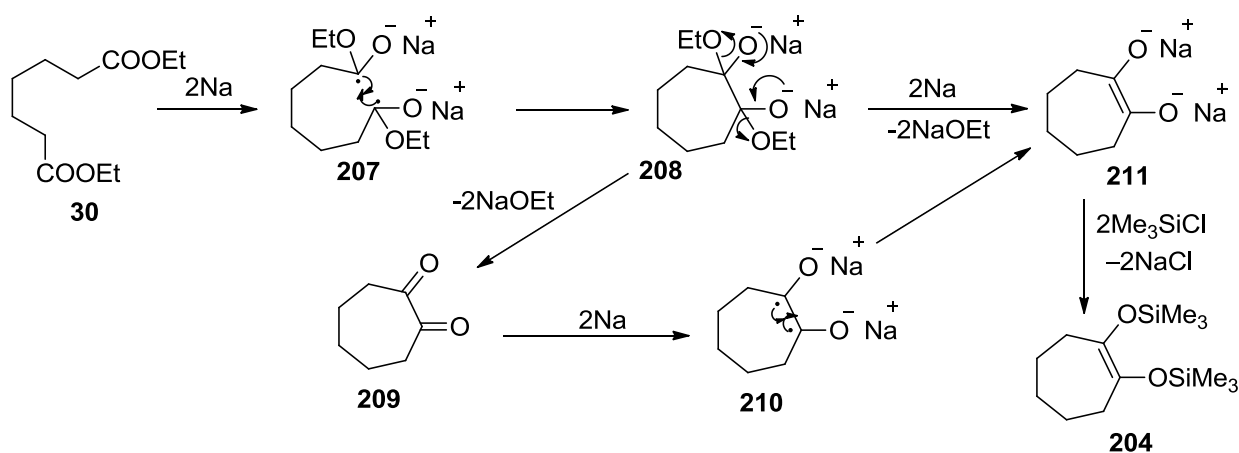


Scheme 7.22: Synthetic pathway for cyclooctandione **206**.

Dicarboxylic esters with carbon chain in the range of 5-9 atoms give cyclic product in excellent yield, especially in the case when α -hydrogen atoms are not replaced by other alkyl groups.^[195] The synthesis of seven membered cyclic heptene **204** was achieved by treatment of

diethylpimelate **30** with triethylsilylchloride in the presence of sodium metal in toluene. For safety reasons, the reaction was conducted behind a glass shield to avoid any damage as a result of possible explosion, which may occur due to heating of Na metal with trimethylsilyl chloride. Initially, the sodium metal was suspended in toluene under vigorous stirring, in the presence of crushed borax test tube and heated up to 90 °C, followed by the addition of trimethylsilylchloride. Finally, the solution of diethylpimelate **30** in toluene was slowly added via a syringe pump over 3 h and the reaction mixture was refluxed overnight. This reaction proceeds very critically and can be monitored by visual observation. The purple color of the suspension indicates successful proceedings of the reaction, on the other hand a yellow or brown coloration of the suspension shows that the reaction has been stalled.^[196] On the completion of the reaction, filtration of the suspension was done over celite in a glass frit, which was a difficult due to very fine particles of sodium chloride. After filtration, the solution was concentrated and the crude product with $R_f = 0.33$ in Pent/DCM (12:1) was subjected to the next step without any purification. The reaction mechanism is given in scheme 7.23, which is considered to take place in four steps:

- oxidative ionization of two sodium atoms on the double bond of two oxygen atoms
- free radical coupling of two carbonyl carbons of the homolytic ester derivative (the elimination of ethoxy groups in the form of sodium ethoxide takes place from both esters functionalities resulting in the synthesis of diketone)
- oxidative ionization of two sodium atoms on both of the carbonyl double bonds, resulting in the synthesis of sodium enediolate
- neutralization with two silylchloride molecules, resulting the removal of two sodium atoms in the form of sodium chloride finally giving cycloheptene silyl enol ether.



Scheme 7.23: Mechanistic pathway for the synthesis of cycloheptene silyl enol ether **204**.

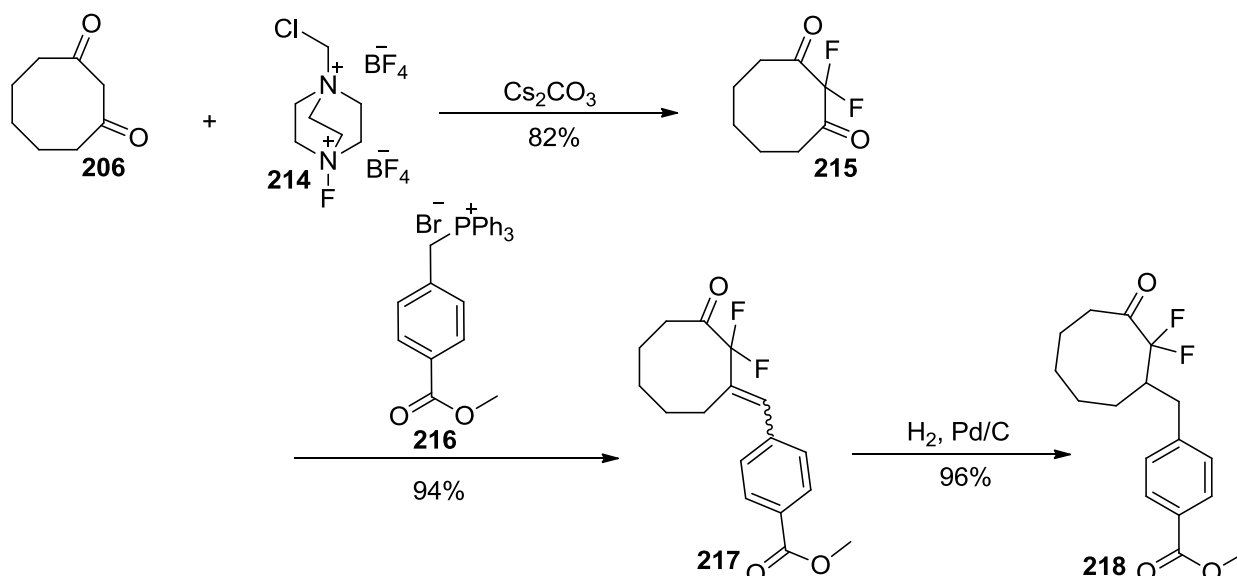
Next step was the cyclopropanation of cycloheptene **204** by treatment with diiodomethane in the presence of diethyl zinc to get bicyclooctane **205**. The reaction proceeds by the catalysis of molecular oxygen,^[197] and carried out in dry toluene by slow addition of diethyl zinc solution over 30 min to the solution of heptene **204** and CH₂I₂ in toluene at -10 °C. The crude product obtained after the evaporation of the solvent was purified by flash chromatography, eluting with Pent/DCM (20:1) to afford a colorless oil 89%, *R_f* 0.32 in Pent/DCM (10:1).

After successful synthesis of bicyclooctane **205**, it was subjected to the oxidative cleavage for ring expansion. It can be achieved by two different reagents; FeCl₃ and H₅IO₆; however, we used iron chloride for this purpose. Although the workup of the reaction with anhyd. FeCl₃ was inconvenient, the reaction proceeds in better yield as compared to oxidation with H₅IO₆. Bicyclooctane **205** was treated with iron chloride under anhyd. conditions by slow addition of FeCl₃ to the solution of bicyclooctane in DMF under nitrogen atmosphere and the dark brown solution was stirred overnight at 60 °C. After 12 h the solution was cooled to rt, quenched with 1% HCl and extracted with CHCl₃. After washing with 1% HCl and brine, the organic phase was dried with MgSO₄ and concentrated. The concentrate was dissolved in DEE and extracted with 3% aqueous KOH solution. The extract was acidified with conc. HCl, saturated with NaCl and back extracted with CHCl₃. The crude product obtained after evaporation of the solvent was purified with silica gel column chromatography, eluting with Pent/DEE (2:1) to afford pure diketone **206** in 59% yield, *R_f* 0.38 in Pent/DEE (1:1). The same reaction can also be performed with H₅IO₆ in abs EtOH; however, in our hands the reaction did not work. The addition of CHCl₃ to the solution during workup gave an explosive reaction leading to the destruction of the product. Another possibility to get cyclooctandione from cycloheptene could be its treatment with bromocarbene formed *in situ* by reaction of bromoform with diethyl zinc.^[198] The oxidative cleavage resulting ring expansion occurs *in situ* and is catalyzed by molecular oxygen. After having 1,3-diketo cyclooctane **206** in hand, next step was the introduction of two fluorine atoms on the ring, which was achieved by treatment with selectfluor **214**, a good handle for direct ionic fluorination of carbonyls. Studies show that compounds existing in the enolic form undergo fluorination more rapidly than those that exist in the keto form. As shown in fig. 7.19, the keto/enol contents of **212** and **213** are 2:1 and 2.4:1 respectively and that is why **213** has less reactivity for selectfluor than **212**.^[199] On the other hand, monofluorinated carbonyls exist in keto form solely and are not easily further fluorinated with selectfluor under neutral conditions. However, the presence of a weak base surprisingly increases the reaction rate.^[200] The use of Cs₂CO₃ for difluorination of diketone by selectfluor improves the reaction rate in such a way that the reaction temperature of 0 °C is sufficient for a good yield.



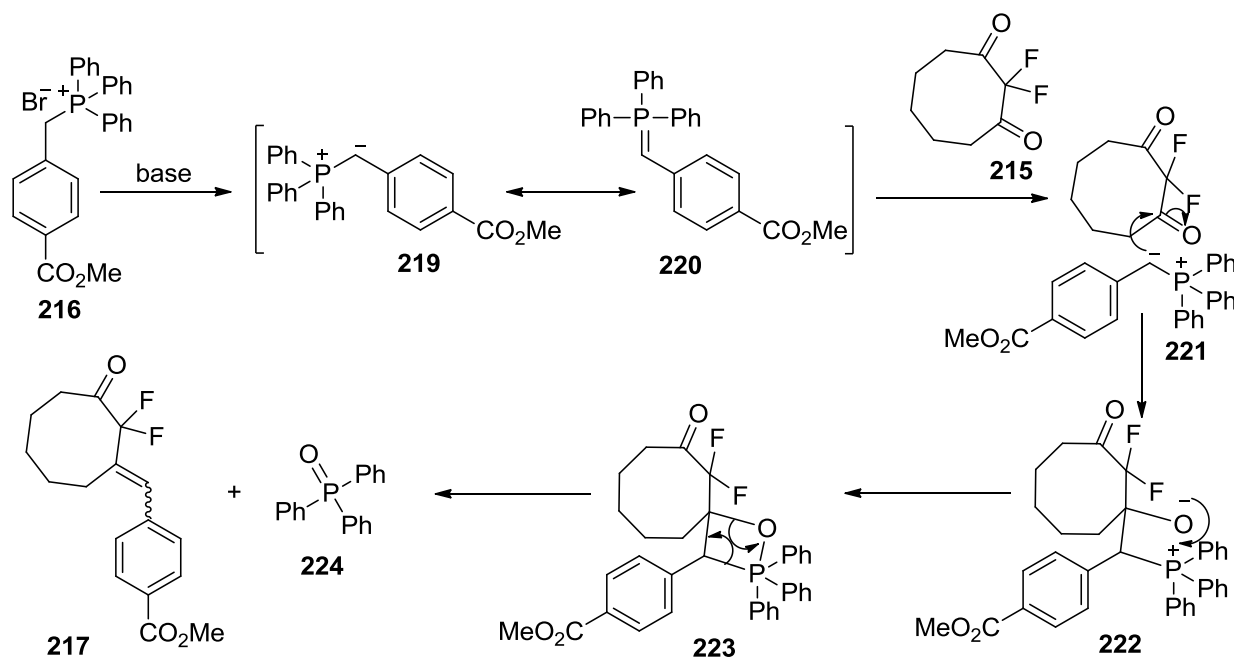
Figure 7.19: Comparative fluorination studies between **212** and **213**.

In the light of all these studies, the fluorination of 1,3-diketone was carried out at 0 °C by the use of selectfluor **214** in the presence of Cs_2CO_3 ^[75] as described in scheme 7.24. The reaction was performed by the treatment of cyclooctan-1,3-dione **206** with cesium carbonate (Cs_2CO_3) in anhyd. MeCN. After stirring for 30 min at rt, the solution was cooled to 0 °C, followed by the addition of selectfluor, allowed to warm to rt after 30 min and stirred for further 2 h. The solvent was evaporated *in vacuo* and the residue was dissolved in 1 N HCl solution. The aqueous phase was extracted with Et_2O and the crude product was purified by flash chromatography, eluting with Hex/EtOAc (8:1) to afford the desired difluorodiketone **215** (82%), R_f 0.38 in Hex/EtOAc (4:1).



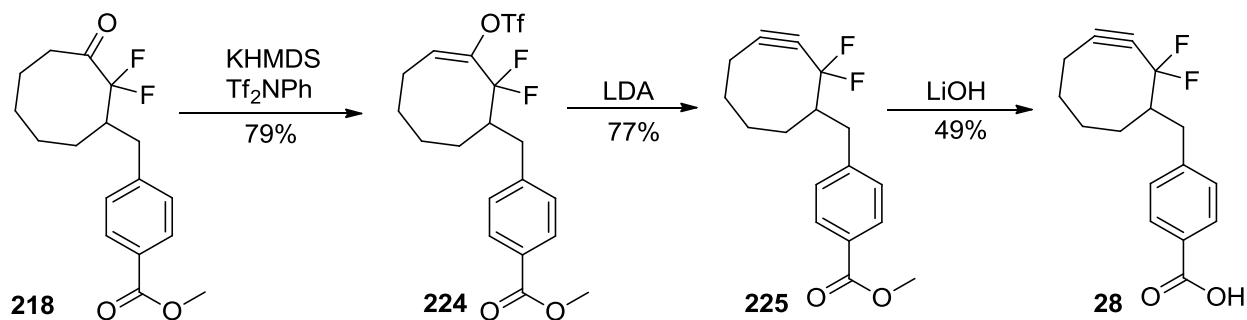
Scheme 7.24: Difluorination of 1,3-diketocyclooctane followed by the Wittig reaction and hydrogenation.

Difluorodiketone **215** was further subjected to Wittig reaction by treatment with Wittig reagent **216** to develop a linker on the cyclooctane ring, illustrated in scheme 7.25. The Wittig salt **216** ([4-(methoxycarbonyl)benzyl]triphenylphosphonium bromide) was obtained by treatment of triphenyl phosphine with methyl 4-(bromomethyl) benzoate in CHCl_3 under reflux for 2 h. After cooling to rt, the solution was filtered and white powder was washed with CHCl_3 , dried (97%) and used without any further purification.



Scheme 7.25: Mechanistic pathway for Wittig reaction.

The Wittig reaction was achieved by treatment of diketone **215** with phosphonium salt **221** of methyl benzoate in THF at 0 °C and in the presence of DBU, which belongs to the amidine class of organic compounds and is a strong base used to catalyze reactions that require the removal of acidic hydrogen.^[201] The purpose of its use in this reaction was to deprotonate the Wittig reagent for the development of a double bond between the phosphorus atom and the adjacent carbon atom to give the ylide **220**. The reaction was stirred for 2 h at 0 °C and warmed to rt followed by further stirring for 48 h. Finally, AcOH was added to the solution to quench the reaction, diluted the reaction mixture with MeOH and concentrated *in vacuo*. The crude product was purified by flash chromatography with Hex/EtOAc (8:1) as an eluent to afford the desired Wittig product **217** in excellent yield (93%). The olefinic double bond of Wittig product **217** was converted to the aliphatic single bond via hydrogenation catalyzed by Pd/C. The reaction was carried out in MeOH under hydrogen atmosphere. After 48 h, the solution was filtered through celite and concentrated to get pure product **218** (99%), R_f 0.6 in Hex/EtOAc (4:1). The successful synthesis of the ketone **218** was followed by the introduction of the triple bond on the ring, which was achieved in two steps as described in scheme 7.26.



Scheme 7.26: Development of the triple bond by base catalyzed elimination of triflate followed by ester hydrolysis.

First step was the synthesis of triflate followed by its removal in second step resulting in the development of an alkyne functionality. The synthesis was achieved by the treatment of ketone **218** with Tr_2NPh in the presence of KHMDS in anhyd. THF. The reaction was carried out by slow addition of ketone solution in THF over 30 min to the chilled ($-78\text{ }^{\circ}\text{C}$) solution of KHMDS in THF. After 50 min Tr_2NPh was slowly added to the reaction mixture via a syringe pump at the same temperature ($-78\text{ }^{\circ}\text{C}$), and after 1 h the solution was warmed to rt. The solution was stirred for further 24 h at rt, quenched with MeOH, concentrated *in vacuo*, and purified by flash chromatography using a gradient of Hex/EtOAc (6:1 to 4:1) to afford the triflate **224** in good yield (85%) as a clear oil, R_f 0.13 in Hex/EtOAc (4:1). Triflate **224** was then treated with LDA, prepared by slow addition of *n*-BuLi to the solution of diisopropyl amine in THF at $-78\text{ }^{\circ}\text{C}$. A portion of LDA solution was slowly added via a syringe pump to the solution of triflate in THF at $-20\text{ }^{\circ}\text{C}$. After complete addition, the reaction solution was warmed to rt over 30 min and quenched with the addition of MeOH. The crude product was purified by flash chromatography, eluting with toluene/Hex (2:1) with 1-4% EtOAc to afford the alkyne **225** (77%) as a colorless oil, R_f 0.29 in toluene/Hex (2:1), which became solid after some time. In the final step, the ester was hydrolyzed to afford a carboxylic acid by treatment of ester **225** with LiOH in dioxane and water at $55\text{ }^{\circ}\text{C}$ for 3 h. The solution was cooled to rt and acidified up to $\text{pH} \geq 2$ with 1 N HCl, extracted with DCM, concentrated under reduced pressure and purified by flash chromatography eluting with a gradient of Hex/EtOAc (8:1 to 4:1) with 1% AcOH to afford DIFO **28** (64%) as a white solid, R_f 0.39 in Hex/EtOAc (4:1) with 1% AcOH. Finally, after 10 steps we were able to isolate the desired product successfully in an overall yield of 11%, which is much better than that of DIFO (I) and also than the aforementioned approach for DIFO (II).

7.5.2.1 Discussion on the spectra of cyclooctyne ester **225**

The intermediates formed in different steps during the synthesis of DIFO (II) **28** were subjected to structure elucidation by both NMR and mass studies. Here we will discuss the NMR spectra of DIFO ester **225** as a reference. The ^1H -NMR spectrum shown in the fig. 7.20 represents an

aromatic ring by two multiplets in the range of $\delta = 7.2$ ppm to $\delta = 7.9$ ppm, each of which possesses two hydrogen atoms indicating the presence of benzoic ester. A singlet at $\delta = 3.82$ ppm is assigned as methyl group of the ester. A multiplet in the range of $\delta = 2.40$ -2.43 ppm along with a doublet at $\delta = 3.07$ ppm collectively possess three hydrogen atoms represent methylene hydrogen atoms of the carbon linking both rings and hydrogen (H-1'') of methylene binding octyne ring with benzoic ester. the rest of the signals are in the form of five multiplets represent 8 hydrogen atoms indicating the four -CH₂- groups of cyclooctyne ring.

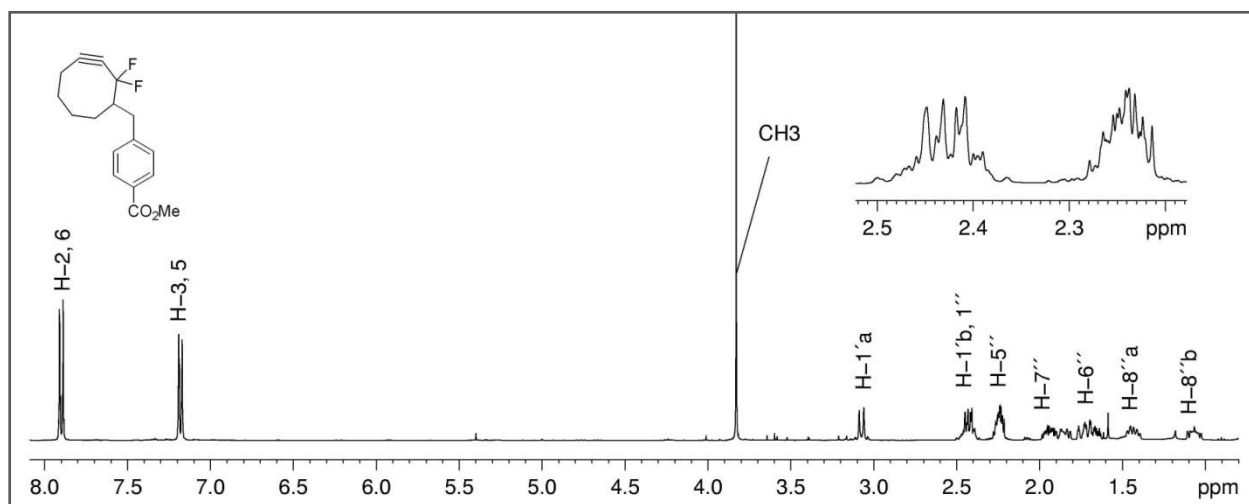


Figure 7.20: ¹H-NMR spectrum of the cyclooctyne ester **225**. (CDCl₃, 400 MHz)

Further analysis of the compound **225** with ¹³C-NMR spectrum shown in fig. 7.21 gives more evidence for the product. A signal resonating at $\delta = 167.14$ ppm is assigned as carbonyl carbon of the ester. A signal at $\delta = 145.43$ ppm represents the para carbon atom of benzoic ester and rest of the five carbon atoms of aromatic ring are reflected by five signals in the range of $\delta = 128.5$ -129.9 ppm. A triplet at $\delta = 117.03$ -121.77 ppm ($J = 238.61$ Hz) indicates a carbon containing two fluorine atoms. The presence of alkynic triple bond is confirmed by two multiplets at $\delta = 109.81$ -109.98 ppm and at $\delta = 84.70$ -85.17 ppm. The ester functionality is confirmed by the presence of a signal at $\delta = 52.13$ ppm and the linkage of the octyne with benzoic ester is confirmed by a triplet at $\delta = 58.11$ ppm with coupling constant $J = 24.24$ Hz, the tertiary nature of which is confirmed by ¹³C-DEPT spectrum. Signals in the range of $\delta = 20.3$ -34.4 ppm indicate five -CH₂- carbons, four of which are of cyclooctyne and one is a methylene carbon establishing a connection between two rings.

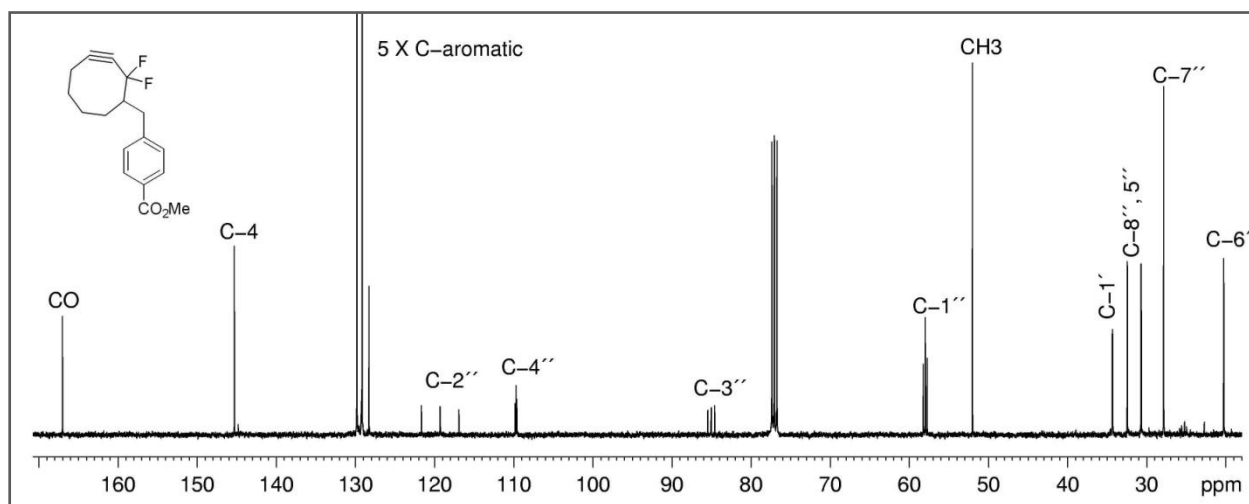
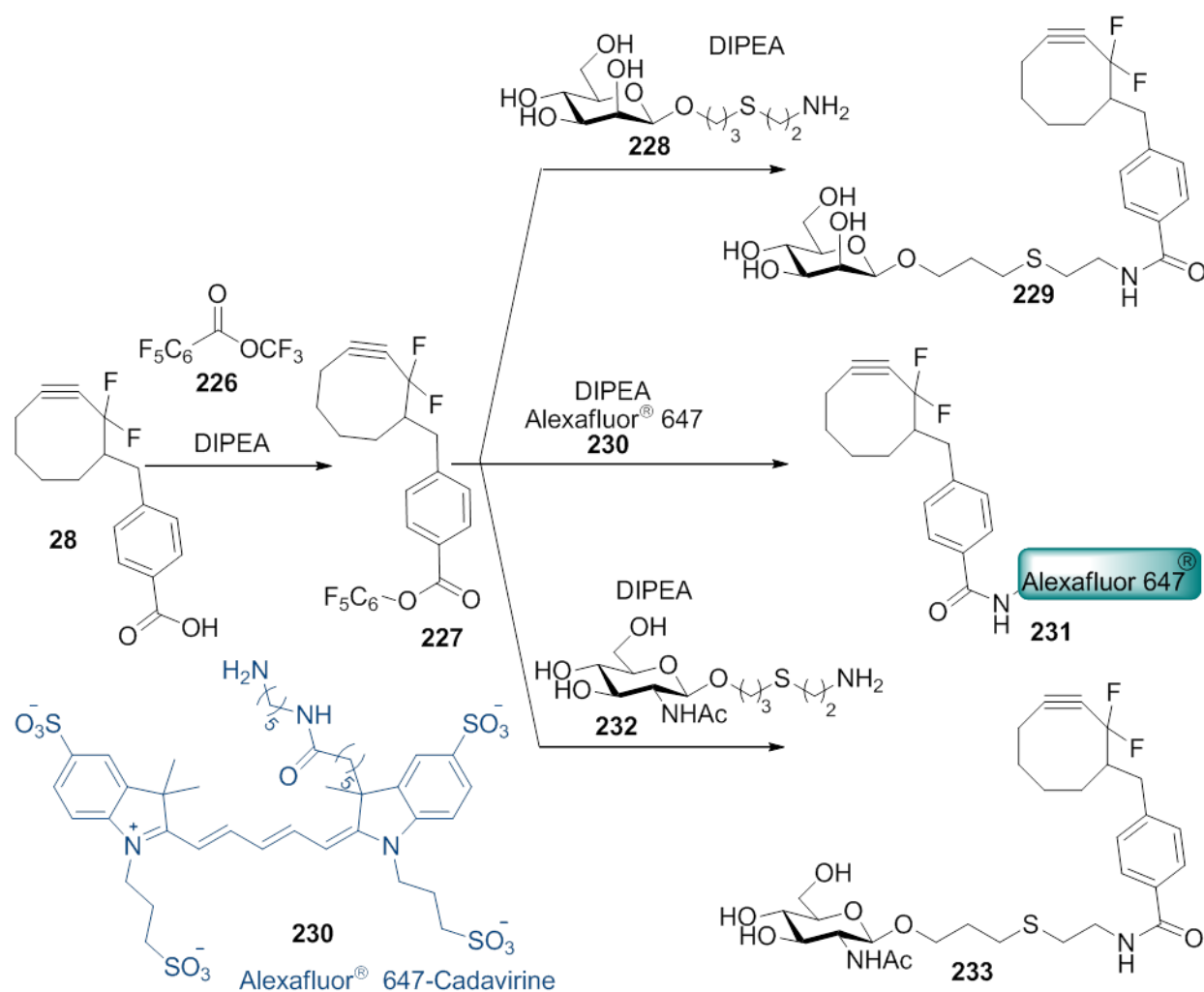


Figure 7.21: ^{13}C -NMR spectrum of the cyclooctyne ester **225** (CDCl_3 , 100 MHz).

The presence of fluorine atoms is confirmed by ^{19}F -NMR analysis and the spectrum obtained shows a doublet at $\delta = -96.16$ ppm ($J = 270.8$ Hz) along with another doublet of doublet at $\delta = -123.77$ ppm ($J = 245.1, 23.4$ Hz). NMR data is further supported by mass analysis via EI-MS, which shows m/z for $[\text{C}_{17}\text{H}_{18}\text{O}_2\text{F}_2 + \text{Na}]^+$ in the range of 315.11, identical to the calculated value 315.11.

7.5.3 Conjugation of DIFO (II) with sugars and fluorescent dyes

In order to enable the cyclooctyne **28** capable of cell surface engineering, the next step was its conjugation with some biologically active or fluorescent molecules. Nonnatural sugars like glucosamin azide (GlcNAz) and mannosamin azide (ManNAz) undergo metabolic process and biotransform into the sialic acid derivatives of azide.^[4] These azides on the cell surface are the target point for octyne molecules to conjugate through Cu free click reaction in our studies. The next step of our strategy, the conjugation of DIFO (II) **28** containing free carboxylic acid can be achieved through amide or ester linkage with an *in situ* catalysis by EDC or DCC.^[202] However, the urea synthesized as a byproduct in the presence of these activating catalysts is difficult to remove. To avoid this difficulty, we planned to follow a two-step strategy described in scheme 7.27. The first step was the activation of the acid by synthesis of pentafluorophenyl ester, followed by conjugation with amine or alcohol in the second step. The conjugation was initiated by the development of a conjugate of Alexafluor-647[®] cadaverine **230** and DIFO (II) **28** through an amide linkage.



Scheme 7.27: Conjugation of different biomarkers with DIFO(II) **28**.

The activation of the acid was achieved by treating DIFO (II) **28** with pentafluorophenyl trifluoroacetate in the presence of DIPEA in anhyd. DCM. All the additions were done at 0 °C and after 15 min the solution was warmed to rt. After 2 h of further stirring, the mixture was filtered through a plug of silica gel, eluting with hexane and the solvent was evaporated *in vacuo* to get a white solid. This solid material was dissolved in anhyd. DMF followed by the addition of alexafluor-647[®] cadaverine **230** at rt, and finally DIPEA. Following day, the solvent was evaporated *in vacuo* and purified two times with flash chromatography, eluting with a gradient of DCM/MeOH (10:1 to 5:1) to afford the conjugate **231** as an indigo colored solid, R_f 0.2 in DCM/MeOH (4:1). The reaction and the workup including the purification of the compound were done under dark as the dye **230** is sensitive to light. After successful conjugation of Alexafluor-647[®] cadaverine, GlcNAc-cystamine **232** and Man-cystamine **228** were also conjugated with DIFO (II) **28** in the same way as described above. The conjugates obtained were confirmed by different analytical techniques including NMR and mass spectrometry. The mass studies were attempted with different techniques like ESI-MS, MALDI, EI-MS and APCI;

however, none of the technique was successful for the accurate mass analysis. The reason for it may be the presence of the fluorine atoms, which are sometime problematic for this kind of analysis. The problem may be solved by the use of FAB-MS; however, we had no access to it.

7.5.4 Synthesis of azacyclooctyne

In order to make the octyne molecule more compatible to the living system and to develop a shorter route, we decided to develop an octyne moiety resembling lactams. The target octyne was afforded through ring closing metathesis (RCM), a good tool for the syntheses of strained i.e. 7 and 8 membered rings.

7.5.4.1 Ring closing metathesis

Ring closing metathesis has paved the path for the synthesis of heterocyclic and carboxylic rings, tolerating different functional groups.^[203] Different mechanisms have been proposed for the reaction, but the most authentic is the Chauvin mechanism, which appears to be more consistent with experimental evidences. As described in fig. 7.22, it comprises two consistent [2+2] cycloadditions and cycloreversions have been suggested, involved in the synthesis of key intermediate metalocyclo-butane, which can undergo either pathway: towards product or back to the reactant.^[204]

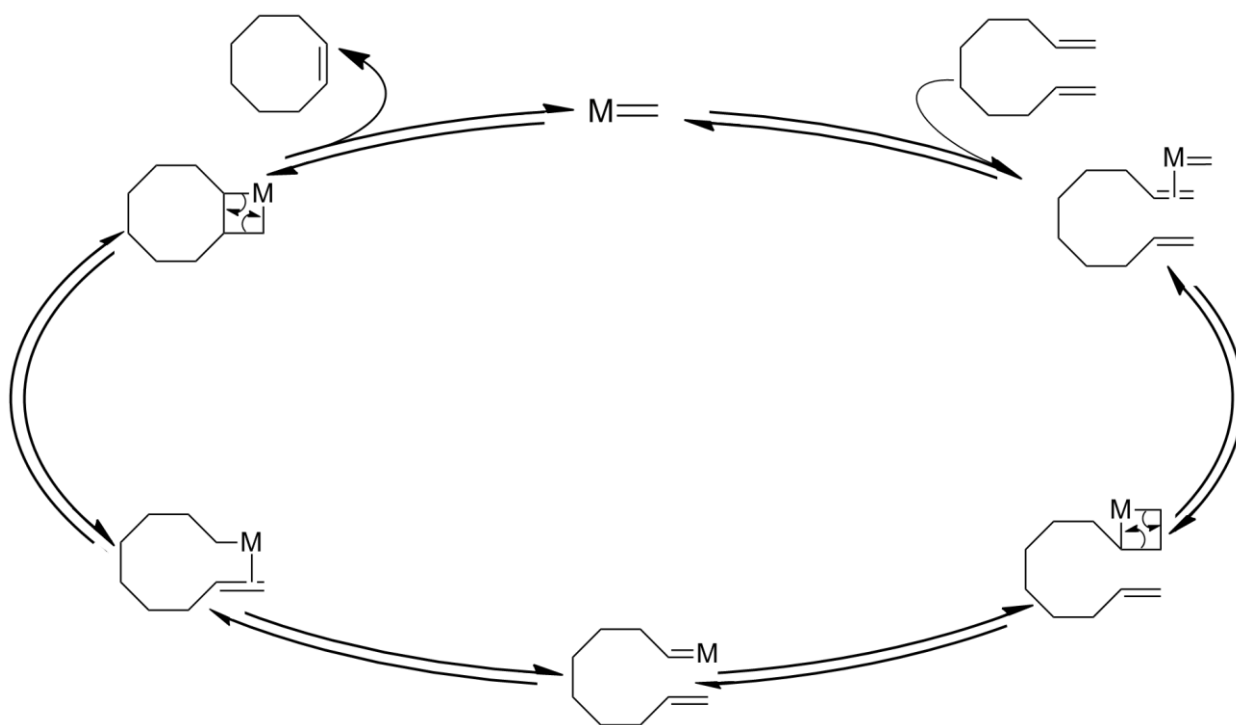
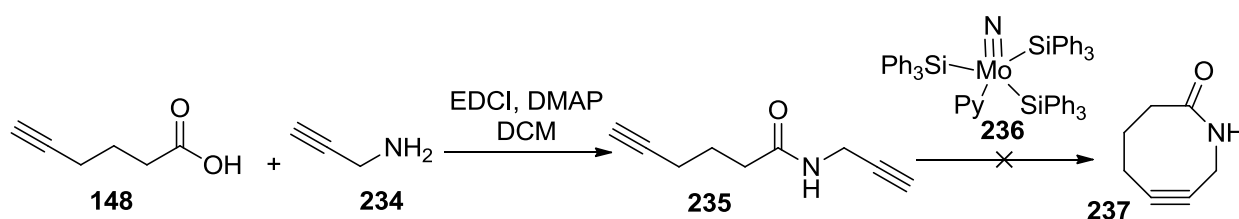


Figure 7.22: Mechanistic pathway for ring closing metathesis proposed by Chauvin.

In the case of terminal alkenes, the driving force for the reaction is elimination of a highly volatile olefin. The reaction is usually performed at high dilution (2-4 μM) to avoid the undesired intermolecular metathesis. Many reports indicate that the decomposition products of the Ru metathesis catalysts, generally Ru-hydride complexes, may be responsible for the side reactions, especially for the isomerization of the olefinic products.^[205]

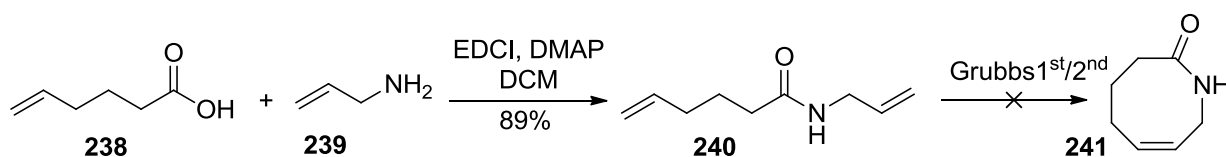
Lactams, the cyclic amides of carboxylic acids have pharmacological importance due to their occurrence in natural molecules.^[205] The β -lactam antibiotics comprise two groups of clinically important therapeutic reagents i.e. penicillins and cephalosporins.^[206] These synthetically valuable applications of lactams motivated us to make the use of them as cyclooctyne moieties. In first attempt, we planned the synthesis of the cyclic octyne through alkyne metathesis. Our strategy shown in scheme 7.28 was initiated by the synthesis of a ten membered acyclic secondary amide **235** with a terminal alkyne by a reaction between 5-hexynoic acid **148** and propargylamine **234** in DCM at -8°C by using EDC and DMAP as catalyst. When the intermediate **235** was subjected to alkyne ring closing metathesis (ARCM) in the presence of a molybdenum catalyst (S1) **236**, no ring closure took place. The reason for this may be the facile synthesis of polymer and the degradation of Schrock alkylidynes by the formation of deprotonated inactive metallacyclobutadienes.^[207-208] To avoid this difficulty, we introduced methyl functionality on the terminal alkynes by treatment with MeI in the presence of NaH, and ARCM was repeated to achieve ring closure but still the reaction did not proceed successfully. We realized that the alkyne functionality is not possible to install in the octyne ring directly by ring closing metathesis due to the ring strain of the molecule.



Scheme 7.28: Synthetic scheme for alkyne ring closing metathesis catalyzed by Schrock's catalyst.

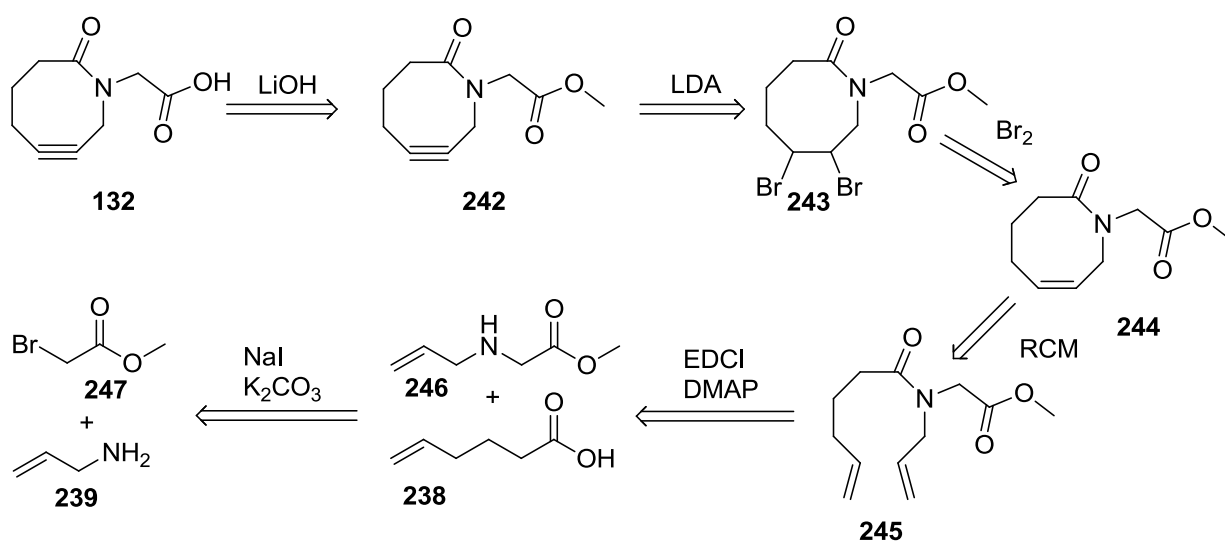
The alternate way of the strategy was then found by olefin ring closing metathesis that is a more facile and an efficient way to develop cyclic molecules. Initially, the synthesis of lactam was planned through RCM of secondary amides and then introduction of a linker through the amide nitrogen for further modifications. For this purpose, the secondary amide **240** was synthesized by treatment of 5-hexenoic acid **238** with allylamine **239** in the presence of EDC and DMAP as described in scheme 7.29. The amide synthesis was achieved excellently (80-85% yield),

however, this secondary amide did not pass through RCM to afford a cyclic octene **241**, even the application of Grubb's 1st and 2nd generation catalyst failed.



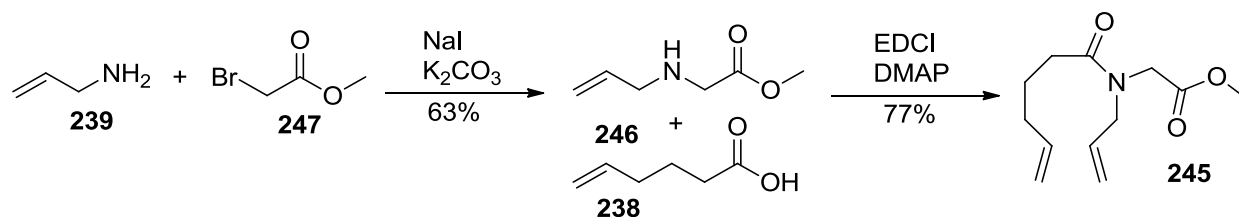
Scheme 7.29: Synthesis of secondary amide **240** and ring closing metathesis to get **241**.

A literature review showed that the secondary amides are unfavorable for ring closing metathesis due to favorable *anti* conformation of amides and less availability of the *syn* isomer at equilibrium between two conformers. The studies also showed that the tertiary amides have both rotamers in equal concentration (1:1) at equilibrium.^[209-210] In addition to conformation, the chelating ability of the amide nitrogen with Grubb's catalyst is another factor that can lead to deactivation of the catalyst^[211]. Keeping in mind these studies, it was envisioned that the protection of the secondary amide or the development of a linker on the amide nitrogen would be useful. To avoid the extra work of protection and deprotection, it was decided to develop an ester functionality to get a tertiary amide. The advantage of the ester was its capability to undergo hydrolysis to give free carboxylic acid, which can be helpful for the conjugation of different linkers to the octyne. Here, we planned to access the target lactam octyne **132** within six steps, which is much simpler than the aforementioned syntheses of cyclooctynes and a retrosynthetic scheme is shown in scheme 7.30.



Scheme 7.30: Retrosynthetic pathway for octyne lactam **132**.

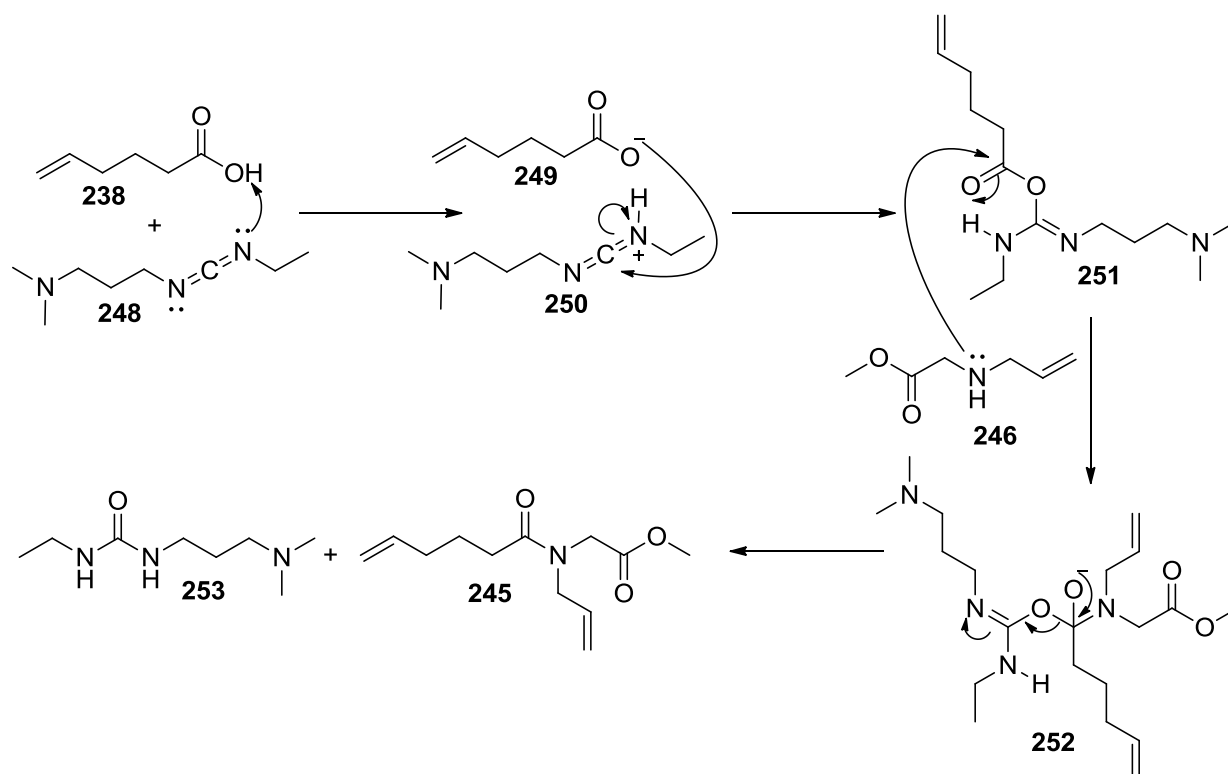
This approach was initialized by the synthesis of the substrate for RCM and achieved in two steps as shown in scheme 7.31. The first step was the synthesis of secondary amine **246** containing an allyl functionality followed by conjugation with hexenoic acid **238** to afford the *tert*-amide **245** in second step. Although, alkylation of primary amines seems to be simple but the synthetic application of the reaction is highly limited due to over alkylation of secondary amine resulting in *tert*-amine and finally quaternary ammonium salts.^[212]



Scheme 7.31: Schematic representation for the synthesis of the *tert*-amide **245** holding terminal alkenes.

To avoid over alkylation, excess of amine was treated with bromide in the presence of NaI at rt. After 30 min, the solution was heated to 50 °C for 1.5 h, cooled to 0 °C, and K₂CO₃ was added to the mixture. The solution was left to stand for 72 h at 5 °C and dissolved in water assuring pH not lower than 10. The product was extracted with Et₂O and purified by flash chromatography eluting with Pent/Et₂O (1:1) to afford a clear oil of the desired secondary amine **246** with an excellent yield 84-86%.

After successful synthesis of the allylamine ester **246**, the next target was its conversion into *tert*-amide with at least two terminal alkene functionalities, which can serve as a RCM substrate. To achieve the synthesis of the desired amide **245**, secondary amine **246** was treated with 5-hexenoic acid **238** in the presence of EDCI and DMAP. The reaction was carried out at –8 °C for 1 h, the solution was allowed to warm to rt and further stirred overnight. Following day, the solution was washed with 0.1 N HCl, concentrated *in vacuo* and purified by flash chromatography, eluting with Pent/Et₂O (1:1) to afford a clear oil of the *tert*-amide **245** with good yield (77-80%), *R_f* 0.47 in Pent/Et₂O (1:1). In the case of secondary amides synthesis, the reaction afforded a racemic mixture of epimers, which are developed as a result of epimerization via the removal of α-H. Initially, this hydrogen is removed to give an enolate and then reprotonates to give racemic mixture of both stereoisomers as described in scheme 7.32.

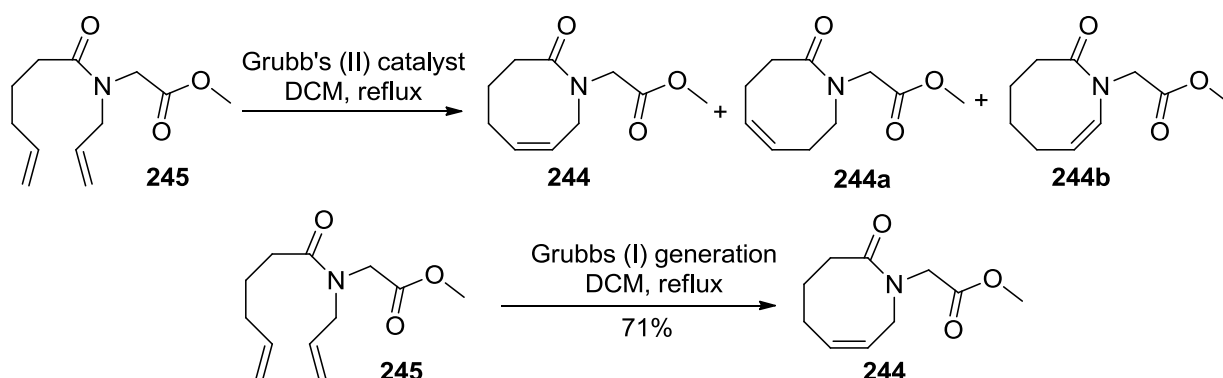


Scheme 7.32: Mechanistic pathway for amide synthesis catalyzed by EDCI and DMAP.

After *tert*-amine **245** was isolated, we focused our attention on the ring closure through RCM. For the synthesis of cyclic octene **244**, *tert*-amide was treated with 2nd generation of the Grubb's catalyst as illustrated in scheme 7.33. However, the reaction rate was very slow and the product isomerized with the passage of time as shown in scheme 7.33. The crude reaction mixture was purified by flash column chromatography and three fractions were obtained due to isomerization, with a double bond between C-5 and C-6 **244a**, C-6 and C-7 **244** and C-7 and C-8 **244b**, along with some other unknown fractions. However, the main fraction was **244** but with the passage of time all the fractions underwent dimerization, confirmed by mass analysis through ESI-MS. It is reported that the Grubb's second generation catalyst undergoes the hydride complexes that are responsible for isomerization of the product.^[213] Another problem during workup was the removal of the catalyst, although the product was purified with column chromatography three times, still traces of the catalyst could be clearly seen by its color.

In order to avoid isomerization, we decided to use the less active 1st generation Grubb's catalyst. The reaction was carried out in DCM at 40 °C. After 2 h of stirring, almost 75% of the educt was transferred to the product, however, further stirring did not give any improvement even further addition of the catalyst was ineffective. Filtration of the reaction mixture over a plug of silica gel and concentration *in vacuo* gave a blackish crude product, which was purified twice by flash chromatography, eluting with Et₂O to afford **244** (63%). The product did not undergo isomerization or decomposition as well as dimerization even at rt for many days. Thus the use of

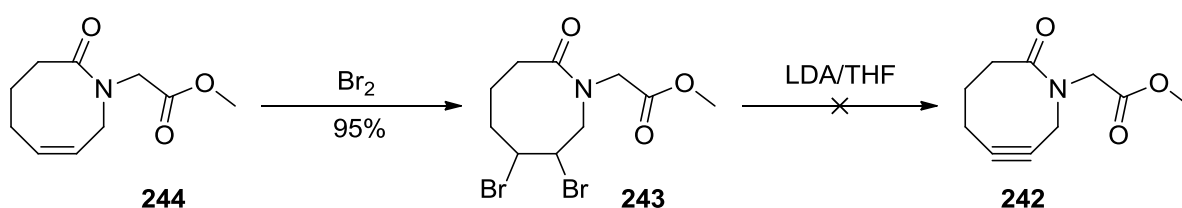
Grubb's 1st generation not only solved the problem of isomerization and purification, it was cost effective too, as Grubb's 2nd generation is ten times more expensive than Grubb's 1st generation.



Scheme 7.33: Different isomers obtained after catalysis of RCM with Grubb's 2nd Generation. To avoid this problem Grubb's (I) was used, which gave good results without any isomerization.

7.5.4.2 Introduction of the alkyne in the lactam ring

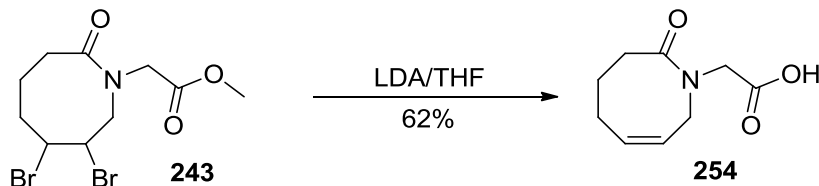
After achieving the successful synthesis of octene **244**, our next target was the introduction of an alkyne group on the ring. The olefin was planned to be brominated followed by dehydrobromination to form alkyne^[214]. The addition of bromine across double bond was achieved by treating olefin **244** with bromine at $-40\text{ }^{\circ}\text{C}$ in DCM as described in scheme 7.34. After completion of the reaction (30 min), aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$ was added to quench bromine and the aqueous layer was extracted with DCM and purified by flash chromatography, eluting with a gradient of Et_2O and EtOAc to afford brominated cyclic amide **243** (95%) as a clear oil, which crystallized at rt.



Scheme 7.34: Reaction scheme for dehydrobromination with LDA.

In the next step, the dehydrobromination for the synthesis of an alkyne was tried by a number of different routes. In first attempt, the dibromo cyclooctanone **243** was treated with freshly prepared solution of LDA in THF at $0\text{ }^{\circ}\text{C}$ in THF. The LDA solution was prepared by slow addition of DIPA in THF to the solution of $n\text{-BuLi}$ in THF at $-78\text{ }^{\circ}\text{C}$. As described in scheme 7.35, only debromination took place affording an alkene along with hydrolysis of the ester instead of the desired alkyne **242**. The product was confirmed by NMR and ESI-MS, which

confirmed the presence of alkene **254**. In a second attempt, the reaction was performed in the presence of *tert*-BuOK in anhyd. THF. However, the analytical investigation of the obtained fractions by column chromatography did not support the presence of the desired product **242**.



Scheme 7.35: Synthesis of alkene **254** instead of alkyne on treatment of dibromide with LDA.

7.5.4.3 Discussion on the NMR spectra of cyclooctene **244**

Synthesis of the lactam octyne was thoroughly monitored by the analysis of the intermediates with NMR as well as mass studies. The ^1H -NMR spectrum of the compound **244**, shown in fig. 7.23, displays a multiplet in the range of $\delta = 5.71$ - 5.87 ppm indicating the presence of olefinic H-6. Another multiplet in the range of $\delta = 5.46$ - 5.53 ppm is assigned as olefinic H-7. Moreover, the same intensity of both signals indicates the presence of an olefin equally substituted from both sides, which is only possible if the RCM has proceeded successfully. The presence of a singlet at $\delta = 4.17$ ppm indicates the methylene hydrogen atoms of the acetyl group linked to the nitrogen of the lactam molecule. Furthermore, a doublet of triplet (dt) at $\delta = 3.93$ - 3.99 ppm ($J = 4.6, 0.8$ Hz) is assigned as 8- H_2 . Another singlet at $\delta = 3.61$ ppm supports the methyl group of ester and a triplet at $\delta = 2.53$ ppm ($J = 6.5$ Hz) indicates the presence of 3- H_2 , next to the carbonyl of the lactam. The presence of two hydrogen atoms (H-5) is confirmed by a multiplet identical to the quartet at $\delta = 2.19$ ppm and another quintet at $\delta = 1.77$ ppm ($J = 6.4$ Hz) assigned as two hydrogen atoms 4- H_2 . All this information supports that the compound **244** is the desired lactam with a double bond developed as a result of RCM. Product **244** was further investigated by ^{13}C -NMR spectrum shown in fig. 7.24 and two signals at $\delta = 175.23$ ppm and $\delta = 172.31$ ppm indicate the presence of two carbonyl carbons. The olefinic double bond is confirmed by two signals at $\delta = 130.54$ ppm and $\delta = 127.42$ ppm and their tertiary nature is approved by ^{13}C -DEPT. A signal of the methyl carbon of ester at $\delta = 49.14$ ppm also exhibits the same behaviour as that of the signals of olefin. The rest of three signals at $\delta = 32.77, 26.19$ and 23.99 ppm indicate the presence of C-3, C-4 and C-5 respectively. ESI-MS showed m/z for $[\text{C}_{10}\text{H}_{15}\text{NO}_3 + \text{H}]^+$ in the range of 198.11, identical to the calculated value 198.24. The m/z for $[\text{C}_{10}\text{H}_{15}\text{NO}_3 + \text{Na}]^+$ was also found at 220.22 in the range of the calculated value 220.09.

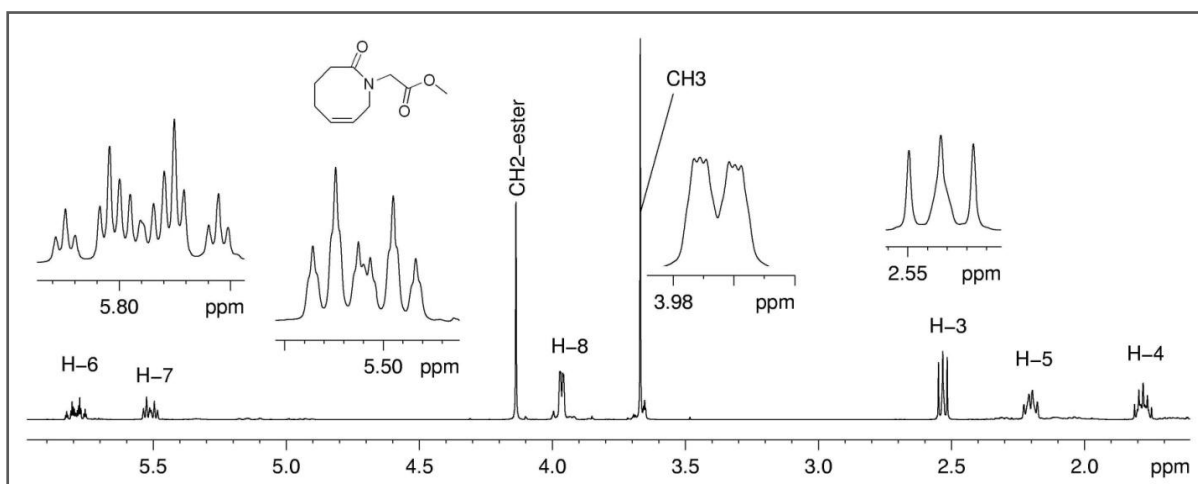


Figure 7.23: ^1H -NMR spectrum of the azocine ester **244** (CDCl_3 , 400 MHz).

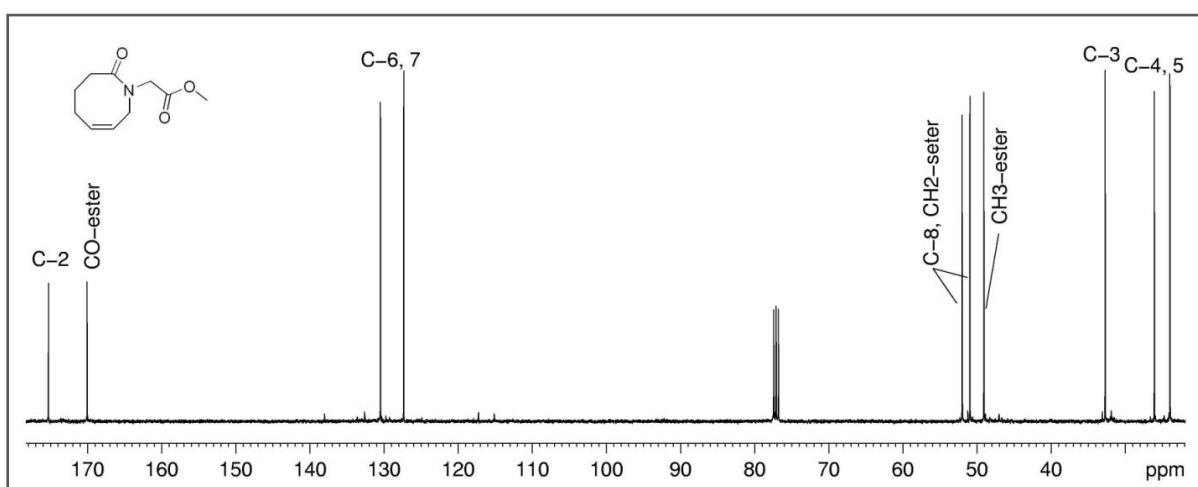
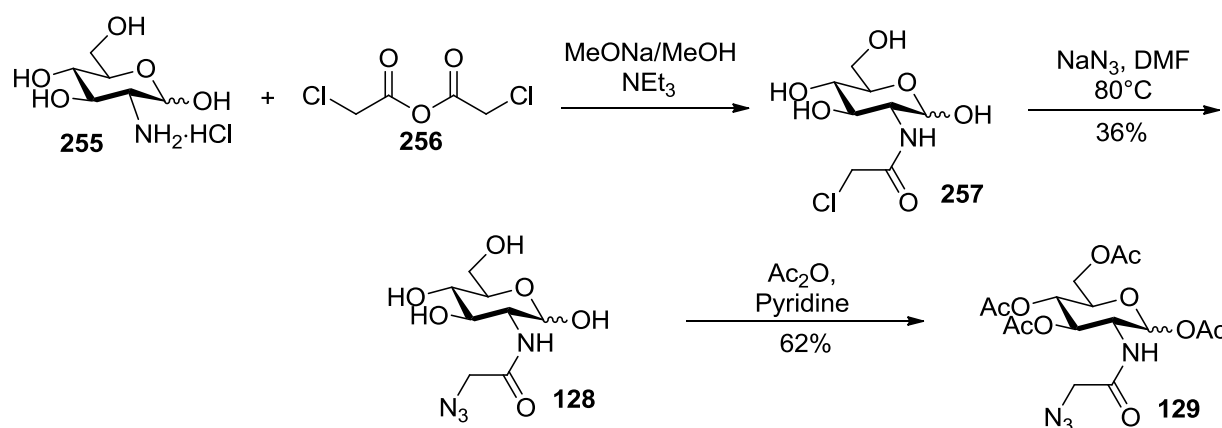


Figure 7.24: ^{13}C -NMR spectrum of the azocine ester **244** (CDCl_3 , 100 MHz).

7.6 *N*-Azidoacetyl Glucosamine

In order to make the strategy of Cu free click reaction applicable for bioorthogonal labeling, the next step after the synthesis of cyclic octynes was the development of their counterpart i. e. the azide moieties. Modified monosaccharides are incorporated into the cell surface and cytosolic glycoconjugates of the mammalian cells by the use of existing biosynthetic pathway.^[34, 128] Acetylated monosaccharides are easily diffused into the cell due to their less polar nature as compare to the nonacetylated polar carbohydrates. Tetraacetyl-*N*-acetyl mannosamine (Ac_4ManNAc), a precursor of sialic acid is incorporated into the cell surface glycoconjugates in the form of Sia through the natural carbohydrate pattern of the cell and can be used as a tool for the introduction of nonnatural glycans.^[215] We selected acetylated glucosamine azide for our further studies on cell surface and the synthesis was achieved in two steps as described in scheme 7.36.



Scheme 7.36: Schematic representation of the synthesis of GlcNAz **128** and **129**.

In the first step, glucosamine hydrochloride **255** was treated with chloroacetic anhydride **256** in MeOH and MeONa. Upon the completion of the reaction, the solvent was removed and the residue was dissolved in DMF followed by the addition of NaN₃ and heated to 80 °C for 2 h. After the completion of the reaction, the mixture was concentrated and purified three times by flash chromatography, eluting with H₂O/IPA/EtOAc (1:3:6) with 1% NH₄OH solution (30%) to afford glucosazide **128** as a light yellow oil (36%), α : β ratio of 1:0.15. GlcNAz **128** was further acetylated by treatment with Ac₂O in pyridine for 12 h and afforded the Ac₄GlcNAz **129** (62%) as a white solid purified by flash chromatography.

7.6.1 Discussion on the spectra of GlcNAz **128** and Ac₄GlcNAz **129**

The ¹H-NMR spectrum of the azide **129** shown in fig. 7.25 confirms four methyl groups assigned by four singlets possessing 12 hydrogen atoms in the range of δ = 2.1-2.2 ppm. A singlet at δ = 3.91 ppm possessing 2 hydrogen atoms represent the methylene group of acetyl bearing azide functionality. A doublet at δ = 6.40 ppm (J = 8.8 Hz) appears from the amide hydrogen and another doublet at δ = 6.24 ppm (J = 3.7 Hz) indicates the presence of H-1 with α conformation. The rest of the signals reflect the presence of the glucose and confirm the compound **129** as a protected glucosazide. Further confirmation of the product is assured by ¹³C-NMR studies shown in fig. 7.26. The presence of five signals in the range of δ = 167.1-171.6 ppm indicates five carbonyl carbons, out of which one is from the amide and rest of the four are acetyl carbonyls directly attached to the ring. Four signals in the range of δ = 20.6-20.9 ppm come from four methyl groups of the acetyl groups and confirm the complete protection of the glucose ring. Furthermore, a signal at δ = 51.37 ppm represent the methylene carbon adjacent to the azide group and another signal at δ = 52.56 ppm reflects the presence of C-2 of the glucose ring. Final proof for the azide **129** comes from ESI-MS, which shows m/z for [C₁₆H₂₂N₄O₁₀+Na]⁺ in the range of 453.1, identical to the calculated value 453.1.

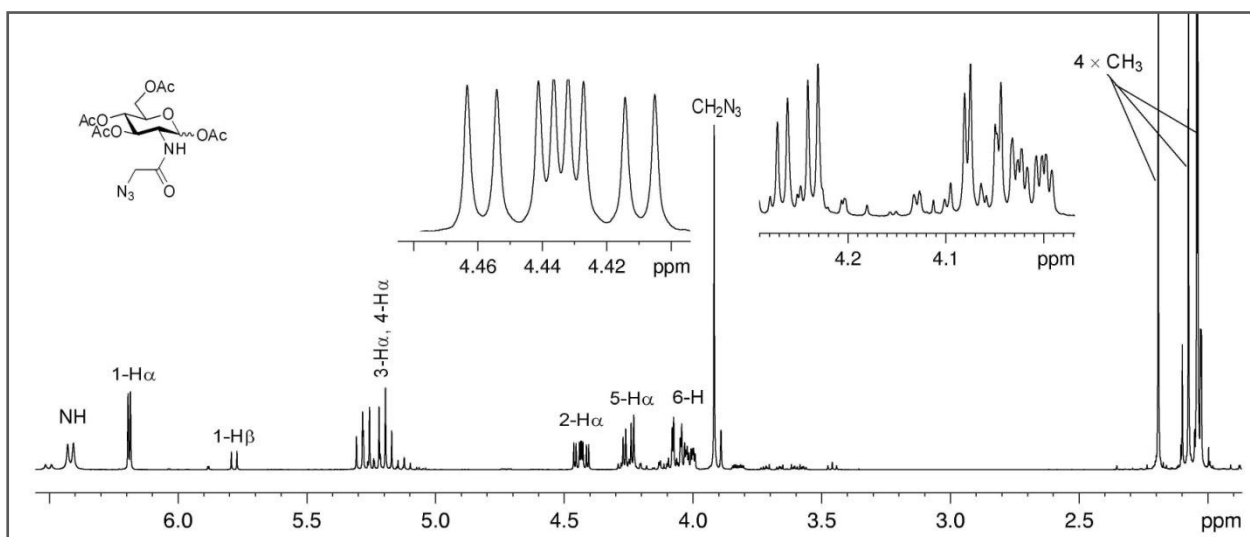


Figure 7.25: ^1H -NMR spectrum of Ac_4GlcNAz **129** (CDCl_3 , 400 MHz).

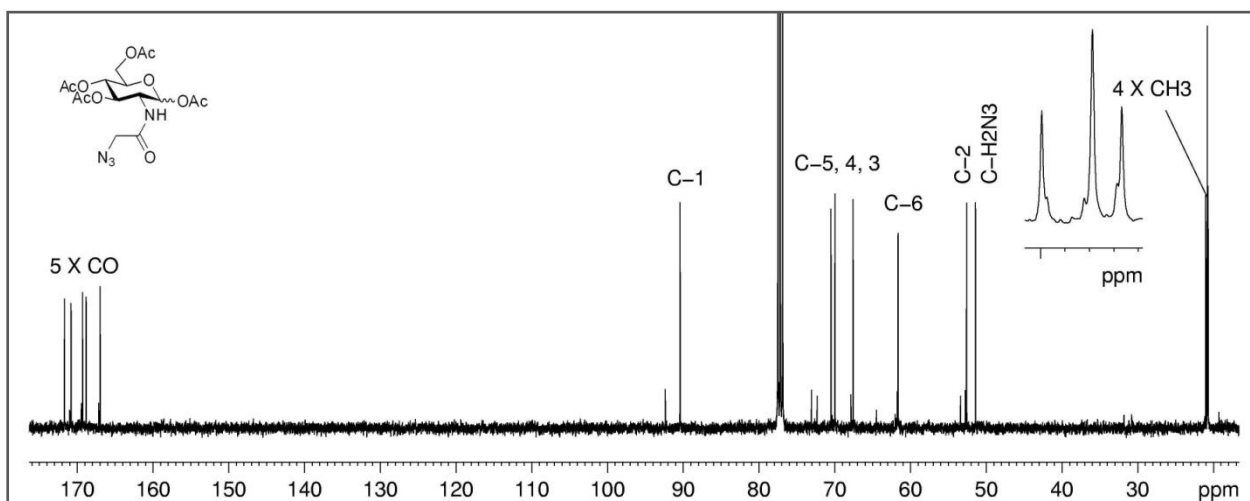
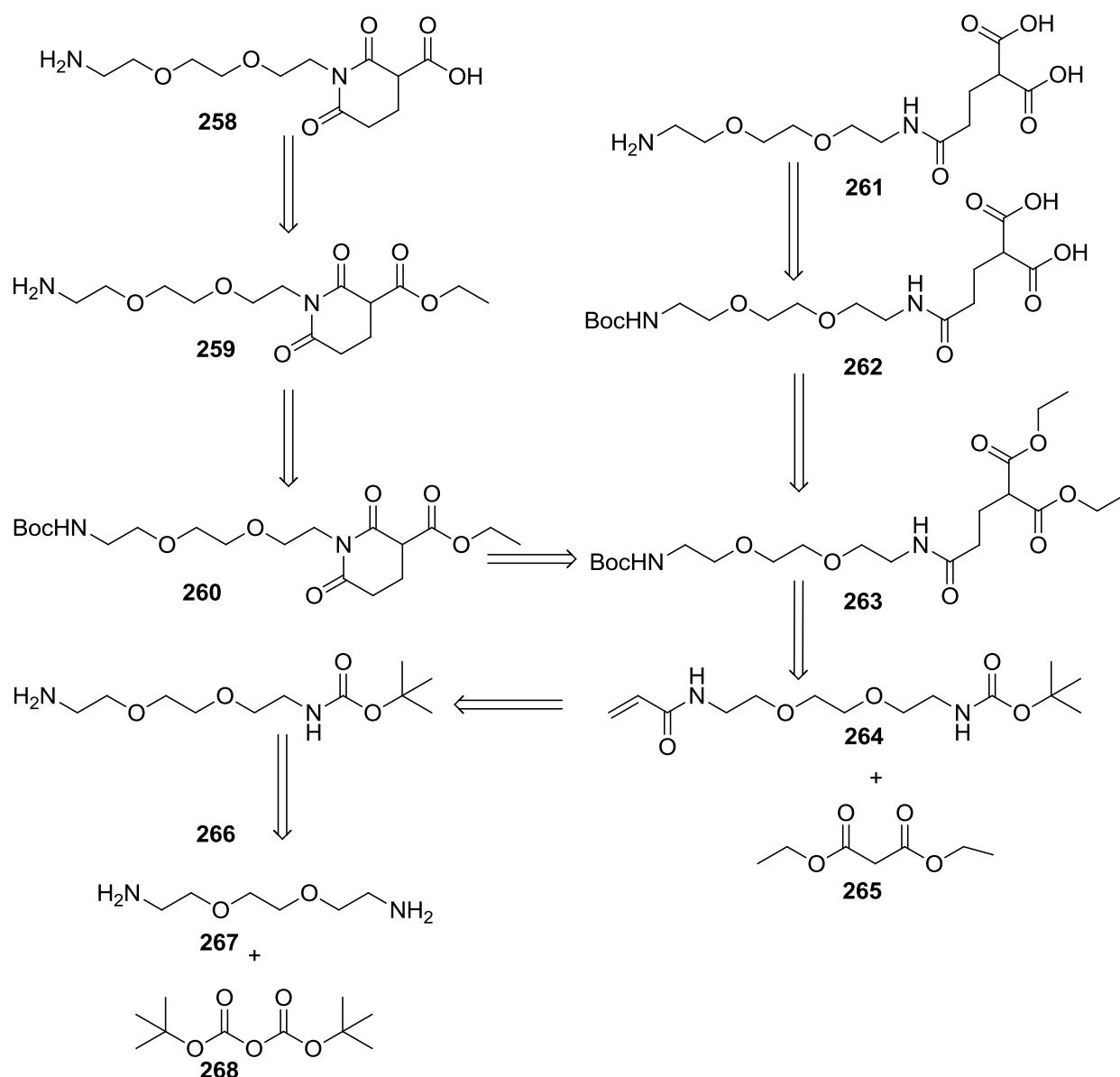


Figure 7.26: ^{13}C -NMR spectrum of Ac_4GlcNAz **129** (CDCl_3 , 100 MHz).

7.7 Synthesis of Dicarboxylic Acid and Glutarimide Derivatives

In search of our second goal, the modification of the cell surface polarity, our next strategy was the development of reagents that can bind to the cell surface directly through their counter partners. The cell surface properties, especially the polarity are vitally important for the behaviour of the living species. An important example comes from trypanosomes, which have a strong negatively charged cell surface due to the carboxylate ions of the cell surface sialic acids. In order to change the cell surface polarity, we planned to develop dicarboxylic acids and glutarimide derivatives with linkers containing polyoxo functionality. A retrosynthetic route for the target molecules is described in scheme 7.37.



Scheme 7.37: Retrosynthetic pathway for dicarboxylic acids and glutarimide derivatives.

7.7.1 Selective protection of amines

To start with diamines, the first task was selective mono protection of diamines. The protecting groups, which may be applied for this purpose, are shown in fig. 7.27. The reactivity of all these groups is different with amines and in some cases harsh conditions are required, e.g. high temperature almost 80 °C in DMF. On the other hand, the deprotection of azide, Fmoc, and Cbz requires H_2 in the presence of Pd/C. After a literature review, we decided to use of Boc functional group because of the ease of application through Boc anhydride (Boc_2O) and its stability towards catalytic hydrogenation and strong basic and nucleophilic conditions.^[216-217]

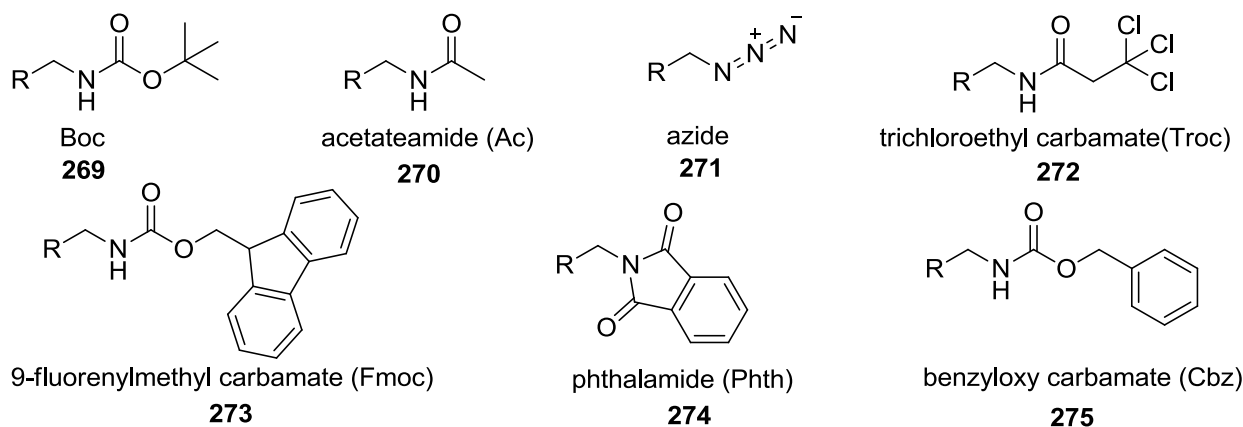
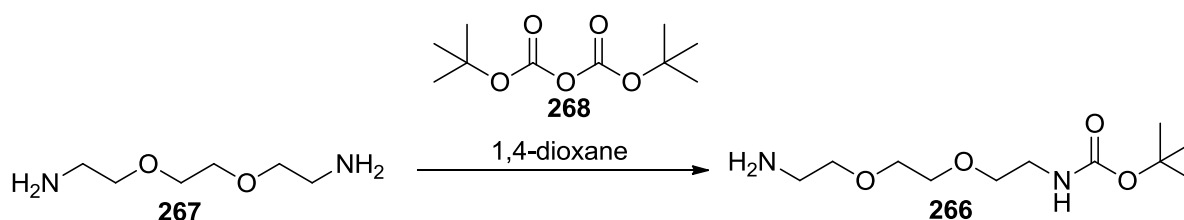


Figure 7.27: Different groups for the protection of amines.

The work was started with mono protection of diamine **267** by treatment with Boc anhydride (di-*tert*-butyl dicarbonate) **268** in dioxane as described in scheme 7.38. The solution of Boc₂O in dioxane was slowly added over a period of 5 h at rt to the solution of diamine. To avoid the protection of both groups, the diamine was used in excess (10 equiv.). The reaction mixture was further stirred overnight and the solvent was evaporated *in vacuo*. The residue was dissolved in water and the product was extracted with DCM. The crude product was purified by flash chromatography, eluting with DCM/MeOH (30:1) with 1% NH₄OH (30%) to afford the carbamate **266** (50-67%) as clear oil.



Scheme 7.38: Synthesis of carbamate by treatment of diamine **267** with Boc anhydride.

Table 7.1: Mono-protection of diamines with Boc anhydride.

Sr. No.	Diamines	Product	Yield [%]
1	$\text{H}_2\text{N}-(\text{CH}_2)_2-\text{O}-(\text{CH}_2)_2-\text{NH}_2$ 267	$\text{BocHN}-(\text{CH}_2)_2-\text{O}-(\text{CH}_2)_2-\text{NH}_2$ 266	50
2	$\text{H}_2\text{N}-(\text{CH}_2)_3-\text{O}-(\text{CH}_2)_3-\text{NH}_2$ 276	$\text{BocHN}-(\text{CH}_2)_3-\text{O}-(\text{CH}_2)_3-\text{NH}_2$ 280	51
3	$\text{H}_2\text{N}-(\text{CH}_2)_2-\text{O}-(\text{CH}_2)_2-\text{NH}_2$ 277	$\text{BocHN}-(\text{CH}_2)_2-\text{O}-(\text{CH}_2)_2-\text{NH}_2$ 281	52
4	$\text{H}_2\text{N}-(\text{CH}_2)_3-\text{O}-(\text{CH}_2)_4-\text{O}-(\text{CH}_2)_3-\text{NH}_2$ 278	$\text{BocHN}-(\text{CH}_2)_3-\text{O}-(\text{CH}_2)_4-\text{O}-(\text{CH}_2)_3-\text{NH}_2$ 282	50
5	$\text{H}_2\text{N}-(\text{CH}_2)_6-\text{NH}_2$ 279	$\text{BocHN}-(\text{CH}_2)_6-\text{NH}_2$ 283	67

7.7.1.1 Discussion on the NMR spectra of Boc amine 266, 80-83

The structures of Boc-protected amines were justified by NMR analysis and a broad singlet at $\delta = 5.12$ ppm in ^1H -NMR spectrum of **266** shown in fig. 7.28 is assigned as a NH of the carbamate. A singlet at $\delta = 1.41$ ppm indicates the presence of nine hydrogen atoms from three methyl groups of Boc. A singlet appearing in the range of $\delta = 3.65$ ppm representing four hydrogen atoms assigned as $1''\text{-H}_2$ and $2''\text{-H}_2$. Two triplets at $\delta = 3.47$ and 3.52 ppm ($J = 5.2$ Hz each) represent $2'\text{-H}_2$ and $1'''\text{-H}_2$. One from the rest of two signals, a dt at $\delta = 3.34$ ppm ($J = 10.4, 5.3$ Hz) corresponds to $1'\text{-H}_2$, on the other hand a triplet (t) at $\delta = 2.85$ ppm ($J = 4.9$ Hz) indicates $2'''\text{-H}_2$. A broad singlet (br s) at $\delta = 1.78$ ppm indicates two hydrogen atoms of amino group. The product was further confirmed by ^{13}C -NMR spectrum shown in fig. 7.29 and a signal at $\delta = 156.46$ ppm indicates the carbonyl group of the Boc. Furthermore, a signal at $\delta = 79.23$ ppm corresponds to the quaternary carbon of carbamate, confirmed by DEPT analysis, and another intense signal at $\delta = 28.51$ ppm represents three methyl groups of Boc. The rest of the signals in the range of $\delta = 44.5\text{--}73.5$ ppm confirm the existence of amine **267**. Thus, both of the NMR spectra indicate the signals of Boc along with diamine and confirm the presence of protected amine **266**. The compounds **280**, **281**, **282** and **283** were also investigated by NMR spectroscopy, which showed the signals of Boc in the same range mentioned in **266** along with the signals of the appropriate diamine. The mono-protection of diamines was further confirmed by mass analysis either by ESI-MS and/or MALDI.

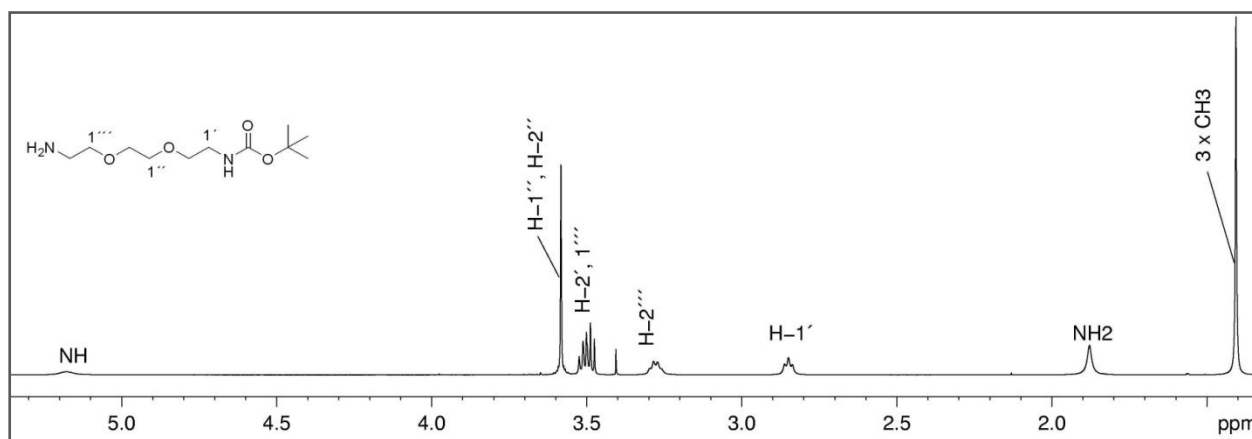


Figure 7.28: ^1H -NMR spectrum of the carbamate **266** (CDCl_3 , 400 MHz).

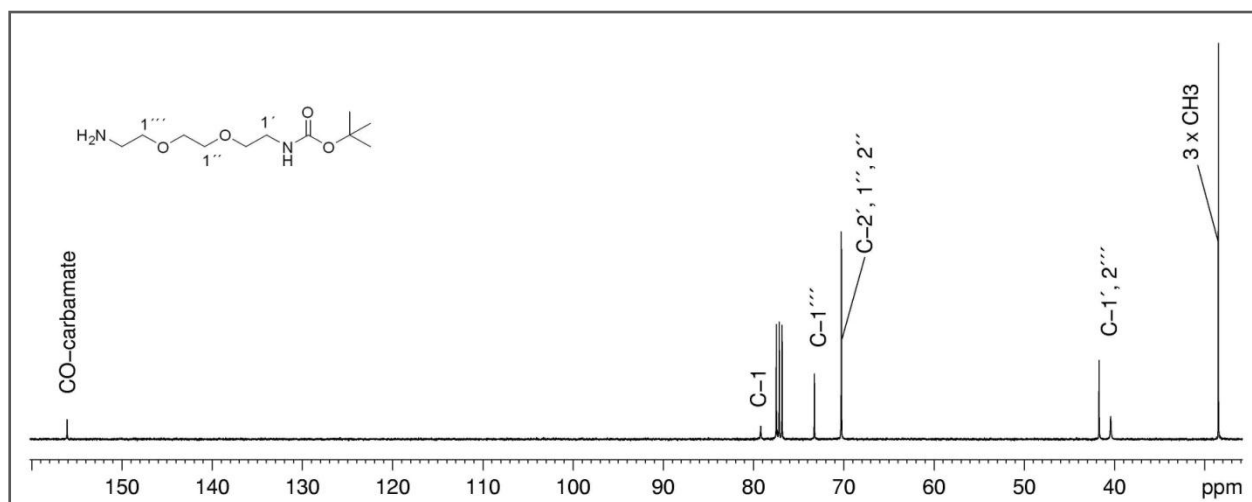
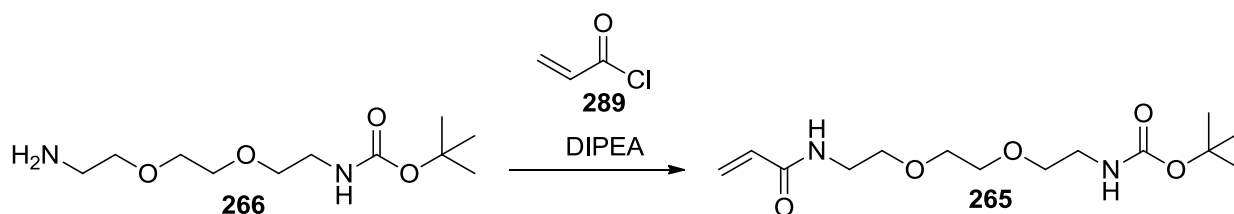


Figure 7.29: ^{13}C -NMR spectrum of the carbamate **266** (CDCl_3 , 100 MHz).

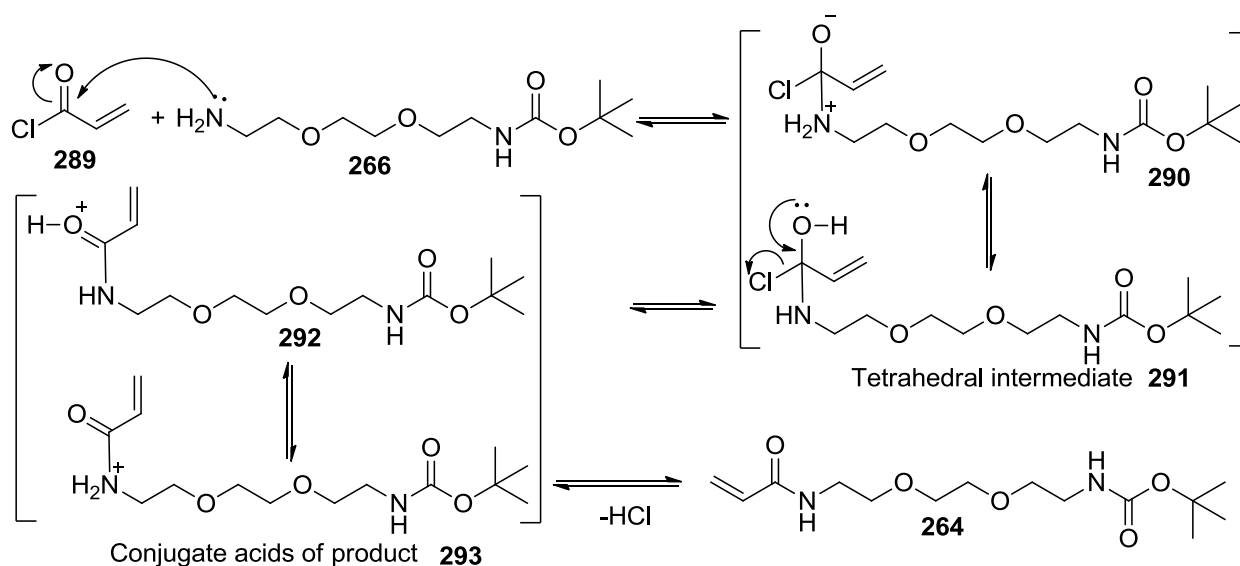
7.7.2 Synthesis of acryloyl amides

Our approach designed for the synthesis of the target glutarimide derivatives and dicarboxylic acids was based on the development of a linker with the diamine chain, which can undergo carboxylic acid synthesis. It was envisioned that the malonic esters are ideal moieties for our approach and the acidic nature of the methylene hydrogen atoms of these esters can be a possible handle for our strategy, which can be exploited for different kind of substitutions on the malonates. A facile and convenient approach may be the use of these acidic hydrogen atoms through Michael addition. However, Michael addition requires α,β -unsaturated carbonyl system in addition to the Michael acceptor i.e. enolate ion. In order to develop α,β -unsaturated carbonyls, the next step after mono-protection of diamines was the generation of acryloyl amide functionality by treatment with acryloyl chloride **289** as shown in scheme 7.39.



Scheme 7.39: Synthesis of acryloyl carbamate **265**.

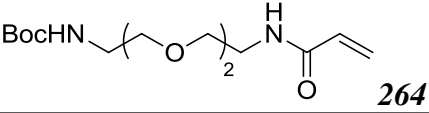
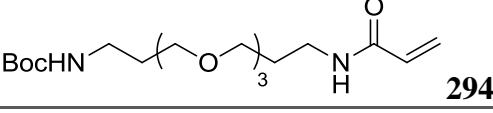
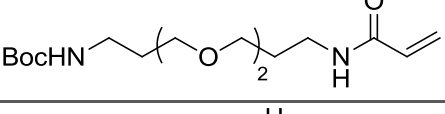
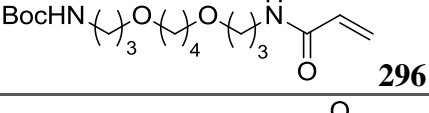
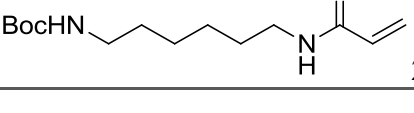
In first step, the attacking nucleophile is added to the carbonyl carbon changing the hybridization of carbon from sp^2 to sp^3 and creates a tetrahedral intermediate as described in scheme 7.40. In second step, elimination of the leaving group takes place and carbon returns from sp^3 to sp^2 hybridization.



Scheme 7.40: Schematic representation for addition elimination mechanism of acryloyl chloride with amine.

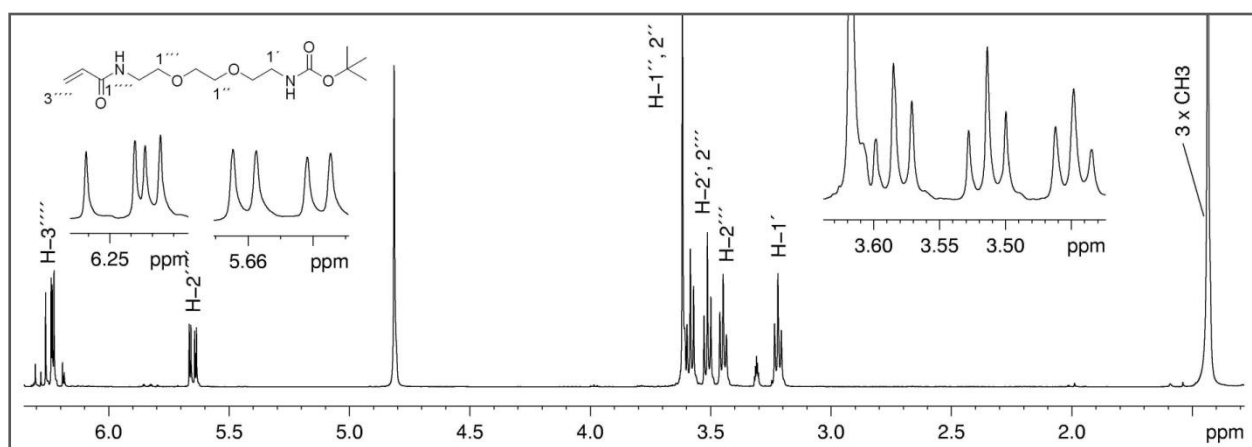
After successful synthesis of the desired acryloyl carbamate **264** the reaction was successfully extended to the synthesis of **294**, **295**, **296** and **297**. However, the reaction time was slightly varied and the detailed informations are listed in table 7.2.

Table 7.2: An overview of the reactivity of acryloyl chloride with amines catalyzed by DIPEA.

Sr. No.	Educt	Acryloyl carbamate	Time (h)	Yield[%]
1	266		2.5	93
2	280		2.0	81
3	281		3.0	97
4	282		2.0	87
5	283		2.5	95

7.7.2.1 Discussion on the NMR spectra of acryloylcarbamate **264** and **294-297**

The ^1H -NMR spectrum of the acryloylcarbamate **264** shown in fig. 7.30, displays a doublet of doublet (dd) in the range of $\delta = 6.25$ ppm ($J = 17.1, 9.2$ Hz) corresponds to $3_{\text{trans}}^{\text{H}_2}$ and another signal (dd) at $\delta = 6.21$ ppm ($J = 17.2, 2.9$ Hz) indicates $3_{\text{cis}}^{\text{H}_2}$. A dd at $\delta = 5.65$ ppm ($J = 9.2, 2.8$ Hz) denotes 2^{H} and these three signals confirm the presence of olefinic double bond of acryloyl functionality. The remaining signals are in the same range as described in carbamate **266** with the only exception being that a triplet at $\delta = 2.85$ ppm in the educt is shifted downfield to $\delta = 3.45$ ppm ($J = 5.5$ Hz) due to the shielding effect of the amide nitrogen.

Figure 7.30: ^1H -NMR spectrum of acryloyl carbamate **264** (CDCl_3 , 400 MHz).

The product **264** was further confirmed by ^{13}C -NMR spectrum shown in fig. 7.31 and a signal at $\delta = 165.73$ ppm shows the presence of the carbonyl carbon of acryloyl amide. Furthermore, a signal at $\delta = 156.03$ ppm indicate the Boc carbonyl carbon. Presence of the acryloyl double bond is confirmed by the signals at $\delta = 130.91$ ppm from the carbon on α position to the amide and a signal at $\delta = 126.13$ ppm from the terminal carbon. The rest of the signals of ^{13}C -NMR are in the same range as that of the educt **266** including a signal at $\delta = 28.34$ ppm assigned as three methyl groups of Boc. A signal at $\delta = 79.22$ ppm represents the quaternary carbon of the Boc, which is further confirmed by ^{13}C -DEPT analysis. The presence of the target acryloyl carbamate **264** was further confirmed by ESI-MS, which showed m/z for $[\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_5 + \text{Na}]^+$ in the range of 325.19. The same analyses were applied for the structure elucidation of the compounds **294**, **295**, **296** and **297**.

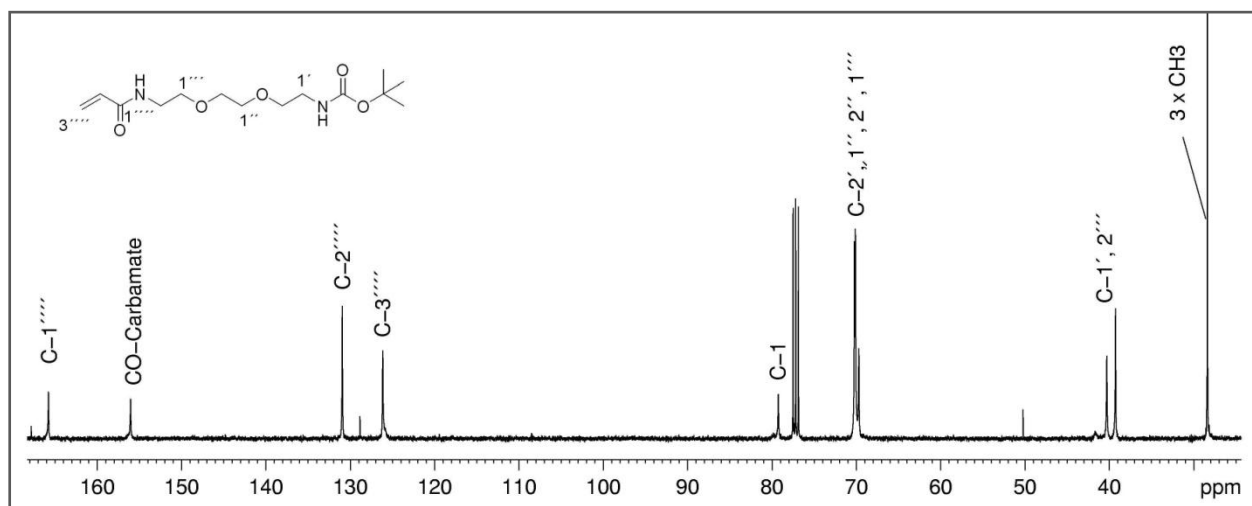
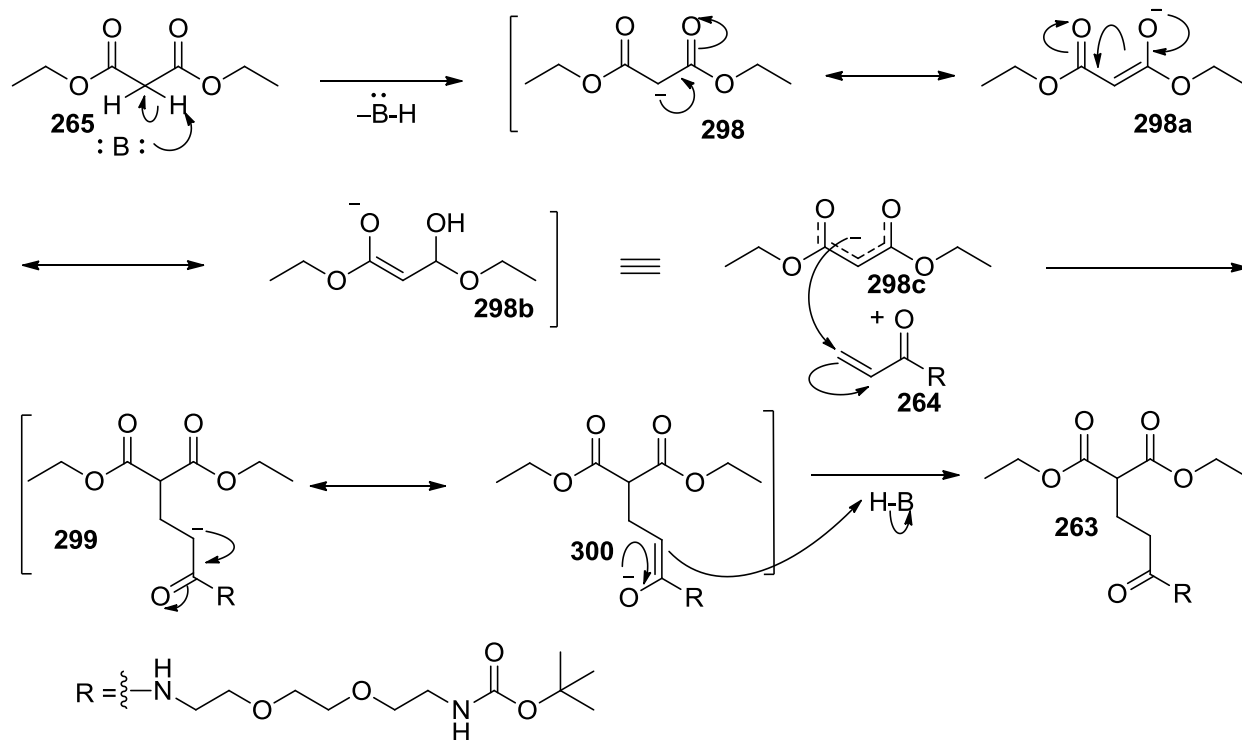


Figure 7.31: ^{13}C -NMR of the acryloyl carbamate **264** (CDCl_3 , 100 MHz).

7.7.3 Michael addition

In order to achieve the desired diamines with dicarboxylic acid and glutarimide functionalities, the next step after successful synthesis of acryloyl amides was their condensation with malonic esters. Malonic esters are the compounds, which can be selectively hydrolyzed to dicarboxylic acids easily under mild conditions. We envisioned that in addition to C-C bond synthesis, malonic esters can also undergo intramolecular aminolysis to give the cyclic molecule. We focused our attention to investigate the proposed reactivity of malonates and to achieve the glutarimide derivatives through a single step Michael addition-cyclization in the domino reaction pattern.^[218-219] Michael addition is a nucleophilic addition of an enolate to α,β unsaturated carbonyl compound, which takes place in the presence of a strong base. Mechanism of the reaction is quite straightforward with each step being in equilibrium with relative strength of base

and the type of the carbonyl moiety. The reaction starts with trapping of acidic α -hydrogen by a strong base, forming an enolate intermediate **298**, stabilized by the resonance contributing structures as shown in scheme 7.41.

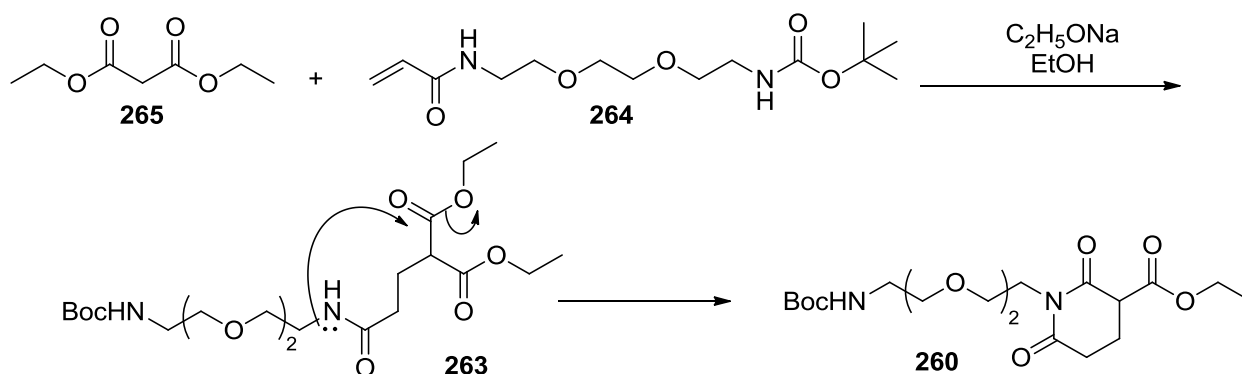


Scheme 7.41: Reaction mechanism of Michael addition catalyzed by a strong base.

In the second step α,β -unsaturated carbonyl **264** is attacked by the enolate, which undergoes a 1,4-conjugate addition to the Michael acceptor and finally the product is stabilized by conjugate acid, formed in first step. The overall driving force for the conjugate addition is enthalpic change that accompanies the replacement of a π bond with σ bond. The selection of a base is also critical and plays a pivotal role in the reaction kinetics, not only for Michael addition but also for the ester aminolysis. There is much evidence that the amines can undergo aminolysis with esters in the presence of strong basic conditions. To achieve domino Michael addition-aminolysis, we tried different bases taking initiative with LiClO_4 both in the presence of solvent and solvent free conditions. However, the reaction did not take place in both cases even different solvents could not improve the results. Our next attempts beyond LiClO_4 were the use of DIPEA and NEt_3 but all these efforts failed. Finally, we decided to increase the basic strength by making the use of some strong bases like sodium alkoxide (MeONa or EtONa). The solvent used for the reaction was corresponding to the alkyl group of the base i.e. MeOH with MeONa and EtOH with EtONa . Initially, the reaction was started with commercially available MeONa in MeOH ; however, the reaction was not successful. Later on, the preparation of EtONa in lab by treatment of EtOH with

Na metal resulted in the desired Michael addition. The reaction afforded two fractions i.e. acyclic **263** (28%) along with cyclic molecule **260** (57%). The synthesis of **260** as a result of the intramolecular aminolysis strengthened our prediction about the ring closure. The ring closure took place by the attack of acryloylamide amine on one of the malonic ester carbonyl carbon leading to the synthesis of a six membered ring with a structural resemblance of glutarimide as described in scheme 7.42. The catalyzed mechanism of aminolysis has been investigated by different groups, revised repeatedly but still without any definitive outcome.^[220-222] Three different mechanisms are expected for the reaction:

- concerted mechanism where transfer of the hydrogen atom from amine to the leaving group takes place simultaneously
- stepwise mechanism where no charged intermediates are present
- stepwise mechanism through zwitter-ionic species.

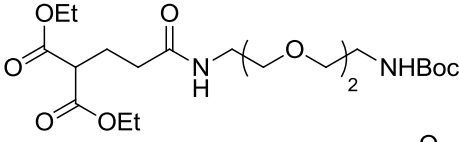
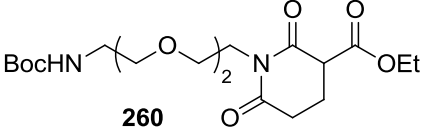
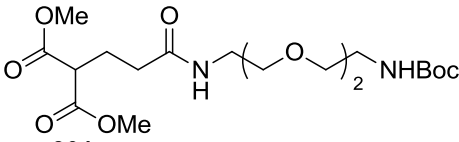
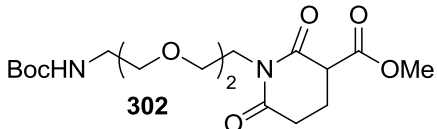
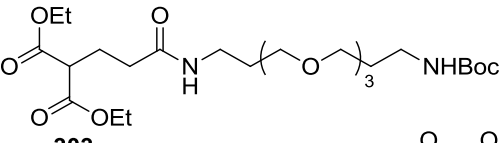
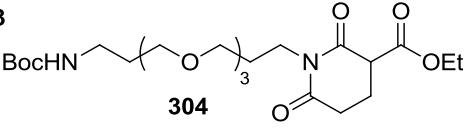
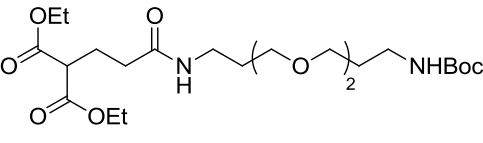
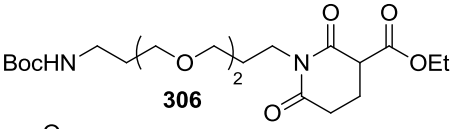
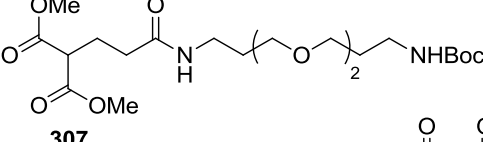
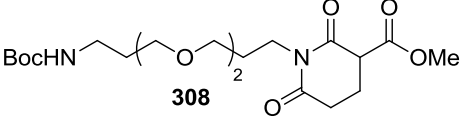


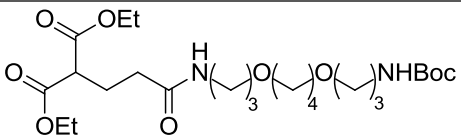
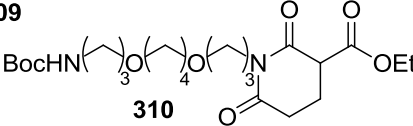
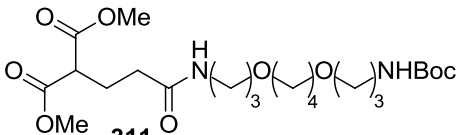
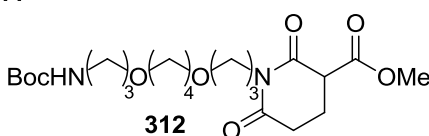
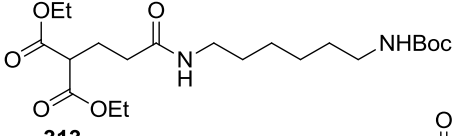
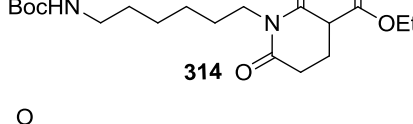
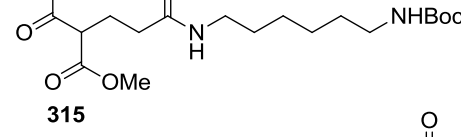
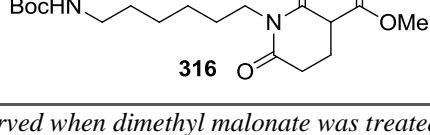
Scheme 7.42: Domino Michael addition-aminolysis giving glutarimide.

We assumed that the over concentration of EtONa (3 equiv.) was responsible for the ring closure and by using the base 1-1.5 equiv. we should be able to get acyclic product exclusively. Our assumption became true when a reaction in EtOH with 1.5 equiv. EtONa and diethylmalonate yielded open-chain product **263** exclusively (75%) rather than the closed ring. After successful synthesis of compound **260** and **263**, we extended the reaction conditions to different acryloyl carbamates and each time we got the mixture of the cyclic and acyclic moieties with different ratios. After investigations of the reaction with EtONa and diethylmalonate, we turned our attention to make the use of dimethyl malonate in the presence of MeONa. Methyl esters, which are easy to hydrolyze/aminolyze as compared to the ethyl esters were assumed to show better results towards ring closure. A reaction between acryloyl carbamate **264** and dimethyl malonate in MeOH/MeONa was performed for 24 h. The concentrate obtained after workup and evaporation of the solvent was subjected to flash chromatography and the product obtained was analyzed by NMR and mass spectrometry. After structure elucidation of the products, the

approach was applied on the carbamates **294**, **295**, **296** and **297**, and the results obtained are summarized in table 7.3.

Table 7.3: Michael addition acryloyl carbamates with diethyl/methyl malonate.

Sr.No.	Educt	Products (Cyclic and acyclic)	Mal.	Base	Product Yield [%]*	
			R	R (equiv.)	Acyclic	Cyclic
1	264	 263	Et	Et (1.5)	263 [75]	260 [n.d]
		 260	Et	Et (3)	263 [28]	260 [57]
		 301	Me	Et (3)	263 [n.d]	260 [85]
		 302	Me	Me (1.5)	301 [77]	302 [n.d]
2	294	 303	Et	Et (1.5)	303 [49]	304 [22]
		 304				
3	295	 305	Me	Et (3)	305 [n.d]	306 [89]
		 306				
		 307	Me	Me (3)	307 [59]	308 [26]
		 308				

4	296	 309	Et	Et (3)	309[64]	310[16]
		 310	Me	Et (3)	309[n.d]	310[83]
		 311	Me	Me (3)	311[25]	312[55]
		 312				
5	297	 313	Et	Et (3)	313[23]	314[61]
		 314	Me	Et (3)	313[n.d]	314[72]
		 315	Et	Me (3)	315[57]	316[28]
		 316				

*Cross esterification was observed when dimethyl malonate was treated with EtONa/EtOH and acryloyl carbamate.

It was confirmed by both studies that the compound obtained was surprisingly an open-chain ester **301**, which contradicted our expected ester aminolysis. It was also observed that the Michael addition in the presence of methyl malonate/MeONa was slower than that of diethyl malonate/EtONa. However, when the reaction was repeated with high concentration of MeONa, ring closure took place as a side product. This low reactivity of methyl malonate was further envisaged in the presence of EtONa/EtOH. For this purpose, we conducted a reaction between the acryloyl carbamate **264** and dimethyl malonate in EtONa/EtOH. This time, the reaction showed the intramolecular aminolysis exclusively without any traces of the open-chain molecule. From this observation, we concluded that the base strength was an important factor to drive the reaction towards the aminolysis leading the molecule to ring closure. This reactivity mode of the aminolysis under mentioned conditions can be explained in the aspect of basic strength. EtONa is a strong base with pKa value 19.2 in EtOH^[223] on the other hand the pKa value of MeONa is

18.3 in MeOH.^[224] This pKa value difference of almost 1 order of magnitude makes EtONa more favorable for aminolysis than that of MeONa. During the reaction of dimethyl malonate in the presence of EtONa/EtOH transesterification was observed to take place solely and the methyl group of the ester was completely substituted by the ethyl group. Thus in the light of all above discussion, it can be concluded that the ring closure was more facile in EtONa, which was facilitated by intramolecular base catalyzed aminolysis.

7.7.3.1 Discussion on the NMR spectra of Michael products **260**, **263** and **301**

The ¹H-NMR spectrum of the first fraction **263** with *R_f* 0.31 in DCM/MeOH (10:1 with 1% NH₄OH, 30% solution) is shown in fig. 7.32. It displayed a quartet in the range of $\delta = 4.13$ ppm (*J* = 6.1 Hz) along with a doublet of triplet at $\delta = 3.95$ ppm (*J* = 7.1, 1.5 Hz), each possessing two hydrogen atoms from 2 × CH₂ of diethyl malonate. The upfield shift of signals of allyl hydrogen atoms in the educt from the range of $\delta = 5.1$ -6.5 ppm to the range of $\delta = 2.1$ -2.7 ppm in product, indicates the disappearance of a double bond as a result of the successful Michael addition. The signal of the methylene hydrogen atoms at the α -position to the ester carbonyls in malonate is very weak due to the interchange of proton with deuteron of deuterated solvent. However, in the case of a nonprotic solvent like CDCl₃, it appears in the range of $\delta = 3.3$ -3.8 ppm in the form of a doublet of doublet (dd).

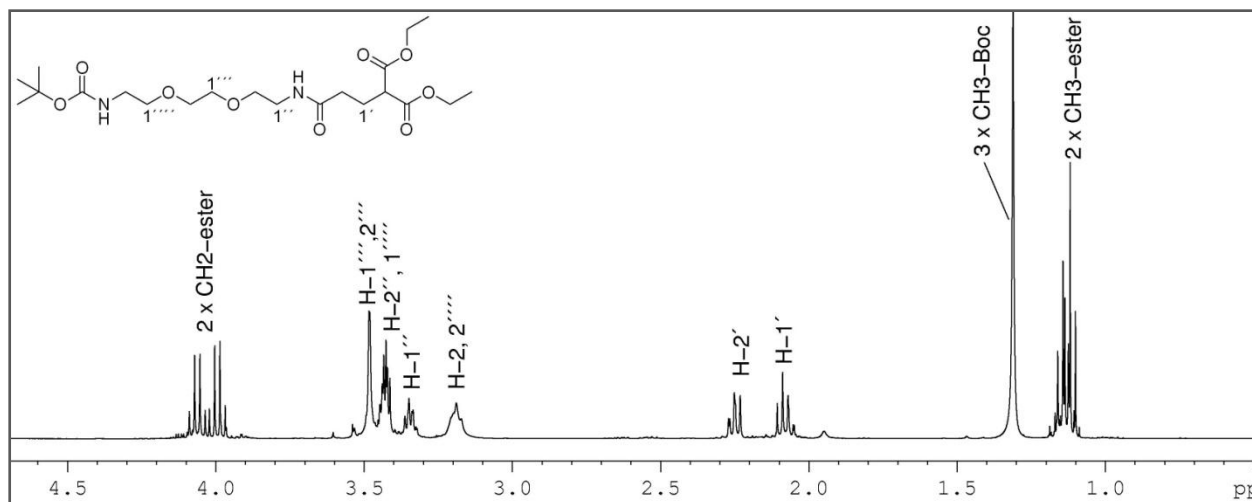


Figure 7.32: ¹H-NMR spectrum of oxo-aza malonate **263** (CDCl₃, 400 MHz).

The product **263** is further confirmed by ¹³C-NMR spectrum shown in fig. 7.33 and two signals in the range of $\delta = 168.6$ -168.9 ppm represent two carbonyl carbons of the malonic ester in addition to the two signals in the range of $\delta = 156.0$ -166.7 ppm from C-3' and CO of the carbamate. A signal at $\delta = 49.21$ ppm represents the tertiary carbon C-2, α to the malonate carbonyls, further confirmed by ¹³C-DEPT spectrum. Two signals in the range of $\delta = 61.5$ -

62.2 ppm come from two CH₂ of ester in addition to a signal at $\delta = 14.19$ ppm, which represent two methyl groups and confirm the acyclic nature of the product. The final proof for acyclic structure of the product **263** comes from mass analysis by ESI-MS indicating m/z for $[C_{21}H_{38}N_2O_9+Na]^+$ in the range of 485.21, identical to the calculated value 485.21.

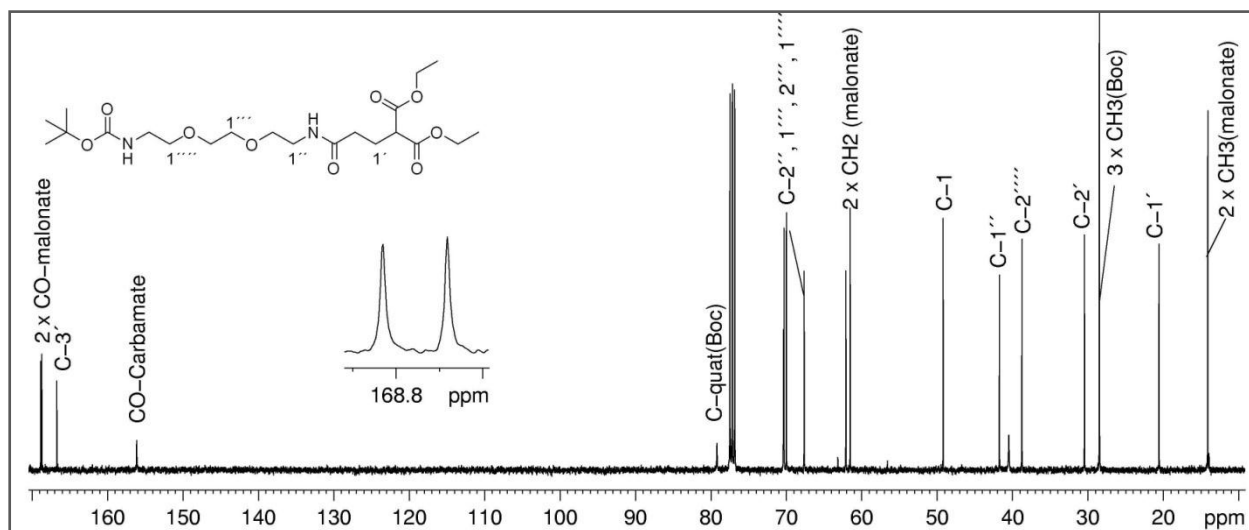


Figure 7.33: ^{13}C -NMR spectrum oxo-aza malonate **263** ($CDCl_3$, 100 MHz).

The 1H -NMR spectrum of the second fraction **260** with R_f 0.19 in DCM/MeOH (10:1 with 1% NH_4OH , 30% solution) is shown in fig. 7.34. A multiplet in the range of $\delta = 4.2$ -4.3 ppm possessing two hydrogen atoms rather than four confirms one ethoxy group of the ester. The cyclic structure of **260** is further confirmed by ^{13}C -NMR spectrum shown in fig. 7.35. It displays only one signal at $\delta = 61.41$ ppm with a single carbon atom instead of two in addition to a signal at $\delta = 13.85$ ppm again with one terminal carbon of the ethyl group of the ester. The remaining signals are similar to those of the first fraction and confirm the cyclic nature of the second fraction. The structure of the compound **260** was further justified by correlation NMR spectroscopy and the six membered cyclic structure of the molecule was confirmed by HMBC spectrum shown in fig. 7.36. It showed a coupling of the two carbonyl carbons with the hydrogen atoms on the carbon adjacent to the nitrogen atom linked to the glutarimide ring. The structural assignment was further confirmed by mass analysis by ESI-MS, which showed m/z for $[C_{19}H_{32}N_2O_8+Na]^+$ 439.2, identical to the calculated value 439.2.

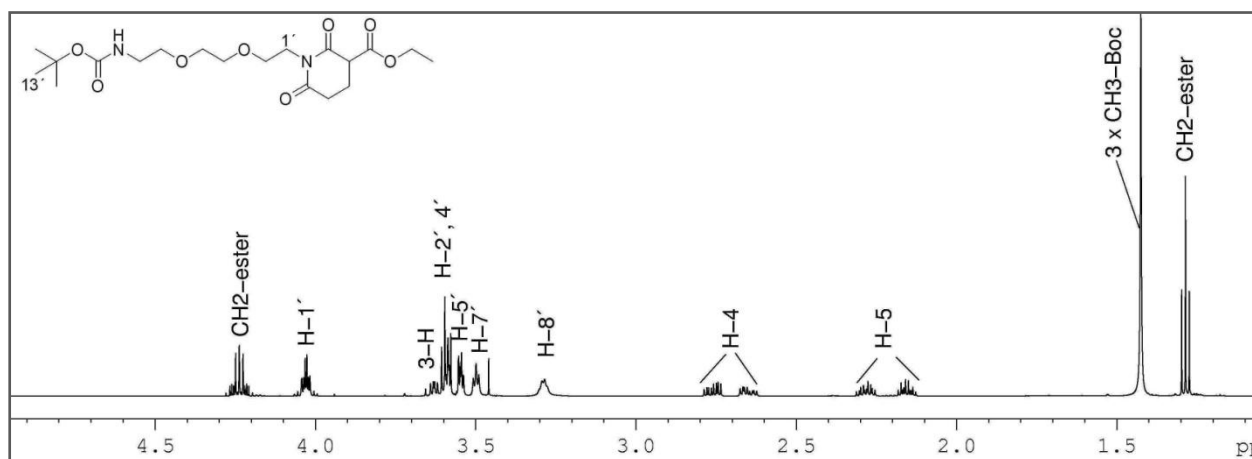


Figure 7.34: ^1H -NMR spectrum of the glutarimide derivative **260** (CDCl_3 , 400 MHz).

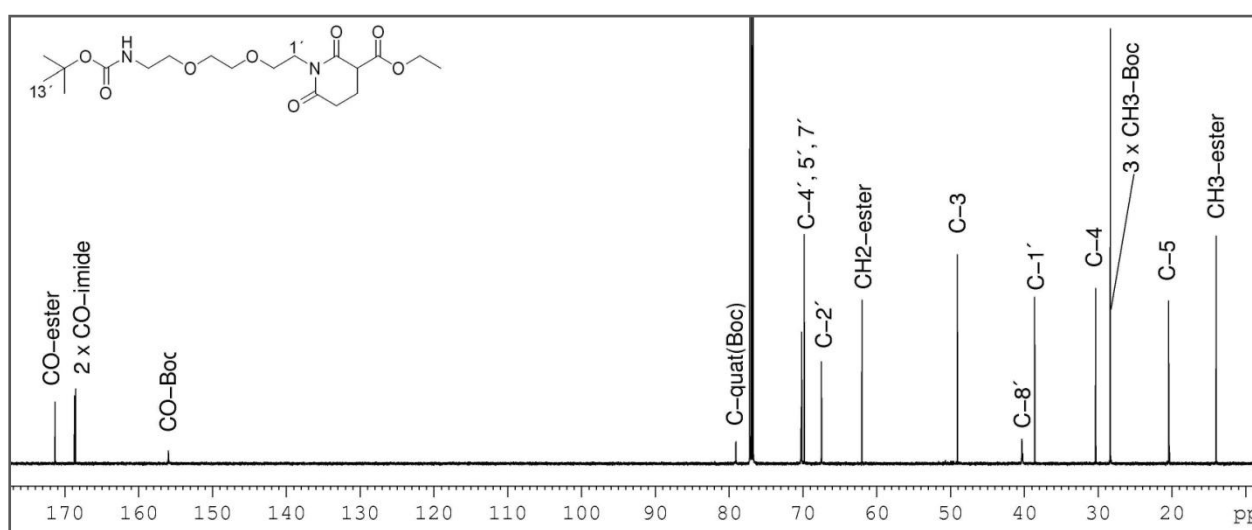


Figure 7.35: ^{13}C -NMR spectrum of the glutarimide derivative **260** (CDCl_3 , 100 MHz).

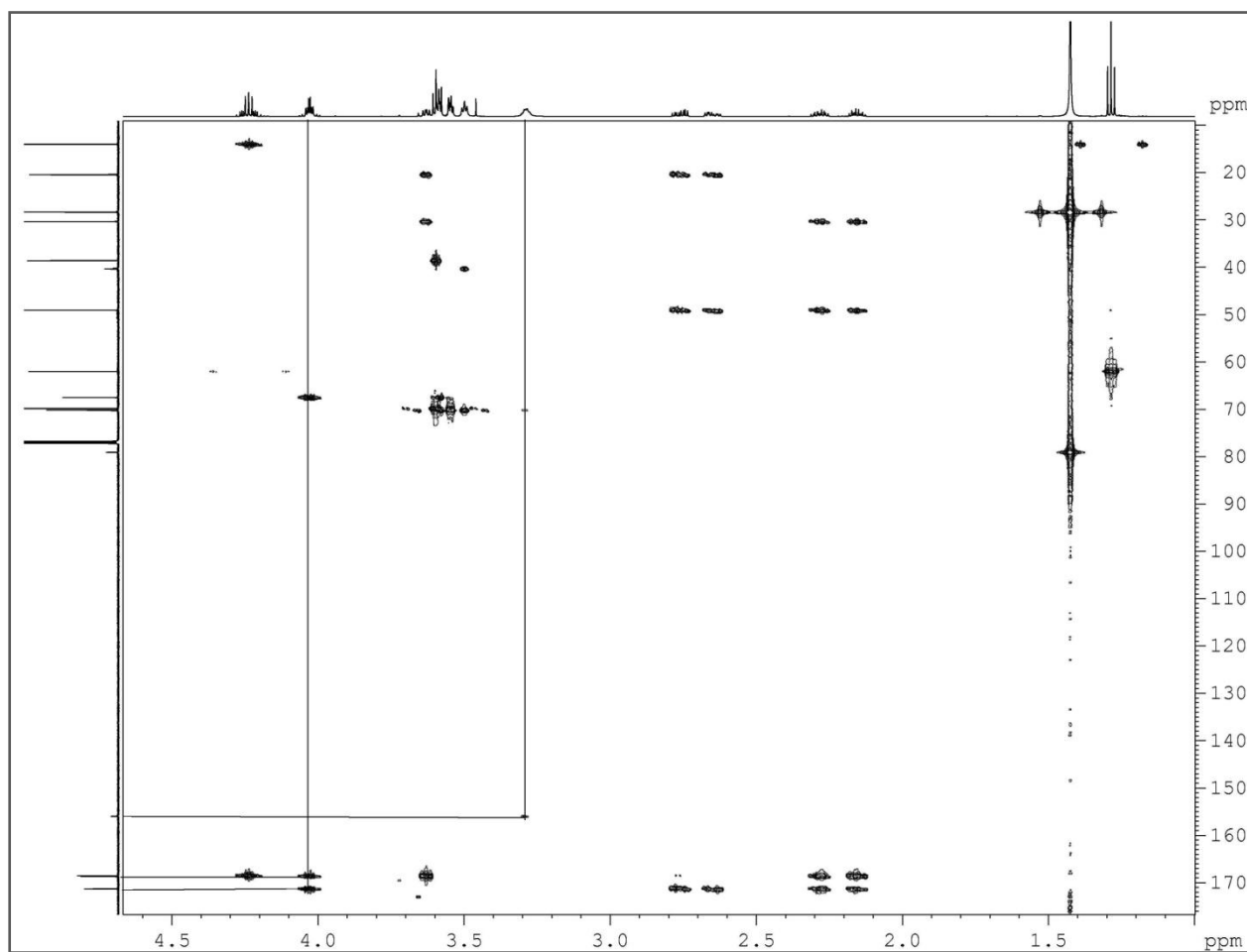


Figure 7.36: HMBC spectrum of the compound **260**.

Similarly, the compound **301** obtained as a result of the reaction between dimethyl malonate and acryloyl carbamate was subjected to the NMR analysis and the ^1H -NMR spectrum of the product revealed the presence of two singlets in the range of $\delta = 3.5$ - 3.6 ppm indicating two methyl groups of the malonate. The rest of the signals are in the same range as that of the acryloyl carbamate **264** as shown in fig. 7.37. The ^{13}C -NMR spectrum of the compound **301** shown in fig. 7.38 displays a signal at $\delta = 173.13$ ppm assigned as two carbonyl carbons of the malonate. A signal at $\delta = 52.31$ ppm indicates the presence of tertiary carbon C-2 on α -position of the malonate carbonyls, which is further confirmed by ^{13}C -DEPT spectrum. Furthermore, two signals in the range of $\delta = 51.3$ - 51.6 ppm come from two methyl carbons of the malonate. The final confirmation of the acyclic nature of compound **301** comes from mass analysis by ESI-MS, giving m/z for $[\text{C}_{19}\text{H}_{34}\text{N}_2\text{O}_9 + \text{Na}]^+$ in range of 457.21, identical to the calculated value 457.21.

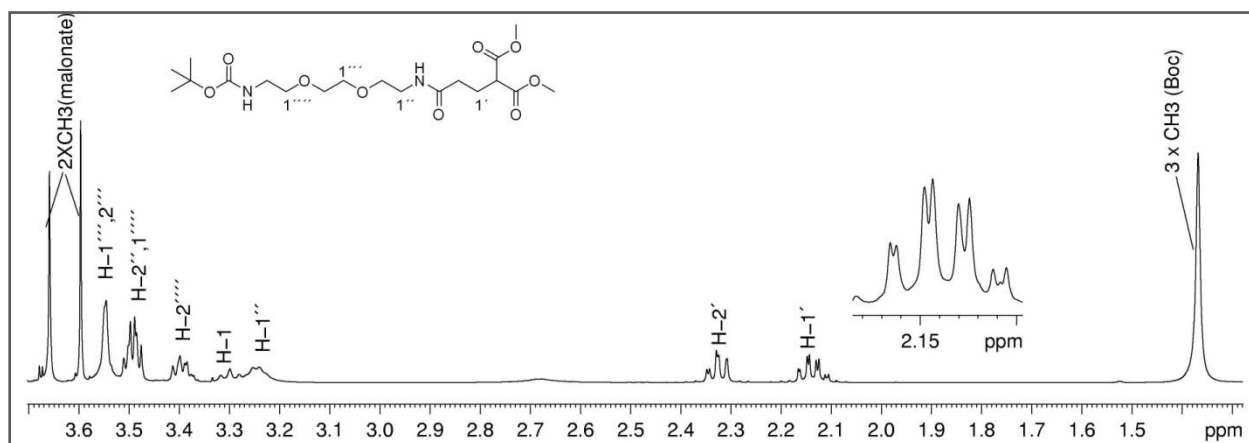


Figure 7.37: ^1H -NMR spectrum of the oxo-aza methylmalonate **301** (CDCl_3 , 400 MHz).

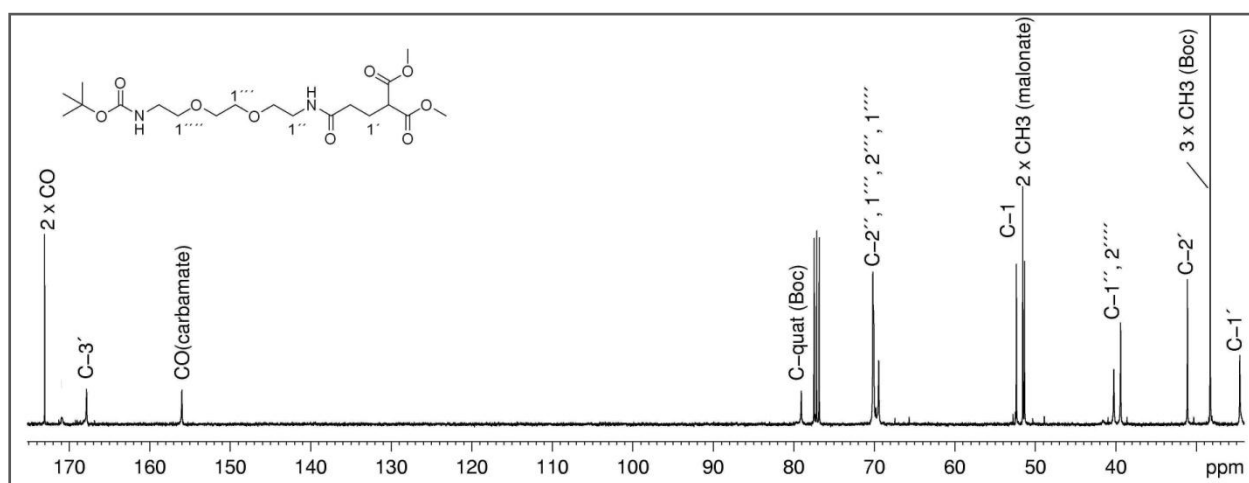
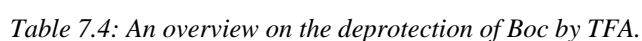


Figure 7.38: ^{13}C -NMR spectrum of the oxo-aza methylmalonate **301** (CDCl_3 , 100 MHz).

7.7.4 Boc deprotection of amines

In order to envisage the effect of acidic media on the ring closure of Michael products, it was planned to investigate the molecules under strong acidic milieu. A domino one-pot Boc deprotection-ring closure through intramolecular aminolysis under strong acidic conditions was designed for our further studies as shown in scheme 7.43. The deprotection of Boc was performed from acyclic ethyl esters and cyclic glutarimides by treatment with 50% TFA in DCM and the reaction was allowed to stir at rt along with continuous monitoring by TLC. On the completion of the reaction with the mixture of **260** and **263**, the solvent was evaporated and the residue was purified by flash chromatography, which afforded the glutarimide derivative **259** as an exclusive product. The reaction confirmed that the esters **260** and **263** underwent aminolysis under strong acidic conditions of Boc deprotection. One possibility under the mentioned reaction conditions might be the interaction between terminal/Boc-protected amine and one of the ester carbonyls of the acyclic molecule **263** to afford a macrocyclic moiety. Another possibility could be the generation of a bicyclic molecule as a result of the interaction between

100

7.7.4.1 Discussion on the NMR spectra of glutarimide ester **259** and acid **261**

The ^1H -NMR spectrum of the compound **259** shown in fig. 7.39 indicates the absence of the singlet at $\delta = 1.37$ ppm in the educts **260** and **263** and shows the disappearance of Boc in the product. The rest of the signals are identical to the educt **260**. As shown in fig. 7.40, the ^{13}C -NMR spectrum also indicates the lack of signals at $\delta = 156.25$ ppm assigned as carbonyl carbon of the carbamate. A signal appearing in the educt at $\delta = 79.09$ ppm representing the quaternary carbon of Boc is also missing. Furthermore, the disappearance of an intense signal at $\delta = 28.39$ ppm, representing three methyl groups of the Boc, confirms the successful deprotection of the amino group. The deprotection was supported by mass analysis (ESI-MS), showing m/z for $[\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_6 + \text{Na}]^+$ in the range of 339.1, in the range of the calculated value 339.1. After the confirmation of the compound **259** by NMR and mass analysis, the approach was applied for the deprotection of more Boc protected amines, one of which was open-chain dicarboxylic acid **262**.

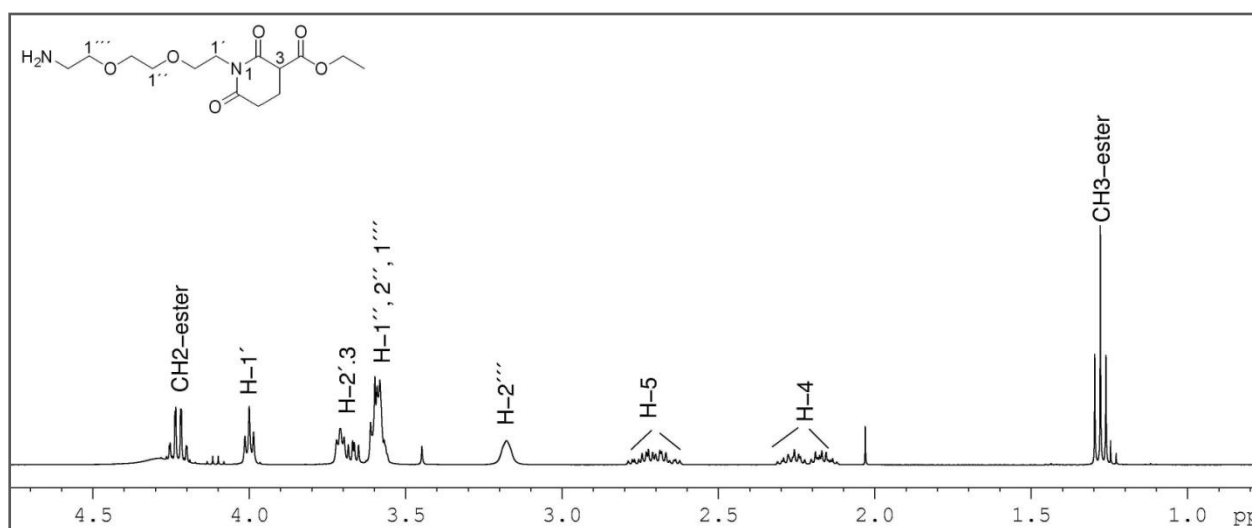


Figure 7.39: ^1H -NMR spectrum of the glutarimide ester **259** (CDCl_3 , 400 MHz).

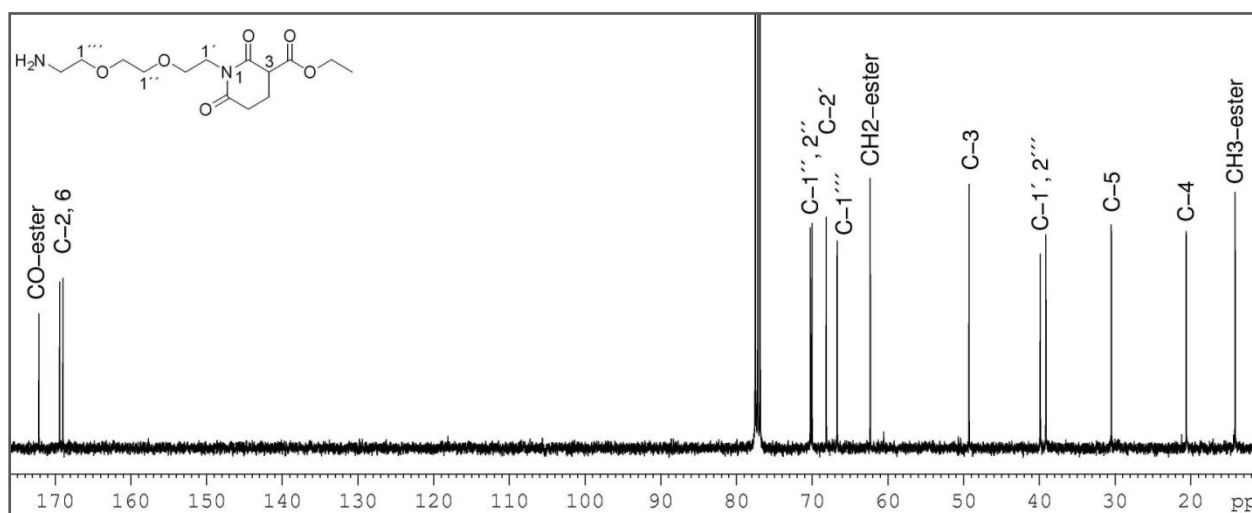


Figure 7.40: ^{13}C -NMR spectrum of the glutarimide ester **259** (CDCl_3 , 100 MHz).

The ^1H -NMR spectrum of **261** as shown in fig. 7.41, lacks a singlet of Boc possessing 9 hydrogen atoms in the educt appeared in the range of $\delta = 1.3$ ppm. However, the presence of the remaining signals, similar to that of the educt **262**, shows that there is no further difference in the structure of the product. During this reaction, no ring closure was observed due to the lack of the reactivity of the carboxylic acids towards aminolysis.

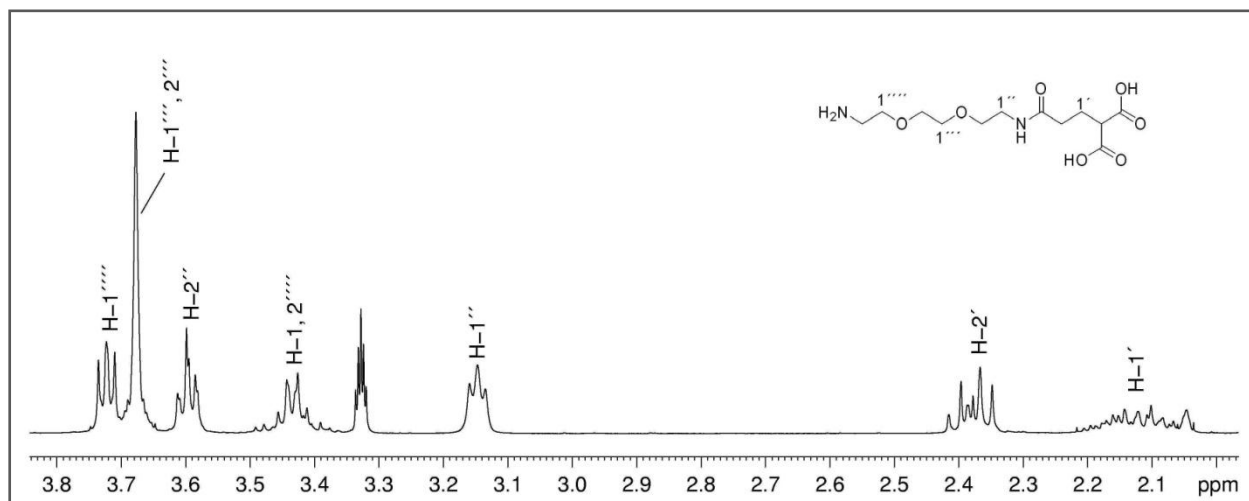


Figure 7.41: ^1H -NMR spectrum of oxo-aza malonic acid **261** (MeOD, 400 MHz).

As shown in fig. 7.42 the ^{13}C -NMR analysis of the molecule **261** gave no signal at $\delta = 156.02$, 79.08 and 28.29 ppm, the characteristic of Boc. Finally the product **261** was confirmed by mass analysis (ESI-MS), showing m/z for $[\text{C}_{12}\text{H}_{22}\text{N}_2\text{O}_7 + \text{Na}]^+$ 329.1 identical to the calculated value 329.1.

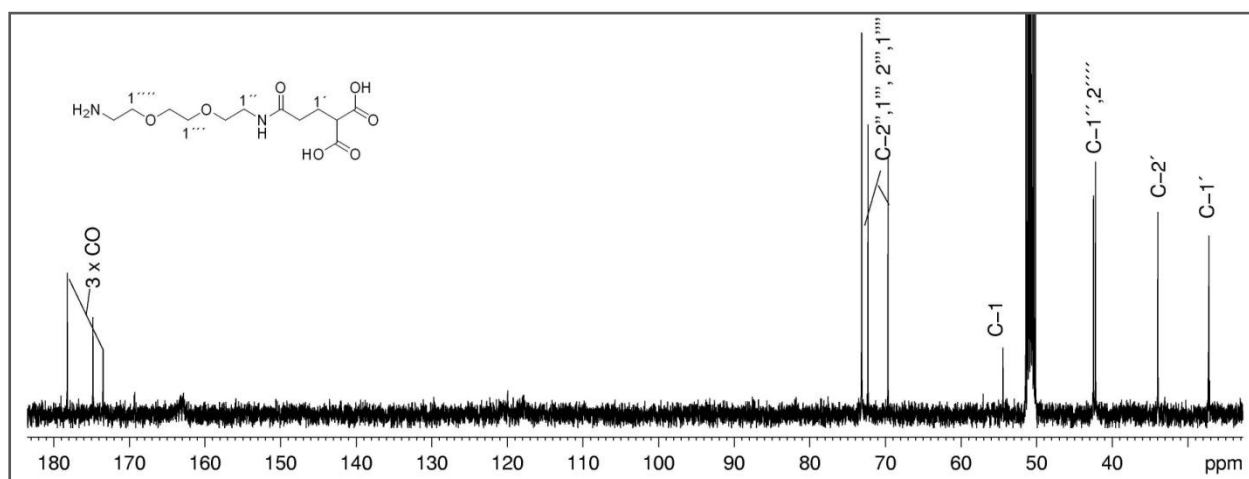
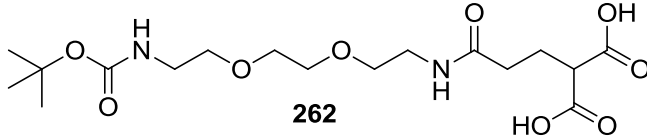
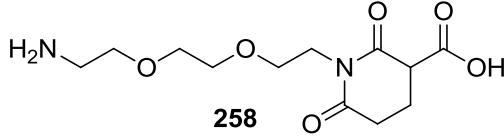
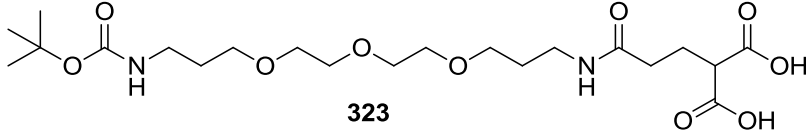
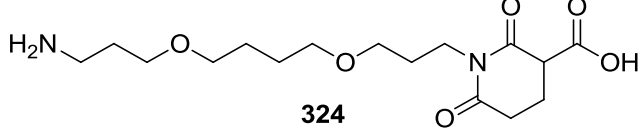
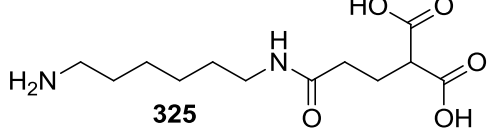
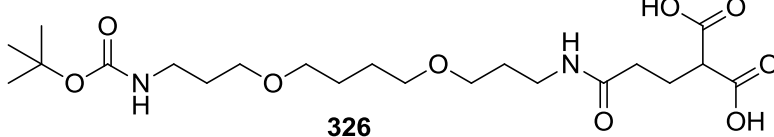


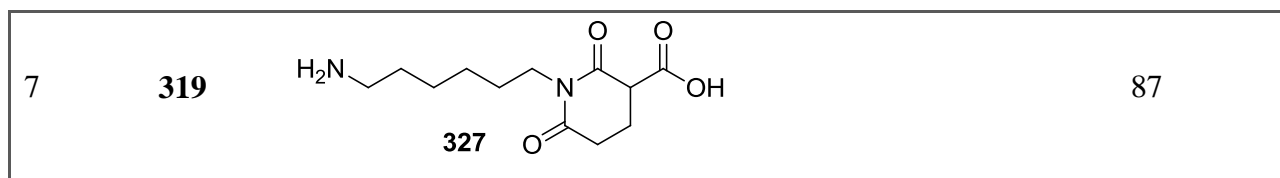
Figure 7.42: ^{13}C -NMR spectrum of oxo-aza malonic acid **261** (MeOD, 100 MHz).

7.7.5 Hydrolysis of the esters

Polarity of the glutarimide derivatives and open-chain esters was enhanced by the conversion of the ester functionality into the carboxylic acid. The ester hydrolysis was carried out in the presence of 1:4 mixture of water and 1,4-dioxane as solvent under mild conditions. LiOH was used in 20 to 25 equiv. to the ester and the reaction was carried out at 55 °C, monitored by TLC. After completion (3 to 4 h), the reaction mixture was cooled to rt and neutralized with 1 N HCl. The aqueous phase was extracted with DCM, and the combined organic phases were dried, filtered and the solvent was evaporated under reduced pressure. The concentrate was purified by flash chromatography and the product was analyzed by different analytical methods including NMR and mass studies. The hydrolysis of different esters is summarized in table 7.5.

Table 7.5: An overview of base catalyzed hydrolysis of the esters.

Sr. No.	Educts.	Carboxylic acids (Products)	Yield [%]
1	301	 262	92
2	259	 258	86
3	303	 323	94
4	318	 324	72
5	315	 325	89
6	311	 326	92



7.7.5.1 Discussion on the NMR spectra of glutarimide acid **258** and **261**

The ^1H -NMR spectrum of the compound **258** shown in fig. 7.43, lacks the signals of the esoteric ethyl group of the educt i.e. a quartet at $\delta = 4.1$ to 4.2 ppm (OCH_2) and a triplet at $\delta = 1.22$ ppm (CH_3). These both signals are the characteristic of the ethyl group of the ester and their absence indicates successful hydrolysis of the ester. The rest of the signals are comparable to the educt **259** and satisfy the presence of the desired product. The product is further confirmed by ^{13}C -NMR analysis given in fig. 7.44. A signal in the range of $\delta = 62.7$ ppm, the characteristic of $-\text{OCH}_2\text{CH}_3$ is missing along with the disappearance of the signal of the terminal carbon of the ethoxy group appeared in the range of $\delta = 14.5$ ppm in the educt. The informations of both spectra reflect that the ethoxy group is missing, which is due to the successful hydrolysis of the ester. The carbonyl signals in the range of $\delta = 172.1$ - 178.2 ppm show the presence of three carbonyl carbons, one from acid and two from glutarimide carbonyls. Mass analysis of the compound confirms m/z in the range of 311.12 for $[\text{C}_{12}\text{H}_{20}\text{N}_2\text{O}_6 + \text{Na}]^+$ identical to the calculated value i.e. 311.12.

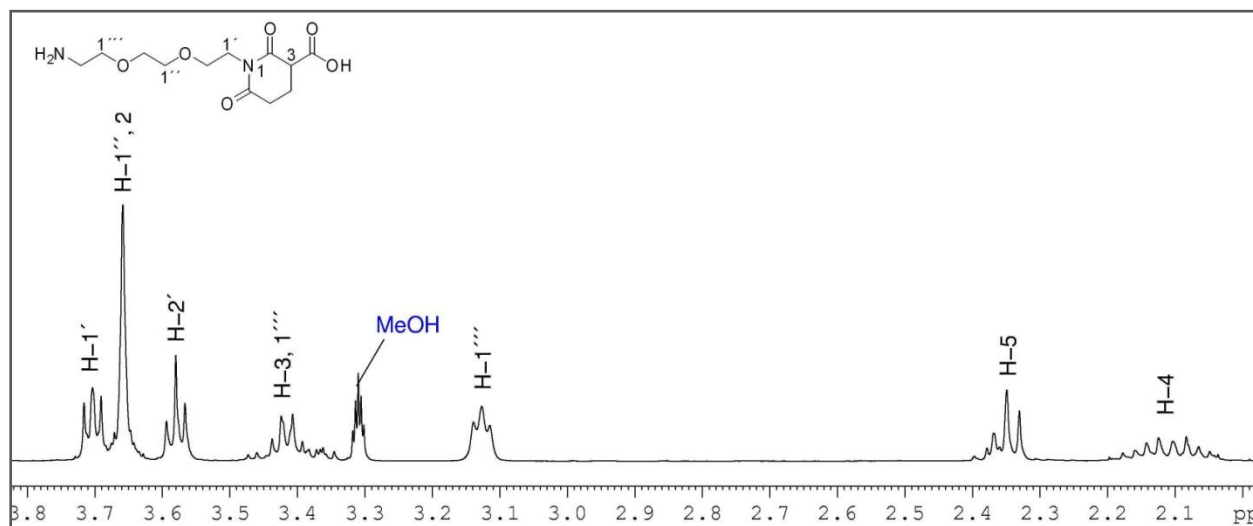


Figure 7.43: ^1H -NMR spectrum of the glutarimide acid **258** (MeOD, 400 MHz).

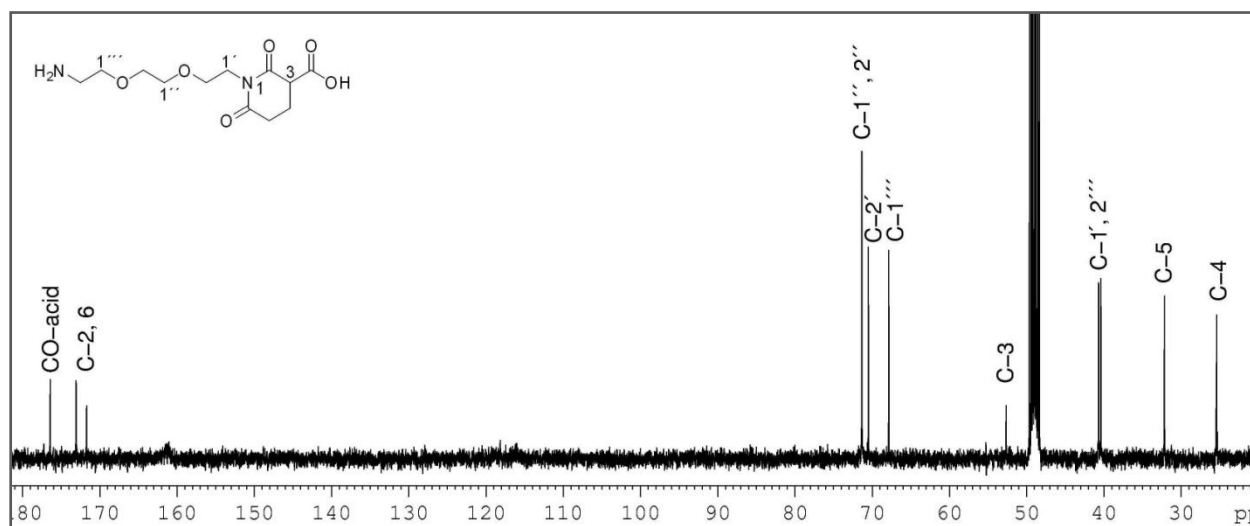
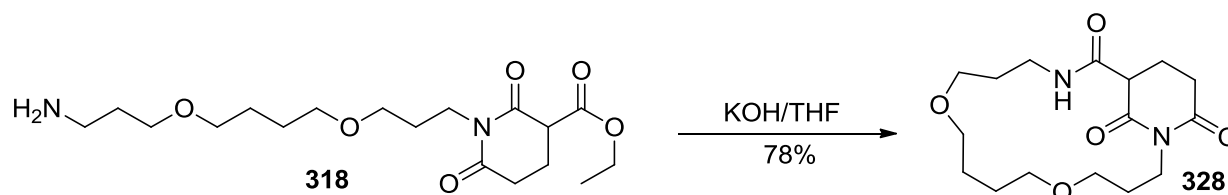


Figure 7.44: ^{13}C -NMR spectrum of the glutarimide acid **258** (MeOD, 100 MHz).

The ^1H -NMR spectrum of dicarboxylic acid **261** also indicates the disappearance of the signal of methyl group, which should appear in the form of singlets in the range of $\delta = 3.5\text{--}3.6$ ppm, and shows the absence of two methoxy groups of methyl malonate. The ^{13}C -NMR spectrum indicates the absence of both methyl groups, which should appear in the range of $\delta = 51.5\text{--}52.9$ ppm. The rest of the signals in both of the spectra are identical to the educt **262** and show that the ester is successfully hydrolyzed, which was further proved by ESI-MS showing mass of the molecule in the range of calculated value.

7.7.6 Synthesis of bicyclic glutarimides

Our current studies toward glutarimide derivatives show that these molecules can be synthesized by domino Michael addition-cyclization under basic conditions. The ease of the molecules to undergo aminolysis was further investigated by the synthesis of bicyclic molecules. For this purpose we planned to monitor the behaviour of these molecules for glutarimide esters with free amino groups. To check this assumption, we subjected the glutarimide derivative **318** to KOH suspension in anhyd. THF under highly dried conditions as shown in scheme 7.44. After overnight stirring the suspension was filtered and the crude product was purified by silica column. Structure elucidation of the compound **328** through NMR studies confirmed bicyclic nature of the molecule and supported our assumption towards the development of bicyclic molecules.



Scheme 7.44: Synthesis of bicyclic molecule **328** by intramolecular aminolysis.

7.7.6.1 Discussion on the NMR spectra of bicyclic glutarimide **328**

The ^1H -NMR spectrum of the compound **328** shown in fig. 7.45 indicates eight hydrogen atoms in the range of $\delta = 3.4$ - 3.6 ppm corresponding to the hydrogen atoms on the carbons directly attached to oxygen in the carbon backbone. A multiplet at $\delta = 3.27$ - 3.29 ppm gives three hydrogen atoms from H-13 and H-16. A HMBC-NMR spectrum of the compound represents a strong coupling between H-13 and the carbonyl carbon C-15, confirming the ring structure of the molecule. Another triplet at $\delta = 3.06$ ppm ($J = 7.0$ Hz) corresponding to H-2 has coupled with two carbonyls C-19 and C-20 in HMBC and confirms the presence of the six membered ring. A strong coupling between a multiplet resonating in the range of $\delta = 2.01$ - 2.12 ppm corresponding to H-17 and the signals resonating at $\delta = 173.97$, 177.35 , and 179.58 ppm corresponding to three carbonyl carbons C-15, 19, and 20 respectively also give proof of cyclic nature of the molecule **328**.

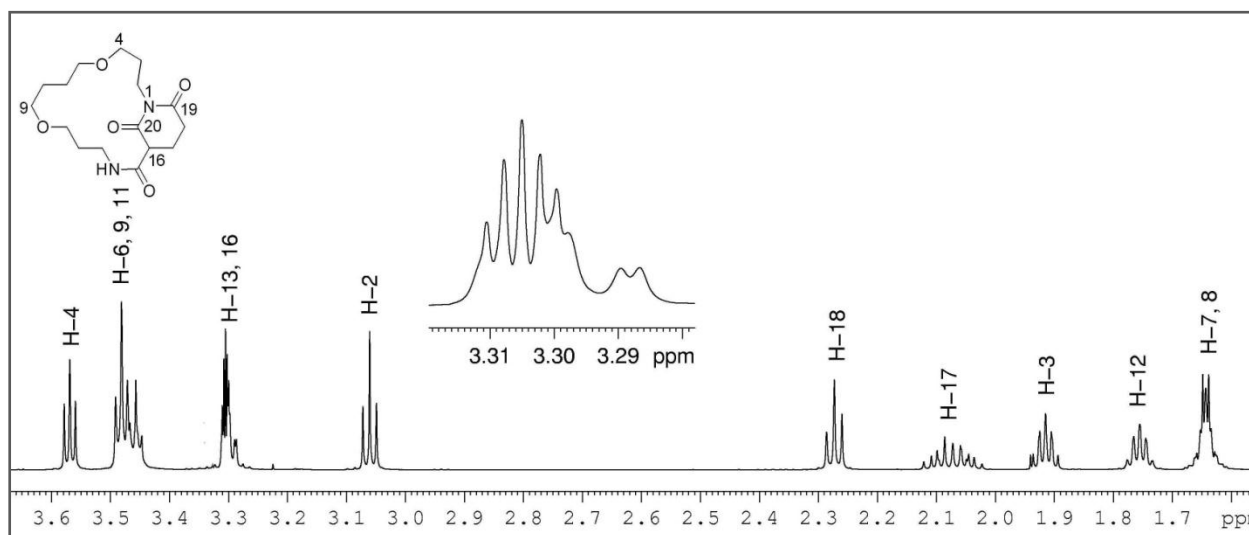


Figure 7.45: ^1H -NMR spectrum of bicyclic glutarimide **328** (MeOD, 400 MHz).

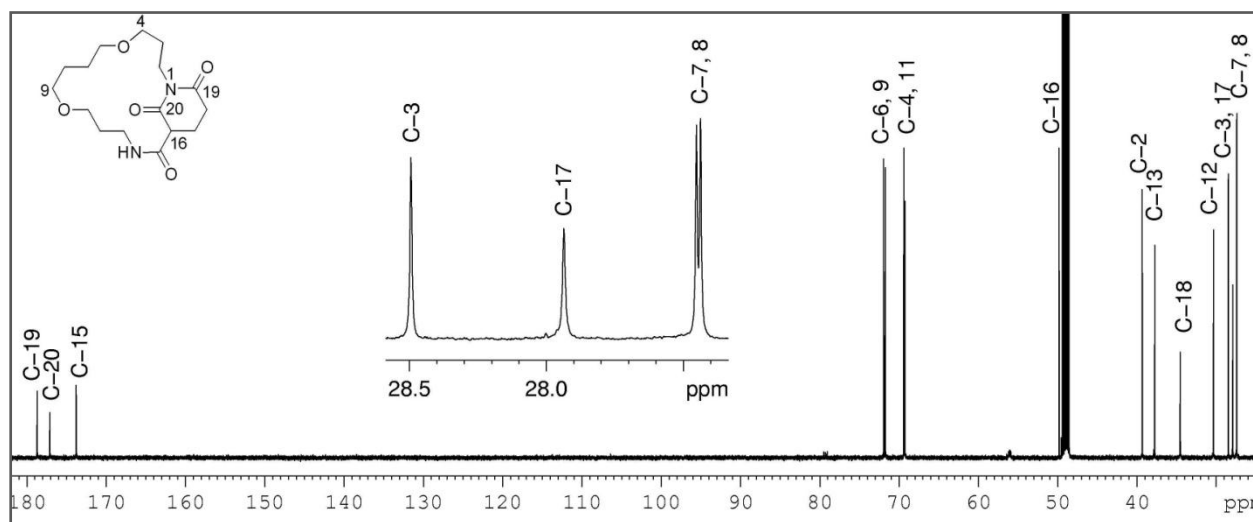
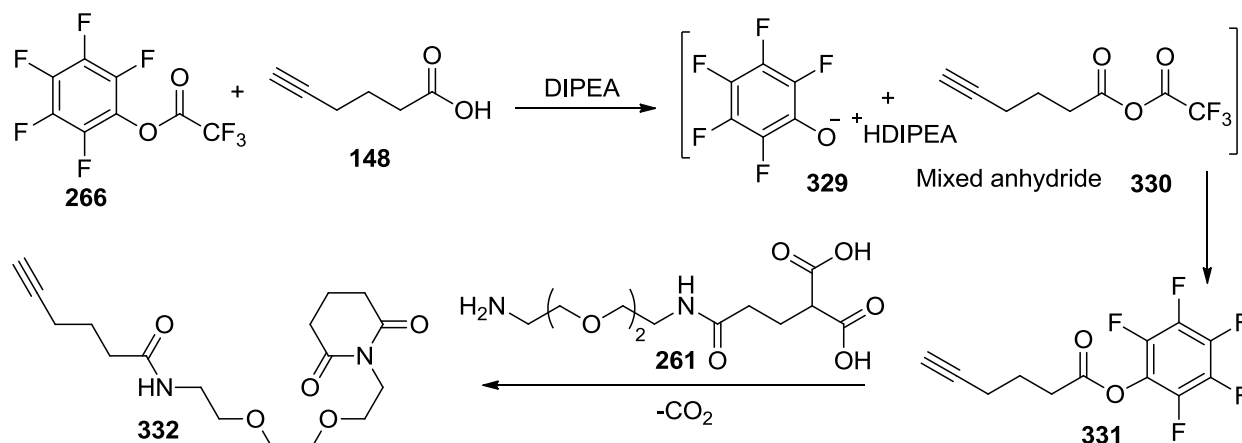


Figure 7.46: ^{13}C -NMR spectrum of bicyclic glutarimide **328** (MeOD, 100 MHz).

7.7.7 Conjugation of dicarboxylic acids

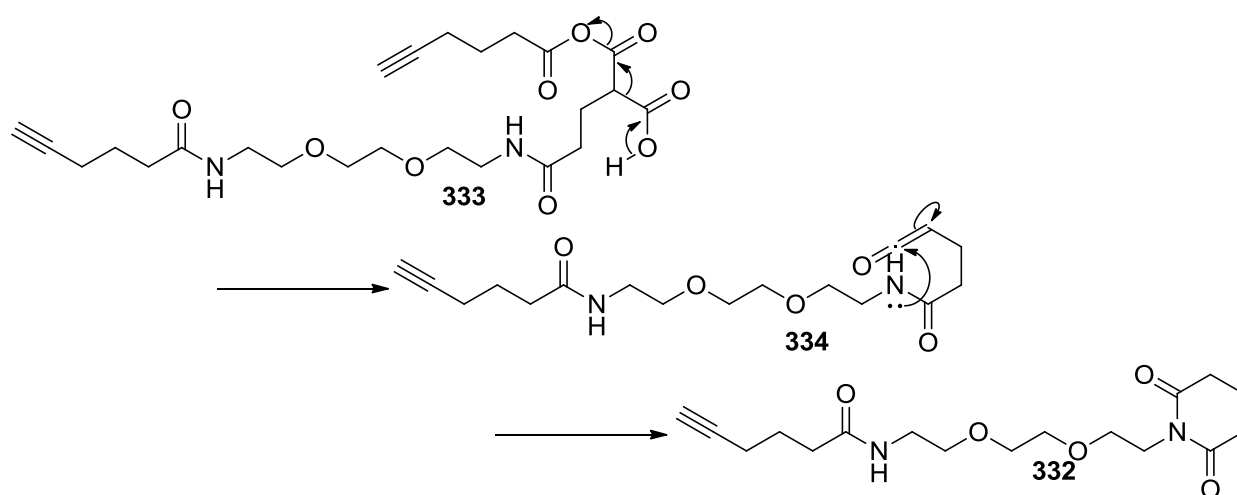
In order to convert the synthesized dicarboxylic acid derivatives capable to the cell surface engineering through labeled cell surface glycoconjugate, the last step of this study was their conjugation with linkers with alkyne or azide functionality. The pattern of acid base conjugation was envisaged to figure out the effect of the reaction conditions on the dicarboxylic acids towards the cyclization. The strategy, to conjugate free amines with carboxylic acids with terminal alkyne functionality could not be achieved directly under *in situ* activating conditions. It was envisioned that the presence of *in situ* activating reagents i.e. EDC or DCC might activate the dicarboxylic acid too and deviate the reaction towards intramolecular conjugation, leading to the development of the cyclic moieties. To avoid this complication, 5-hexynoic acid **148** was activated separately by the treatment with pentafluorophenyl trifluoroacetate (PFP-TFA) **266** in the presence of DIPEA. Another factor behind designing this strategy was to study the behavior of the activated esters on the dicarboxylic moiety. It was envisioned that in excess of the activated ester, dicarboxylic acid may suffer from activation through anhydride synthesis under basic environment. We assumed that the activated ester of dicarboxylic acid will be able to develop a cyclic molecule through intramolecular aminolysis by a domino decarboxylation-cyclization reaction. PFP-TFA is an excellent PFP-ester used for the simultaneous protection and activation of carboxylic acids including amino and thio carboxylic acids as well.^[225] The reaction scheme 7.45 indicates that the reaction proceeds through the synthesis of a mixed anhydride. We synthesized the activated ester **331** by the treatment of PFP-TFA **266** with 5-hexynoic acid **148**, using DIPEA as base in DCM. The reaction was conducted at rt, stirred for 2 h and the solvent was evaporated followed by purification by flash chromatography. The next goal after the synthesis of activated ester **331** was its conjugation through peptide linkage by treatment with

amino dicarboxylic acid **261**, which was achieved successfully in the presence of DIPEA and anhyd. DMF. The reaction mixture was stirred for 24 h, evaporated the solvent and the residue was subjected to flash chromatography for purification. The eluted fractions were analyzed by NMR and mass spectrometry.



Scheme 7.45: Pathway for the synthesis of peptide bond by the use of activated ester (PFP-ester).

Analytical data of the obtained fractions showed that the molecule suffered from decarboxylation under basic conditions to develop glutarimide derivative **332**, and supported our assumption. This cyclization is assumed to take place through a domino decarboxylation-cyclization through a ketene intermediate followed by nucleophilic attack of amine on the carbonyl carbon leading to the formation of a six membered ring. A proposed mechanism for the obtained molecule **332** is described in scheme 7.46.



Scheme 7.46: A proposed mechanism for domino decarboxylation-ring closure.

Aminolysis of the second nitrogen to develop a macrocycle was also possible, but the correlation NMR spectroscopy assured that the obtained molecule was a six membered ring and not a

macrocycle. It is believed that the reaction takes place through an anhydride intermediate, which then suffers from elimination, decarboxylation and finally ring closure by intramolecular aminolysis.

7.7.7.1 Discussion on the NMR spectra of the acid conjugate of glutarimide amine 332

The ^1H -NMR spectrum of the compound **332** shown in fig. 7.47 displays a downfield triplet at $\delta = 3.97$ ppm ($J = 6.1$ Hz) representing two hydrogen atoms from H-1'. A multiplet in the range of $\delta = 3.14$ -3.19 ppm indicates the presence of six hydrogen atoms of ethoxy region along with a triplet at $\delta = 3.51$ ppm ($J = 5.5$ Hz) indicating the ethoxy hydrogen 2'-H₂. A triplet at $\delta = 2.66$ ppm ($J = 6.5$ Hz), possessing four hydrogen atoms, corresponds to the hydrogen atoms (H-3, 5) adjacent to the carbonyl functionalities of the ring. The presence of the terminal hydrogen of the alkyne (H-6''') from hexynoic acid substituent is appeared as a triplet ($J = 2.6$ Hz) at $\delta = 1.96$ ppm.

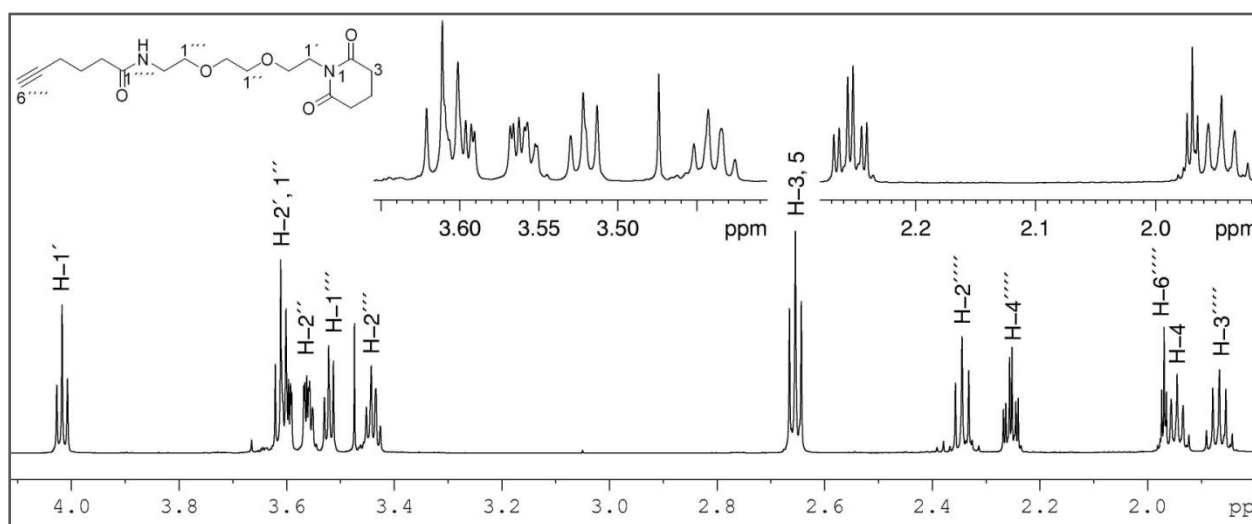


Figure 7.47: ^1H -NMR spectrum of hexynoic acid conjugate of glutarimide amine **332** (CDCl_3 , 400 MHz).

The presence of an alkyne functionality is further confirmed by ^{13}C -NMR by a signal at $\delta = 84.14$ ppm indicating the quaternary carbon (C-5''') of alkyne triple bond, which is further confirmed by ^{13}C -DEPT spectrum shown in fig. 7.48. It also indicates the presence of terminal carbon of the alkyne (C-6''') by a signal in negative intensity at $\delta = 70.1$ ppm. Two quintets at $\delta = 1.94$ ppm ($J = 6.8$ Hz) and at $\delta = 1.86$ ppm ($J = 7.2$ Hz) in ^1H -NMR spectrum represent 4-H₂ and 3'''-H₂ respectively. The presence of three signals in the range of $\delta = 174.9$ -175.5 ppm in ^{13}C -NMR indicates three carbonyl carbons, out of which two come from glutarimide ring and the third represents hexynoic acid conjugate. The rest of the signals in ^{13}C -NMR and ^1H -NMR

spectra are representing the corresponding $-\text{CH}_2-$ carbon of the target conjugate. The final proof comes from ESI-MS, which gives m/z for $[\text{C}_{17}\text{H}_{26}\text{N}_2\text{O}_5\text{-H}]^-$ in the range of 337.18, identical to the calculated value 337.18.

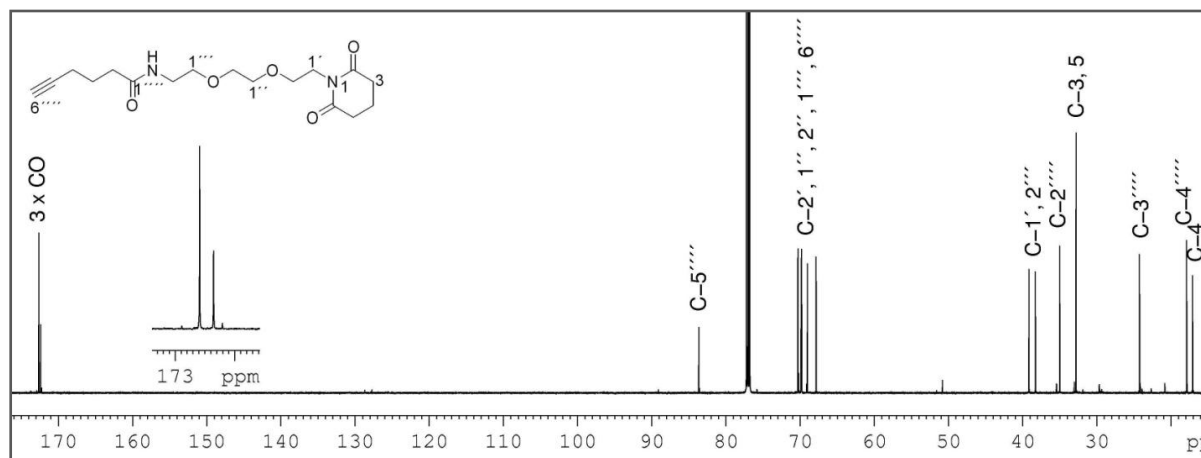
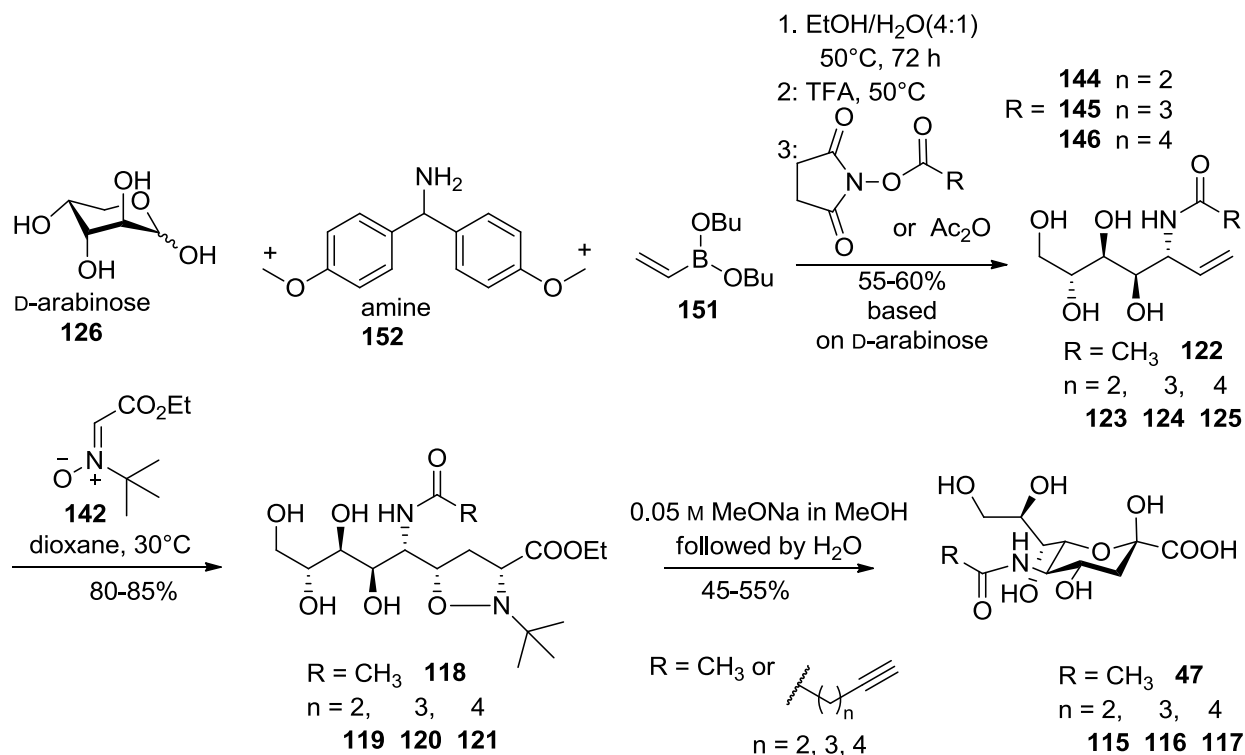


Figure 7.48: ^{13}C -NMR spectrum of the hexynoic acid conjugate of glutarimide amine **332** (CDCl_3 , 100 MHz).

8 Summary

In chemical biology the modification on the cellular level by means of a non-native chemical functionality normally known as a bioorthogonal chemical reporter is achieved either by metabolic or by protein engineering. These modified molecules, incorporated into biomolecules of a living organism are subsequently utilized for tagging and visualizing the biomolecules by molecular imaging. In this thesis, the synthesis of different motifs for bioorthogonal labeling through click reaction, a 1,3-dipolar cycloaddition of azides and alkynes to give triazoles has been reported. When the modified sugars with alkyne or azide functionality are introduced into the culture media of cell lines, they enter into the cell, pass through metabolic processes and appear themselves as glycoconjugates of the cell surface in the form of sialic acids, carrying these bioorthogonal chemical reporters.

Sialic acids are recognized as most common ligands for pathogenic and non-pathogenic viruses, bacteria and protozoa. Due to their exposed position on cell surfaces, Sia have evolved not only to shield cells from the environment, but also as recognition markers in multicellular organisms. Being the outermost glycan of cell membrane glycoconjugates, they can be exploited by click reaction to achieve bioorthogonal labeling. In order to make use of modified Sia in bioorthogonal labeling, we synthesized a series of Sia derivatives with a linker holding a terminal alkyne group. The targeted Sia derivatives were synthesized through a three-step strategy, the first step of which is a modified Petasis reaction as shown in scheme 8.1. It makes use of dibutyl vinyl boronic ester **151**, which facilitates the introduction of a vinyl group to an unprotected D-arabinose **126**. Amine **152** was used for the introduction of an amino group, with which different substituents could be introduced. For our investigations towards the synthesis of Sia derivatives, we used carboxylic acid moieties with terminal alkyne functionalities, after activation with *N*-hydroxy succinimide (NHS) in the presence of DCC. The reaction was achieved by stirring for 72 h at rt followed by the addition of TFA, stirred for further 24 h and finally the activated esters of carboxylic acids/ Ac_2O and NaHCO_3 were added after replacement of the solvent with dried MeOH. The next step was a highly diastereoselective 1,3-dipolar cycloaddition, that enabled the facile conversion of the vinyl group to the isoxazolidine derivatives **118** and **119-121** as a result of an addition reaction with nitron **142**. The conversion of isoxazolidines into the desired Sia derivatives **52** and **115-117** was achieved by a base-catalyzed ring-opening reaction followed by hydrolysis.



Scheme 8.1: Synthesis of Sia derivatives with alkyne functionality.

All three steps are highly compatible with unprotected hydroxyl groups in carbohydrates. Consequently, by this modified efficient approach different novel Sia derivatives were synthesized and can be used in advance for the synthesis of further Sia derivatives with different functionalities from common inexpensive materials. Thus, applying this approach, we synthesized D-*N*-acetylneuraminic acid **52**, D-*N*-pent-4-ynoylneuraminic acid (Neu5Pent) **115**, D-*N*-hex-5-ynoylneuraminic acid (Neu5Hex) **116**, and D-*N*-hept-6-ynoylneuraminic acid (Neu6Hept) **117**. The approach utilized is highly flexible, as different aldose sugars in addition to a variety of activated esters with different functional groups can be used and the substituents on the Sia nitrogen can be altered for the synthesis of a wide range of Sia derivatives.

The next step was the synthesis of azide moieties, through which the Sia alkynes could be conjugated by click reaction. For this purpose, the synthesis of different azides i.e. acetylated galactose azide **127**, glucosamine azides **128**, **129**, fluorescein azide **130** and rhodamine azide **131** was achieved successfully as shown in fig. 8.1.

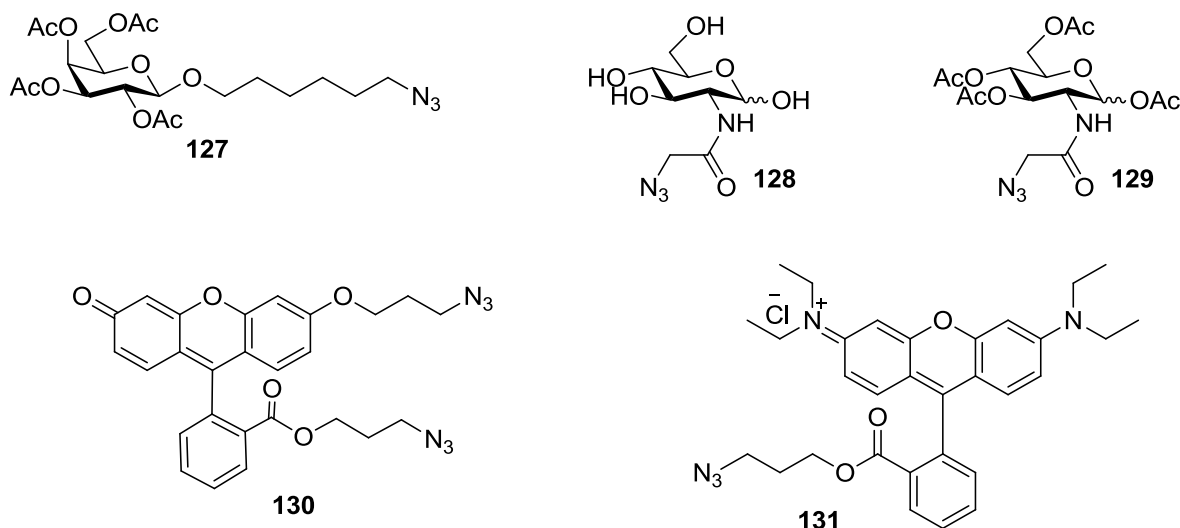


Figure 8.1: Different azide molecules for the investigation of click reaction and for bioorthogonal conditions.

Conjugation of Sia derivatives through click chemistry can be an interesting approach in drug discovery, due to the terminal position of sialic acids on glycoconjugate chain, masking the cell. Thus, the approach can be of great value in future's possible detection of tumors, inflammations or HIV. At the time when alkyne and azide functionalities were in our hand, the next step was their conjugation through click reaction. We initiated our study with the synthesis of triazole **185** through the Sharpless approach by the treatment of azide **127** with 5-hexynoic acid **148** in the presence of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate in the mixture of *tert*-BuOH and water (1:1) as solvent. Similarly, the reaction route was further confirmed by the synthesis of triazoles via the treatment of simple azides and alkynes. After confirmation of the reaction conditions, the approach was applied on the conjugation of Neu5Hex **116** and acetylated galactose azide **127**. However, the reaction mixture after the purification showed very weak signals of the required triazole when monitored by NMR and mass spectrometry. We observed that the Sia derivatives decomposed under the mentioned reaction conditions. In order to avoid this complication, we made use of CuI and DIPEA as promoter in MeOH, which gave an excellent yield of the product. The triazoles synthesized in the context of the investigation of the click reaction are summarized in fig. 8.2.

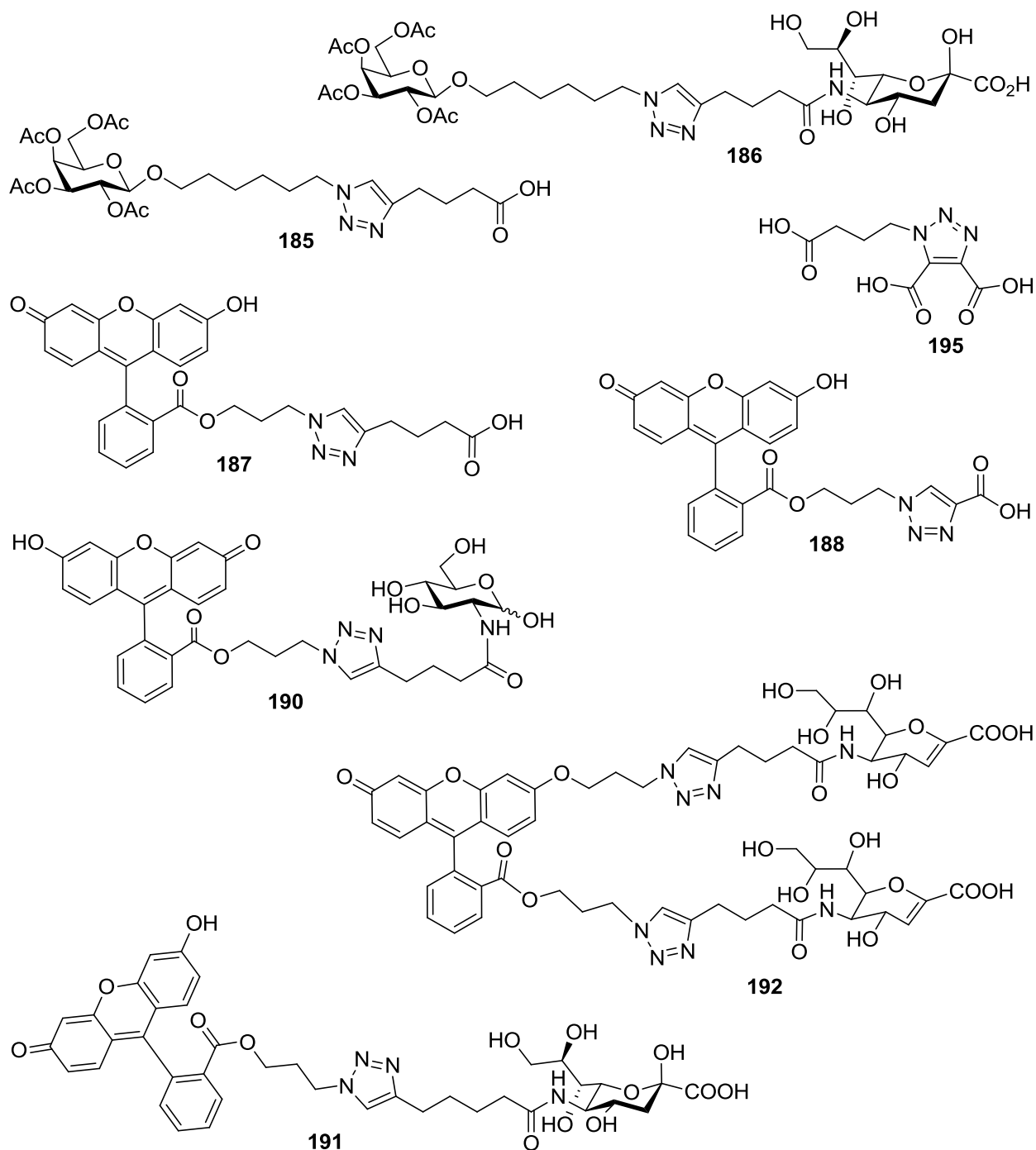


Figure 8.2: Different triazoles synthesized under Sharpless and modified conditions of CuAAC.

After solving the problem of the reactivity of Sia derivatives towards click reaction, they were conjugated with different moieties like fluorescein azide and galactose azide. The investigations on the synthesis of different triazoles were followed by the application of the strategy on the cell surface, which was achieved successfully under optimized conditions. The cell imaging was performed by incorporation of GlcNAz **129** and Neu5Hex **116** differently into the glycoconjugates of the HEp-2 cell lines. It was then followed by tagging the labeled cell surface

glycoconjugates with alkynylated TAMRA and fluorescein azide **130** respectively. The labeled cells were imaged by fluorescent microscopy as shown in fig. 8.3.

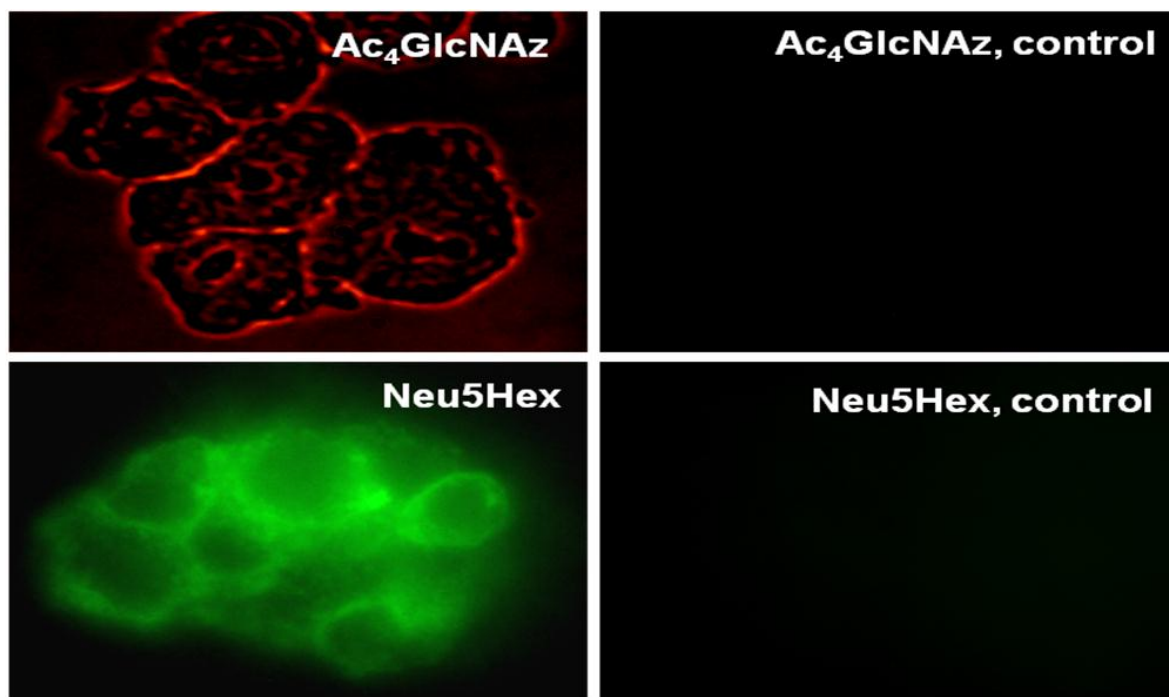


Figure 8.3: Top left: HEp-2 cells incorporated with Ac₄GlcNAz, labeled with alkynylated TAMRA at 580 nm. Bottom left: HEp-2 cells with incorporated Neu5Hex, labeled with fluorescein azide at 525 nm. On the right: background and unspecified staining controls at the same wavelength as corresponding pictures on left.

Although, labeling of the cell lines with Sia derivatives and GlcNAz was done successfully by CuAAC in the presence of TBTA followed by the imaging of the cells by fluorescent microscopy, the toxicity of copper was still a disadvantage of the introduced approach. To avoid copper toxicity, the approach was extended to the synthesis of cyclooctynes in form of DIFO (I) and DIFO (II). Ring strain of the cyclooctyne, in addition to the electron withdrawing groups, facilitates the click reaction to take place at rt even in the absence of the copper catalyst. After the synthesis of DIFO (II) **28**, it was conjugated with different motifs including mannosamine **228**, glucosamine **232** and Alexafluor 647 **230** as shown in fig. 8.4

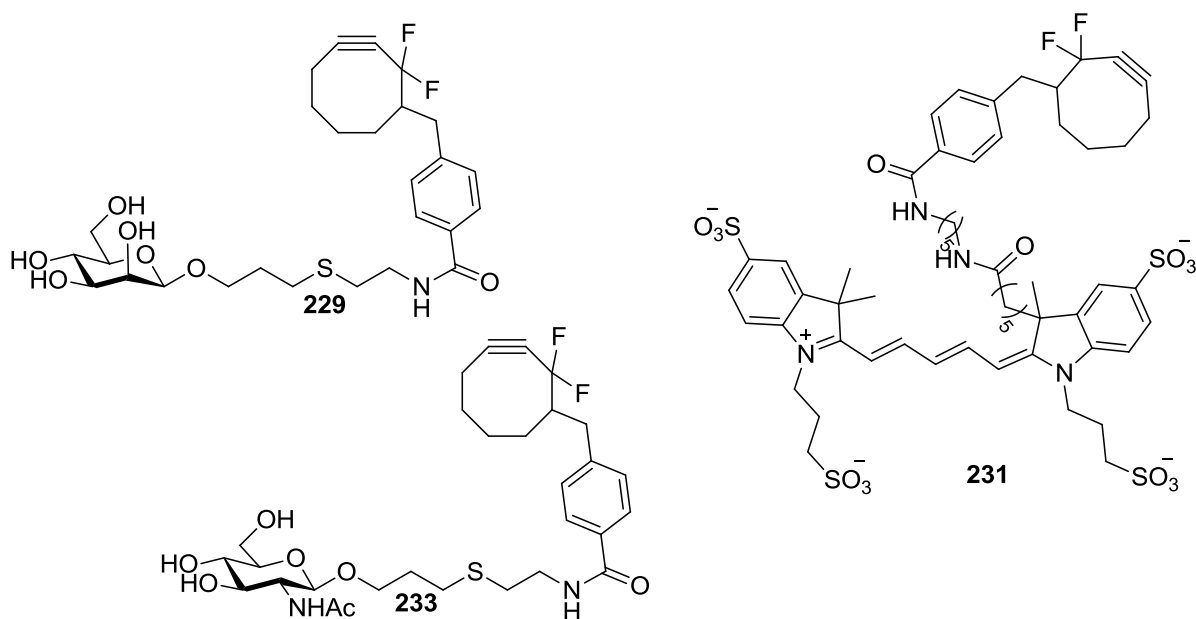
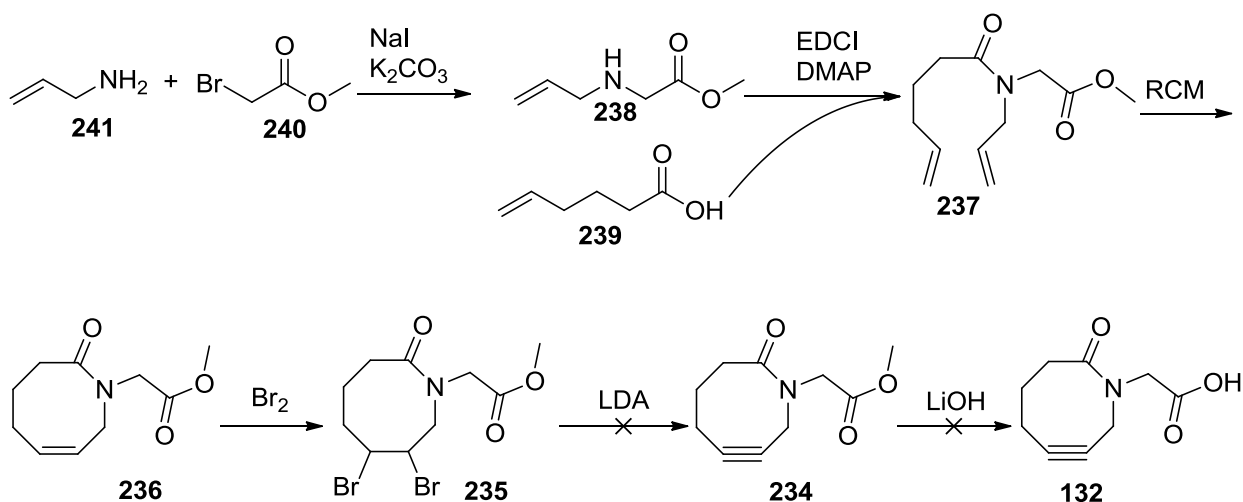


Figure 8.4: Conjugates of DIFO (II) with fluorescent and nonfluorescent motifs.

The syntheses of cyclooctynes reported up to now are complicated and long e.g. the synthesis of DIFO (I) takes place in 13 overall steps and DIFO (II) in 9 steps. Thus, there was need to improve the synthetic route and the reactivity of the cyclic octynes. We attempted to synthesize an octyne with lactam structure, which could be accomplished in six steps. Cyclic octene **236**, obtained as a result of the ring closing metathesis, was subjected to bromination followed by dehydrobromination to obtain triple bond as shown in scheme 8.2. All steps of the synthetic route worked nicely; however, the dehydrobromination was not successful.



Scheme 8.2: Synthesis of azocycyne derivative **132**.

This thesis also covers the synthesis of highly polar oxo diamines containing carboxylic functionality both in cyclic and acyclic forms. This part of the thesis was designed to make investigations on the negatively charged cell surface of the parasites. The parasites i.e.

trypanosomes are resistant to human antibodies due to the negative charge of the carboxylate ions of Sia covering the cell surface. There is need to change the surface polarity of the parasites, so that they might be targeted by the antibodies. For this purpose, the diamine derivatives holding dicarboxylic acid functionality were synthesized, which are still under investigation for biological activity. In addition to the dicarboxylic acid diamines, we synthesized glutarimide derivatives in an efficient way through a domino Michael addition-cyclization reaction. Glutarimide derivatives have emerging scope in pharmacological science, and many glutarimide derivatives are in clinical practice for years. Their syntheses were achieved in different mode of reaction patterns i.e. base catalyzed domino Michael addition-cyclization, acid catalyzed domino Boc deprotection-ring closure and domino decarboxylation-cyclization as a result of an intramolecular aminolysis during conjugation of free amino group with carboxylic acid in the presence of Huenig base. In addition to the glutarimide derivatives, the synthesis of bicyclic glutarimide derivative **328** was also achieved as a result of base catalyzed aminolysis between free amine and carbonyl carbon of the ester **318** described in fig. 8.5.

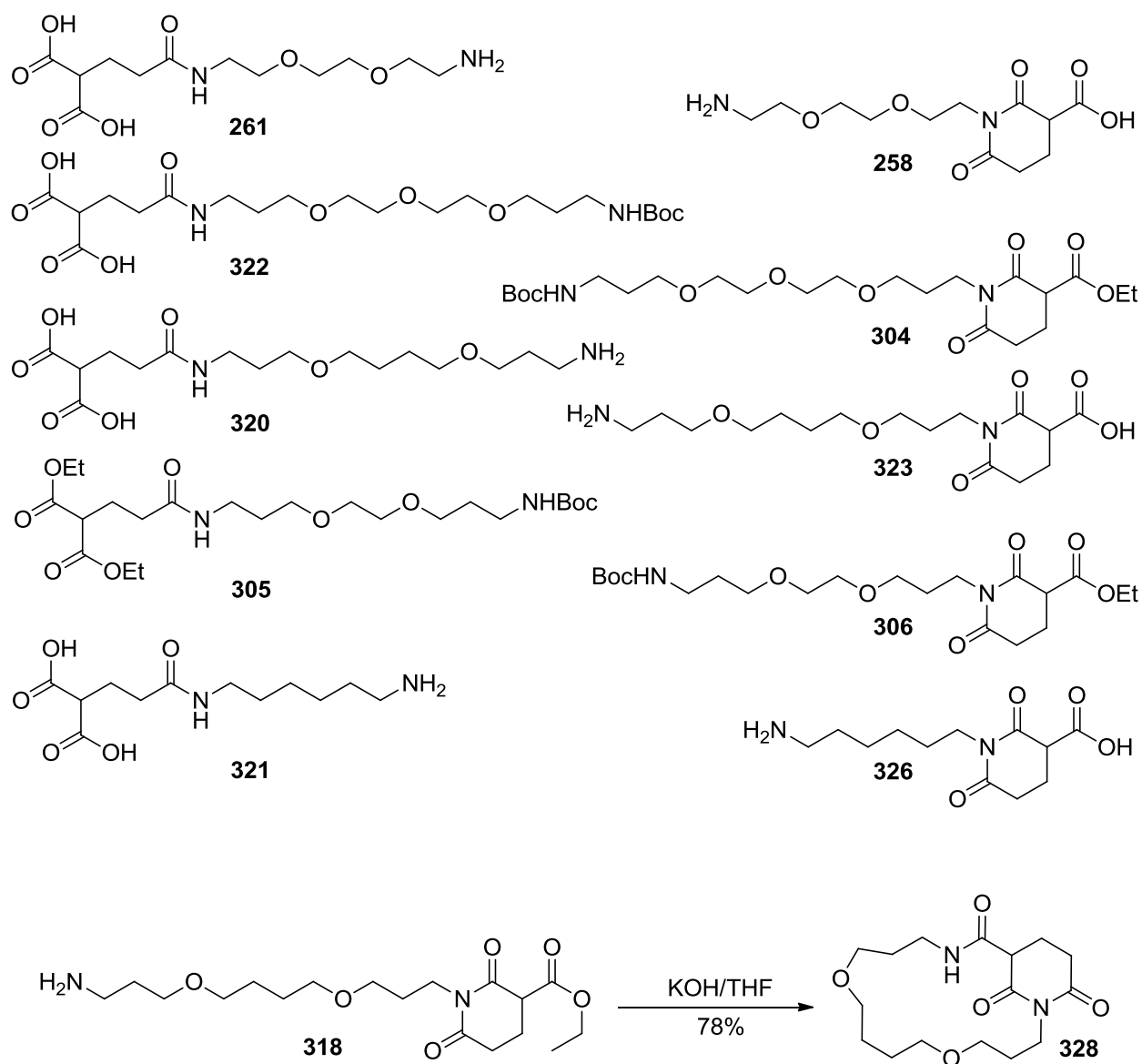
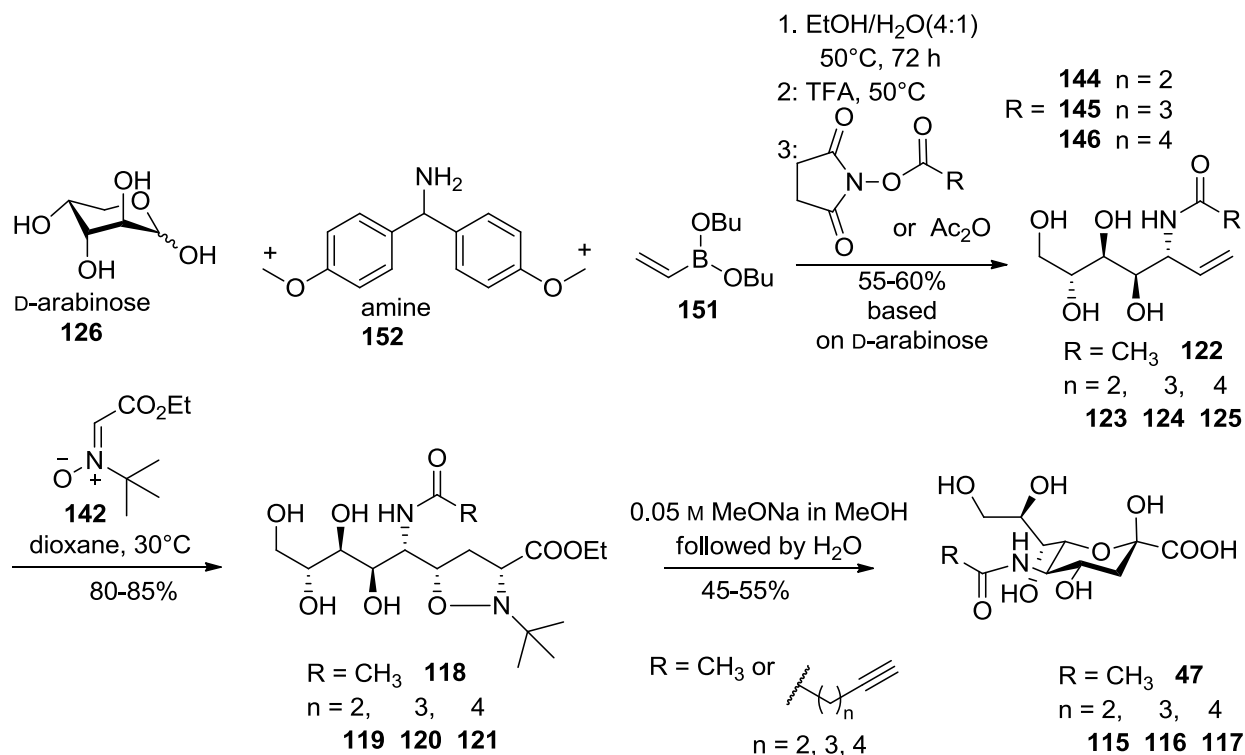


Figure 8.5: Structure of different cyclic and acyclic diamine structures with carboxylic acid functionality.

Zusammenfassung

In der chemischen Biologie werden Modifizierungen auf zellulärer Ebene in Form von nichtnativen chemischen Funktionalisierungen, sogenannten bioorthogonalen Reportern, durch Stoffwechsel- oder Proteinengineering ermöglicht. Eingebaut in Biomoleküle eines lebenden Organismus können die modifizierten Moleküle angesteuert und zur Visualisierung der entsprechenden Biomoleküle in der molekularen Bildgebung eingesetzt werden. In dieser Arbeit wurde über die Synthese verschiedener bioorthogonaler Markierungsmotive berichtet, die in 1,3-dipolaren Cycloadditionen (Click-Reaktion) zwischen Azid und Alkin Triazol bilden. Zucker mit einer solchen Funktionalisierung können von Zellen aus dem Nährmedium aufgenommen und metabolisiert werden, sodass sie schließlich als Sialinsäuren in Glykokonjugaten auf der Zelloberfläche präsentiert werden-ausgestattet mit einem bioorthogonalen chemischen Reporter. Sialinsäuren dienen als prominentes Erkennungsmotiv für pathogene und nichtpathogene Viren, Bakterien und Protozoa. Aufgrund ihrer exponierten Lage auf Zelloberflächen tragen Sia einerseits zur Abschirmung der Zellen von ihrer Umgebung bei, andererseits wird ihnen eine Funktion in Erkennungsprozessen multizellulärer Organismen zugesprochen. Als äußerste Kohlenhydratstruktur von Glykokonjugaten der Zellmembran sind sie deshalb für Click-Reaktionen zur Einführung einer bioorthogonalen Markierung äußerst attraktiv. Um Manipulationen auf der Zelloberfläche unter Einsatz von modifizierten Sia Strukturen zu realisieren, wurde eine Reihe von Sia Derviaten synthetisiert, welche über einen Linker mit einer terminalen Alkingruppe funktionalisiert sind. Die Zielstrukturen wurden in einer dreistufigen Synthese dargestellt, mit einer Variante der Petasis Reaktion in der ersten Stufe (Schema 8.1). Mit Dibutylvinylboronsäureester **151** wird die Einführung einer Vinylgruppe in ungeschützte D-Arabinose **126** erleichtert, während über das Amin **152** eine Aminogruppe eingebaut wird, über die eine Funktionalisierung mit verschiedenen Substituenten erzielt wird. Für unsere Untersuchungen wurden als Substituenten Carbonsäuren mit terminaler Alkinfunktion eingesetzt, die zuvor mit *N*-Hydroxysuccinimid (NHS) in Anwesenheit von Dicyclohexylcarbodiimide (DCC) aktiviert wurden. Die Reagenzien **151**, **126** und **152** wurden in einem Ethanol/Wassergemisch für 72 h bei rt gerührt, bevor Trifluoressigsäure (TFA) zugegeben und für weitere 24 h gerührt wurde. Nach Entfernen des Lösemittels und Resolvatation in Methanol wurde schließlich der aktivierte Carbonsäureester bzw. Essigsäureanhydrid und NaHCO₃ hinzugefügt.



Schema 8.1: Synthese von Sia Derivaten mit Alkinfunktionalisierung.

Der nächste Schritt stellte eine hoch diastereoselektive 1,3-dipolare Cycloaddition dar, bei der die Vinylgruppe unter Ausbildung der Isoxazolidinderivate **118** und **119-121** mit dem Nitron **142** reagierte. Die Konversion der Isoxazolidine in die gewünschten Sia Derivate **52**, **115-117** erfolgte über eine basenkatalysierte Ringöffnungsreaktion und einer abschließenden Hydrolyse. Alle drei Stufen sind ohne Einschränkungen kompatibel mit ungeschützten Hydroxylgruppen in Kohlenhydraten, so dass in diesem modifizierten und effizienten Herangehen neuartige Sia Derivate dargestellt werden konnten und zukünftig eine Vielzahl von Sia Derivaten mit verschiedenen Funktionalisierungen aus herkömmlichen, günstigen Vorstufen darstellbar ist. So wurden mit diesem Ansatz, D-N-Acetylneuraminsäure **52**, D-N-pent-4-ynylneuraminsäure (Neu5Pent) **115**, D-N-hex-5-ynylneuraminsäure (Neu5Hex) **116** und D-N-hept-6-ynylneuraminsäure (Neu6Hept) **117** synthetisiert. Die verfolgte Synthesestrategie ist hochflexibel und erlaubt den Einsatz verschiedener Aldosen sowie eine Variation der aktivierten Ester bzgl. der funktionellen Gruppe am Stickstoff, so dass eine große Bandbreite an Sia Derivaten zugänglich ist. Nach erfolgreicher Synthese der alkinfunktionalisierten Sia Strukturen wurden Azidoverbindungen dargestellt, die über eine Click-Reaktion mit den Sia Derivaten konjugiert werden sollten. Zu diesem Zweck wurden acetylierte Azidogalactose **127**, Azidoglucosamin **128**, **129**, Azidofluorescein **130** und Azidorhodamin **131** dargestellt (Abb. 8.1).

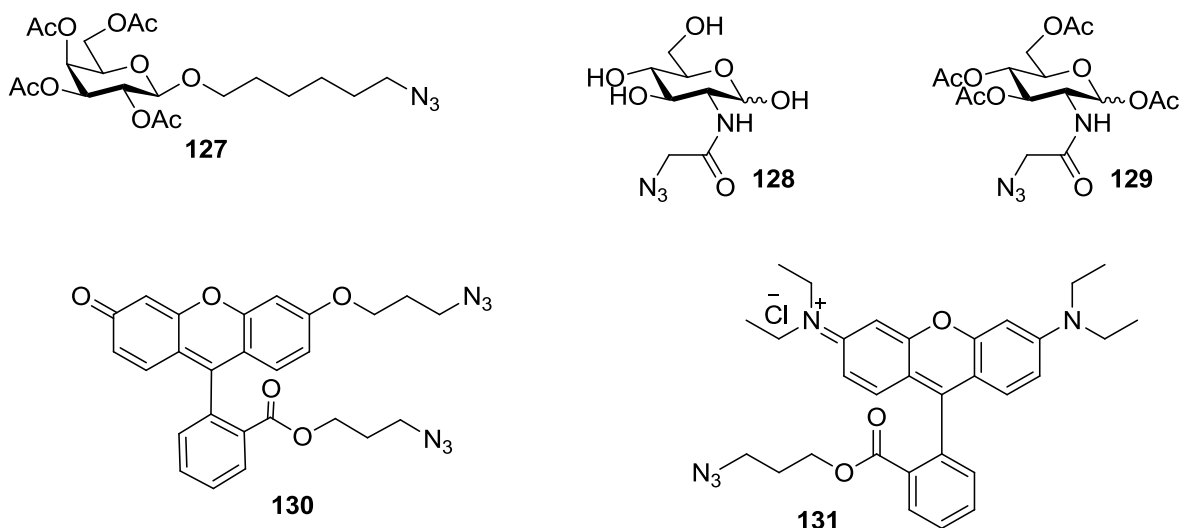


Abbildung 8.1: Verschiedene Azidoverbindungen zur Untersuchung von Click-Reaktionen unter bioorthogonalen Bedingungen.

Eine Konjugation von Sia Derivaten mittels der Click-Chemie ist ein interessanter Ansatzpunkt in der Wirkstoffforschung, da diese Kohlenhydratstrukturen terminale Positionen von Glykokonjugaten einnehmen und somit die Zelle maskieren. Folglich steckt in diesem Ansatz ein großes Potential für zukünftige Detektionen von Tumoren, Entzündungen oder HIV.

Im nächsten Schritt mussten die erfolgreich synthetisierten Azid- und Alkinderivate konjugiert werden. Dafür wurde zunächst unter Sharpless-Bedingungen das Azid **127** mit 5-Hexinsäure **148** in Anwesenheit von $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ und Natriumascorbat in einem *tert*-BuOH/Wassergemisch zu Triazol **185** umgesetzt. Auf ähnliche Weise konnte die Reaktionsdurchführung durch die Synthese von Triazolen mit einfachen Aziden und Alkinen bestätigt werden. Mit den optimierten Bedingungen wurde dann versucht, Neu5Hex **116** mit der acetylierte Azidogalactose **127** zu konjugieren. Die Reaktionsmischung zeigte nach der Aufreinigung jedoch sehr schwache Signale des gewünschten Triazol in NMR- und Massenspektren.

Diese Beobachtung konnte auf eine Zersetzung der Sia Derivate unter den oben beschriebenen Reaktionsbedingungen zurückgeführt werden. Um diese Komplikation zu vermeiden, wurde CuI und DIPEA als Promoter in MeOH eingesetzt, was zu exzellenten Ausbeuten des Produkts führte. Mit Kenntnis der Reaktivitätsprobleme der Sia Derivate und unter Einsatz der optimierten Bedingungen wurden diese mit verschiedenen Strukturen wie Azidofluorescein und Azidogalactose konjugiert, einige davon werden in Abb. 8.2 gezeigt.

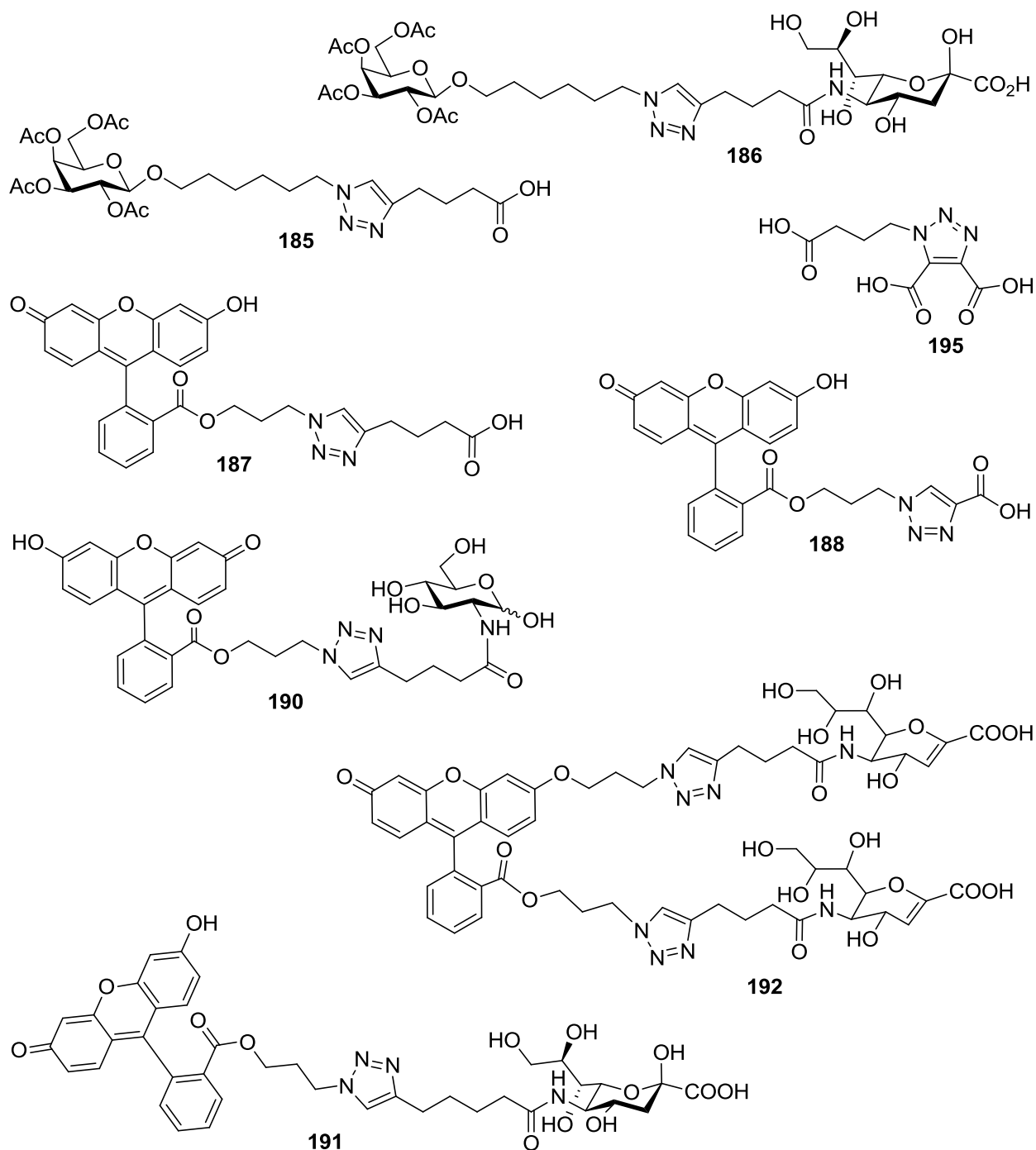


Abbildung 8.2: Verschiedene Triazole, die nach Sharpless und modifizierten CuAAC Bedingungen synthetisiert wurden.

Der Ansatz wurde im Weiteren auf Reaktionen mit der Zelloberfläche übertragen, wobei zunächst GlcNAz **129** und Neu5Hex **116** in Glykokonjugate von Hep-2 Zellen eingebaut und anschließend mit alkinyliertem Tetramethylrhodamine (TAMRA) bzw. Azidofluorescein konjugiert wurden. Auf diese Weise konnte der Einbau modifizierter Kohlenhydratstrukturen in lebende Zellen mit der Fluoreszenzmikroskopie visuell verfolgt werden (Abb. 8.3). Trotz der erfolgreichen Konjugation von metabolisiertem GlcNAz mit einem Fluoreszenzmarker unter

Einsatz von CuAAC und TBTA stellt die hohe Toxizität von Cu eine gravierende Einschränkung des Ansatzes für Anwendungen an lebenden Systemen dar.

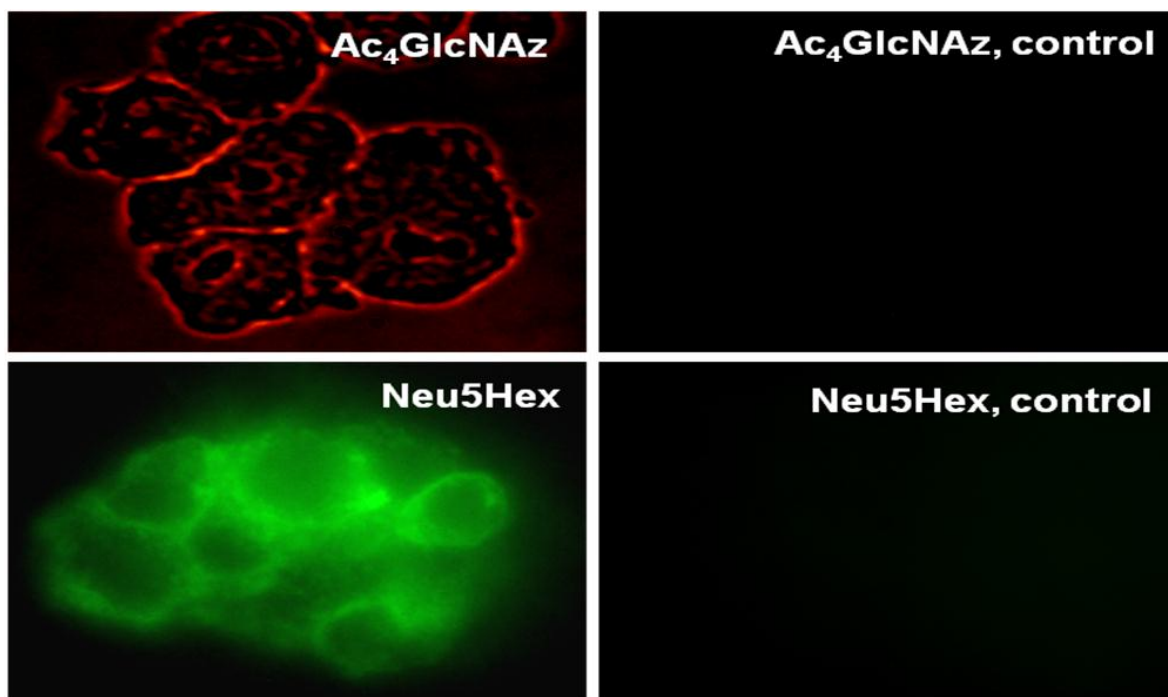


Abbildung 8.3: Links oben: HEP-2 Zellen mit eingebautem Ac₄GlcNAz, markiert mit alkinyliertem TAMRA bei 580 nm. Links unten: HEP-2 Zellen mit eingebautem Neu5Hex, markiert mit Azidofluorescein bei 525 nm. Rechts: Hintergrund mit Kontrollen zu unspezifischen Färbungen bei gleichen Wellenlängen wie bei den entsprechenden Bildern links.

Um dieses Problem zu umgehen, wurden mit DIFO (I) und DIFO (II) zwei Cyclooctine synthetisiert, die aufgrund der hohen Ringspannung sowie der elektronenziehenden Substituenten ohne Einsatz von Cu und bei rt mit Aziden Triazole bilden. DIFO (II) **28** wurde anschließend mit verschiedenen Motiven wie Mannosamin **228**, Glukosamin **232** und Alexafluor 647 **230** konjugiert (Abb. 8.4). Letzteres wird in Experimenten der molekularen Biologie zur Bildgebung eingesetzt.

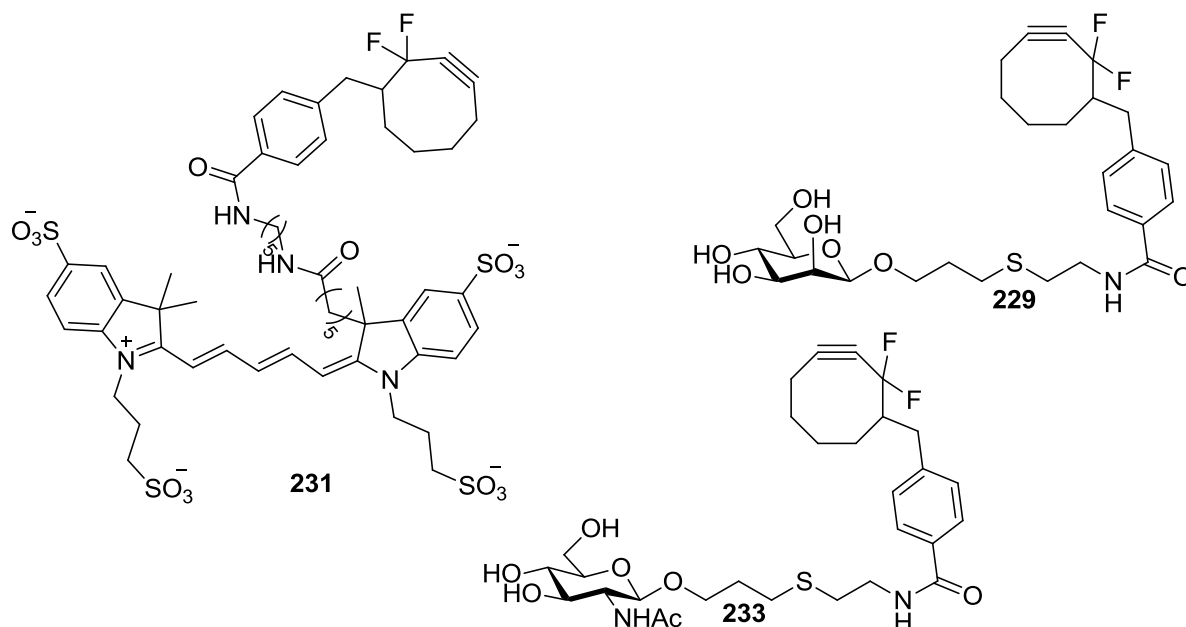
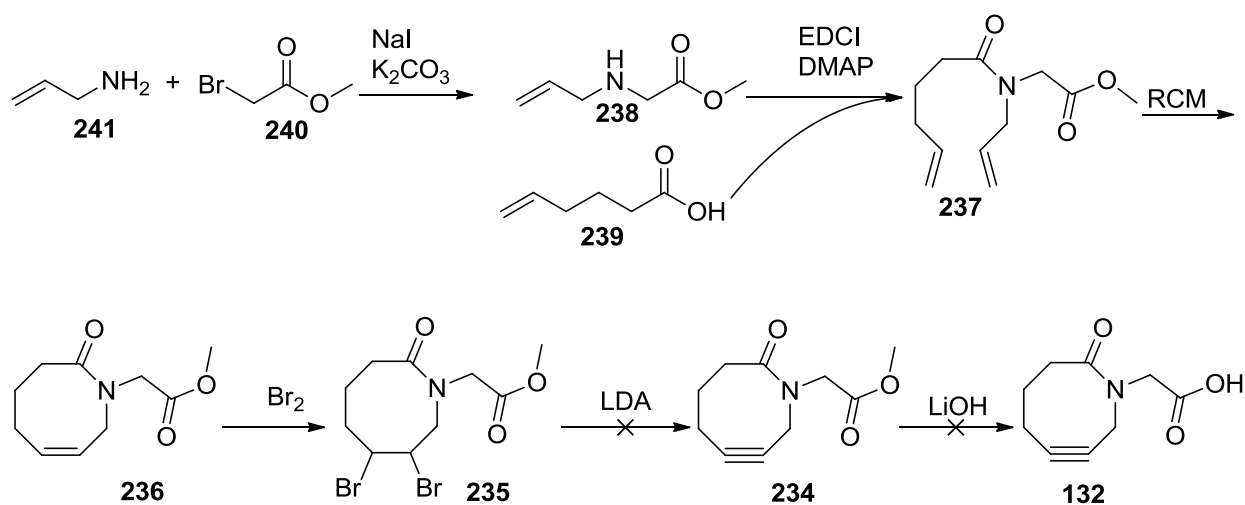


Abbildung 8.4: Konjugate von DIFO (II) mit fluoreszierenden und nichtfluoreszierenden Motiven.

Die bisher beschriebenen Synthesen von Cyclooctinen sind kompliziert und langwierig, so sind z.B. zur Darstellung von DIFO (I) 13 Stufen erforderlich und 9 Stufen für die Synthese von DIFO (II). Folglich besteht die Notwendigkeit, die Syntheseroute zu Cyclooctinen zu verbessern und dabei gleichzeitig eine gesteigerte Reaktivität zu erreichen. Um diesen Anforderungen gerecht zu werden, wurde ein Octin mit Lactamstruktur gewählt, welches in nur sechs Stufen darstellbar sein sollte (Schema 8.2). Eine Bromierung des cyclischen Octens **236**, gefolgt von einer Dehydrobromierung sollte die Dreifachbindung liefern. Bis auf letztere Reaktion funktionierten alle Stufen dieser Syntheseroute problemlos.



Schema 8.2: Synthese des Azocinderivat **132**.

In dieser Arbeit wurden außerdem Synthesen von hoch polaren Oxodiaminen mit Carboxylfunktion sowohl in cyclischer als auch acyclischer Form vorgestellt. Dieser Teil der Arbeit adressiert Studien zu negativ geladenen Zelloberflächen von Parasiten. Parasiten wie Trypanosome sind resistent gegen humane Antikörper, da die Carboxylationen von Sia auf der Zelloberfläche eine negative Ladung präsentieren. Eine Veränderung der Oberflächenpolarität könnte zu einer Erkennung von Antikörpern führen und eine Bekämpfung der Parasiten ermöglichen. Aus diesem Grund wurden Diaminderivate, welche zusätzlich eine Dicarbonsäurefunktionalität tragen, synthetisiert (Abb. 8.5). Diese Strukturen werden in aktuellen Arbeiten auf ihre biologische Aktivität getestet. Desweiteren konnten Glutarimidderivate über eine Domino-Michael-Additionscyclisierungsreaktion effizient dargestellt werden. Glutarimidderivate haben in der pharmakologischen Forschung stetig an Bedeutung gewonnen und viele dieser Verbindungen sind seit Jahren in der klinischen Anwendung. Die Darstellung gelang nach verschiedenen Reaktionsstrategien, darunter eine basenkatalysierte Domino Michael Additionscyclisierung, eine säurenkatalysierte Domino Boc-Entschützungsringchlussreaktion sowie eine Domino-Decarboxylierungscyclisierung als Resultat einer intramolekularen Aminolyse zwischen einer freien Aminogruppe und einer Carbonsäure in Anwesenheit der Hünig-Base. Zusätzlich konnte erfolgreich das bicyclische Glutarimidderivat **328** in einer basenkatalysierten, intramolekularen Aminolyse von **318** synthetisiert werden.

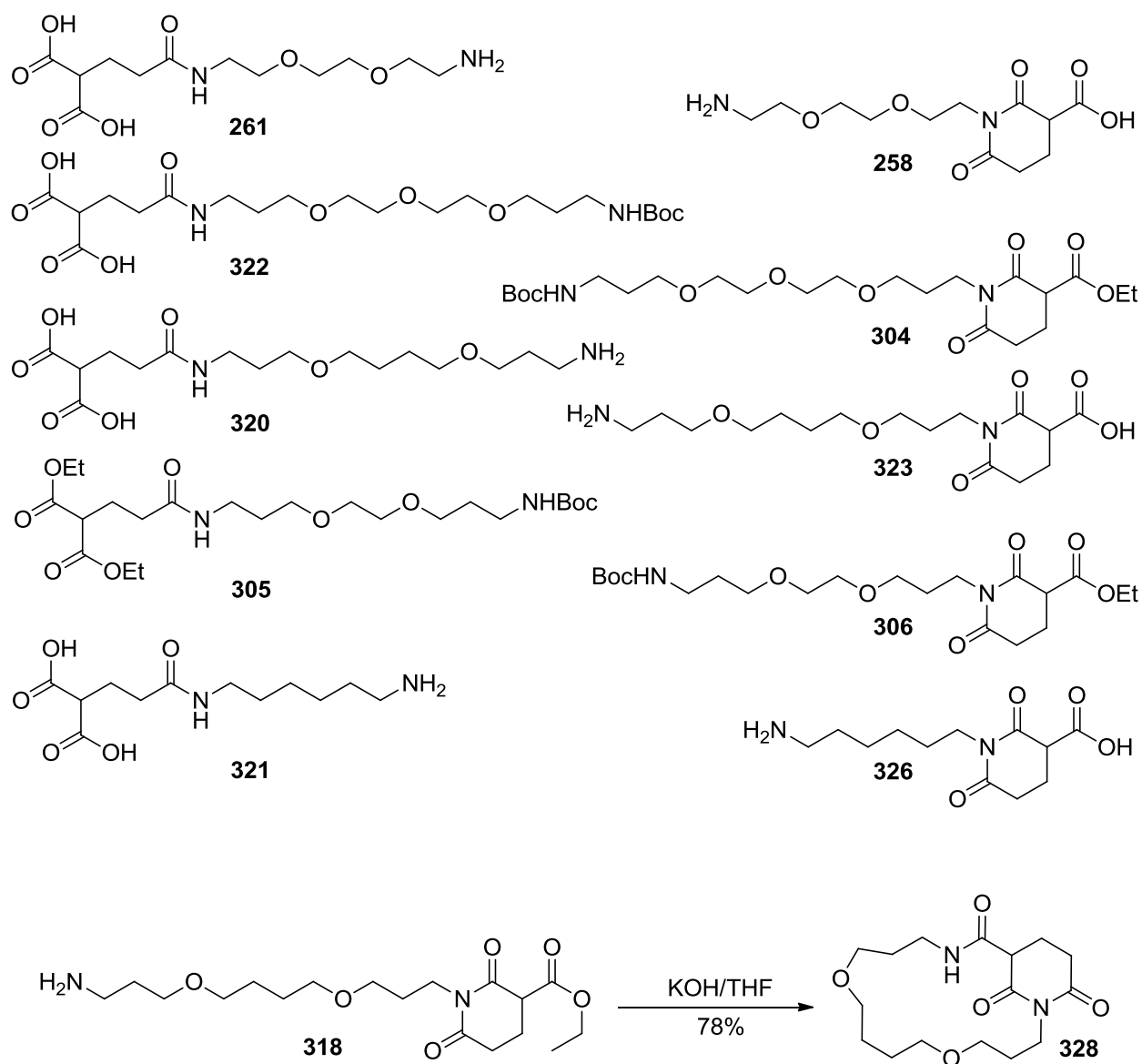


Abbildung 8.5: Strukturen verschiedener cyclischer und acyclischer Diamine mit Carbonsäurefunktionalität.

9 Outlook

The way of *in vitro* studies of biomolecules has been changed by bioorthogonal chemistry; however, challenges, i.e. reagents toxicity and acceleration of the reaction rate, remain for the future. Looking in advance, there is a need for investigations on the optimization of existing approaches and introduction of new methods to overcome the challenges. Azide alkyne cycloaddition in the form of a triazole is one of the approaches introduced so far that has proved to be a useful tool for bioorthogonal labeling. I anticipate that the adaptation of the venerable work by Staudinger, Huisgen, Sharpless, Bertozzi, Wittig and Krebs, and its further optimization will be pursued for the development of entirely new biocompatible chemical reactivities. Click reaction should be considered as an ideal approach for future investigations on live animals, not only due to its bioorthogonality, but also due to the facile availability and development of the functionalities involved. The approach followed in this work for the synthesis of alkynoic Sia derivatives has great potential for the development of a wide range of Sia derivatives. The modified petasis reaction in our approach enables facile introduction of different functionalities by using activated esters with a variety of functional groups, especially alkyne and azide. Synthesis of alkynes with high potential for click reaction, i.e. cyclooctynes with electron withdrawing groups, is another challenge for chemists, and the strategies introduced in this work may be helpful for the researchers working in this field. A simplified approach for the synthesis of azacyclooctynes has been introduced, which will open a new way for biologically compatible cyclooctynes due to their resemblance with natural lactams. In addition, the search of entirely new biocompatible chemical reactivities with improved bioavailability and bioorthogonality may provide fruitful new discoveries. A new class of molecules, oxo-aza dicarboxylic acids, introduced in this work, may have potential to change the cell surface properties, especially polarity. In addition, a new approach for the synthesis of glutarimide derivatives may be able to solve the configuration problems of the molecules, and smooth their clinical applications by minimizing the congenital effects. In all these efforts, the collaboration of chemists and biologists will be very important for the tailoring of unanswered questions. I suggest that new discoveries in this regard should be understood not merely as tool-making for biologists, but as a bona fide intellectual challenge for the chemists who must think outside the round-bottom flask to design robust chemical reactions that can operate *in vivo*, and teach us about living systems for decades in the future.

10 Experimental Work

10.1 Materials and Methods

All the reactions were performed under inert conditions unless otherwise described, using nitrogen or argon gas. The glassware was dried over flame or on heat gun and cooled to rt in the presence of an inert gas. Reagents were transferred by using syringe and the needles were pre-dried under phosphorus pentaoxide (P_2O_5) atmosphere in a desiccator if required. Organic solvents for both workup and column chromatography were cleaned by simple distillation over “Büchi R-114 Rotavapor” fitted with a water condenser, a Büchi water bath, and a “Vacuubrand MZ 2C” oil free diaphragm pump fitted with a “Vacuubrand CVC2” vacuum controller. The solvents for chemical reactions, even of HPLC grade from suppliers, were dried by us. anhyd. Et_2O , toluene and THF were obtained by distillation on sodium/benzophenone ketyl under an atmosphere of nitrogen immediately prior to use. Similarly anhyd. DCM was dried by refluxing over phosphorus pentaoxide (P_2O_5) under an atmosphere of nitrogen. anhyd. *N,N*-dimethylformamide (DMF) was prepared by distillation under reduced pressure and stored over molecular sieves. anhyd. MeOH was obtained by distilling over Mg metal in the presence of inert gas and the purification of MeCN was achieved by distillation over calcium hydride (CaH_2). When necessary the solvents (H_2O) were degassed by evacuating.

Commercially available reagents were purchased from Acros Organics, TCI, Alfa Aesar, Merck Chemicals, and Sigma-Aldrich Chemical Co. and used as received or after pre-treatment if necessary. Reactions were carried out under dry N_2 when necessary. The combined organic layers, after solvent extraction were dried over $MgSO_4$ or Na_2SO_4 and filtered over whatman filter papers.

The evaporation of the solvents from the reaction mixture or pure product after column chromatography was achieved by the use of Büchi Rotavapor and a Büchi 461 water bath and an Edwards E2M5 high vacuum pump. The last traces of the solvents were removed using a Trivac D4B high vacuum. Water was lyophilized on an Edwards Modulyo freeze-dryer.

Thin layer chromatography (TLC) was carried out on 0.2 mm silica gel, Macherey-Nagel (Düren) pre-coated plastic sheets (PolyGram[®] SIL G/UV₂₅₄) using UV light as the visualizing agent for conjugated systems. UV inactive substances were stained on TLC plates by the use of different staining reagents.

Alkaline $KMnO_4$: Universal staining reagent, especially sensitive to unsaturated systems i.e. alkenes, alkynes and carbonyl compounds. The reagent was prepared by the addition of $KMnO_4$ (1.50 g), K_2CO_3 (10.00 g) and NaOH (1.25 mL, 1% solution) in 200 mL of water.

Vanillin: An acidic solution of vanillin is a universal staining reagent, and gives different colors depending upon the nature of the functional group. For example, with alkyne functionality it gives green color. It was prepared by the addition of vanillin (12 g) in EtOH (200 mL) and conc. H_2SO_4 (2.25 mL).

Ninhydrin: Specific to amines, prepared by the addition of ninhydrin (400 mg), to BuOH (200 mL) followed by the addition of 10% AcOH (5 mL).

***N*-(1-Naphtyl)-ethylendiamin-dihydrochlorid:** It is a good staining reagent for alcohols and carbohydrates. It was prepared by the addition of *N*-(1-naphthyl)-ethylendiamin-dihydrochloride (5% w/w) and H_2SO_4 (5%) in methanol.

Column chromatography was carried out with Silica gel 60 (230-400 mesh ASTM) purchased from Macherey-Nagel (Düren). If required, deactivated silica gel was prepared by rinsing in 1% NEt_3 solution of the solvent system used as eluent. Size exclusion chromatography (SEC) was performed by the use of biogel eluting with degassed Milli-Q water.

^1H -NMR spectra were recorded on Bruker AC-300 (300 MHz), Bruker WH-400 (400 MHz) and Bruker DRX-600 (600 MHz) spectrometers supported by NOESY and COSY. Chemical shifts (δ_{H}) are reported in parts per million (ppm) downfield of TMS (0 ppm), or using the residual non-deuterated solvent as internal standard CDCl_3 (δ_{H} = 7.26 ppm, singlet, δ_{C} = 77.00 ppm, central of triplet) or MeOD (δ_{H} = 3.31 ppm, central of quintet, δ_{C} = 49.0 ppm, central of septet) and are quoted as the signal then in brackets integral, multiplicity and coupling constants. Coupling constants are expressed in Hertz (Hz). Multiplicities are assigned as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quin), doublet of doublet (dd), triplet of doublet (td), doublet of triplet (dt) and multiplet (m). ^{13}C -NMRs were recorded on Bruker AC-300 (75 MHz), Bruker WH-400 (100 MHz) and Bruker DRX-600 (150 MHz) spectrometers. Carbon spectra assignments are supported by DEPT editing if necessary. ^{19}F -NMR spectra taken by using CFCl_3 as an internal standard are supported by both coupled and decoupled analysis.

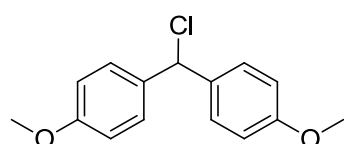
Mass spectra were recorded with Finnigan MAT-8430 spectrometer using the electron spray ionization (ESI), matrix assisted laser desorption/ionization (MALDI), electric impact ionization (EI) and atmospheric pressure chemical ionization (APCI). Optical rotations were recorded on a Dr. Kernchen Elektronik Automation Sucromat digital automatic saccharimeter.

All the safety precautions were adopted during the handling of chemicals in accordance with the safety instructions outlined in “Good Laboratory Practice” and the compounds were named according to the IUPAC nomenclature system.

10.2 Sialic Acid and Derivatives

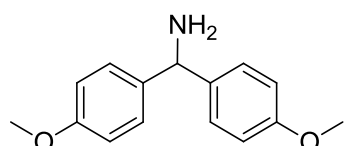
The synthesis of benzhydrylamine was achieved in two steps, the substitution of the hydroxyl group by chloride followed by the exchange of chloride by an amino group.

10.2.1 4,4'-Dimethoxybenzhydrylchloride **157**



Dimethoxybenzhydrol **156** (5.00 g, 20.51 mmol), dissolved in Et₂O (100 mL) was saturated with dry HCl gas. The mixture was evaporated to dryness and the residue was redissolved in Et₂O (70 mL) decanted from approximately 1 mL aqueous phase and reevaporated to yield 4,4'-dimethoxybenzhydrylchloride as an oil, which quickly solidified.

10.2.2 4,4'-Dimethoxybenzhydrylamine **152**



4,4'-Dimethoxybenzhydrylchloride **157** (8.54 g, 35.02 mmol) dissolved in DCM (5 mL) was added to DCM (100 mL) under nitrogen atmosphere and the solution was saturated with NH₃ with continuous stirring at rt. The solution was stirred for 3 h and concentrated *in vacuo*. The crude product was purified by flash silica gel chromatography using Et₂O as eluent to afford the amine **152** (4.15 g, 17.12 mmol) as a slightly yellow oil, which was solidified at rt.

Yield: 48%.

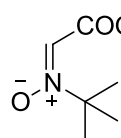
R_f: 0.34 (Et₂O).

¹H-NMR (300 MHz, CDCl₃): δ 7.13-7.09 (m, 4H, 3-H, 5-H, 3'-H, 5'-H), 6.74-6.68 (m, 4H, 2-H, 6-H, 2'-H, 6'-H), 4.91 (s, 1H, CHNH₂), 4.73 (s, 2H, NH₂), 3.63 (s, 6H, 2 × CH₃).

¹³C-NMR (75 MHz, CDCl₃): δ 160.03 (C-4, C-4'), 138.92 (C-1, C-1'), 129.02 (C-1, C-1', C-6, C-6'), 114.74 (C-3, C-3', C-5, C-5'), 59.51 (CNH₂), 55.68 (2 × OCH₃).

MS (ESI): *m/z* [M+Na]⁺ calcd for [C₁₅H₁₇NO₂+Na]⁺ 243.13, found 243.12.

10.2.3 Ethyl (2*E*)-[*tert*-butyl(oxido)imino]acetate (Nitrone) **142**



In a dried three neck flask equipped with nitrogen supply, *N-tert*-Butyl hydroxylamine hydrochloride **158** (1.25 g, 10.00 mmol), ethyl glyoxylate **159** (1.02 g, 50% in toluene, 10.00 mmol), and NaHCO₃ (1.68 g, 20.03 mmol) were stirred in toluene (20 mL) at rt for 12 h. The solid was filtered and the filtrate was concentrated to afford nitrone **142** (1.55 g, 8.95 mmol), which was pure enough as shown by spectral studies and used directly without any purification.

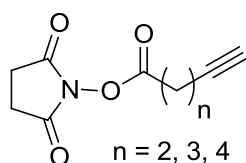
Yield: 90%.

R_f : 0.25 (Et₂O/Pent, 1:1).

¹H-NMR (400 MHz, CDCl₃): δ 7.22 (s, 1H, N=CH), 4.20 (q, J = 7.1 Hz, 2H, CH_{2,ester}), 1.47 (s, 9H, 3 \times CH_{3,tert-Butyl}), 1.25 (t, J = 7.1 Hz, 3H, H_{3,ester}).

¹³C-NMR (100 MHz, CDCl₃): δ 160.69 (CO_{ester}), 121.25 (C=N), 74.57 (CH_{2,ester}), 60.74 (C_{qua,tert-Butyl}), 28.04 (3C, 3 \times CH_{3,tert-Butyl}), 14.10 (CH_{3,ester}).

10.2.4 General procedure (A) for the synthesis of active esters of carboxylic acids **144**, **145**, **146**



Carboxylic acids **147**, **148**, **149** (20.00 mmol) were treated with *N*-hydroxysuccinimide **150** (20.00 mmol) and DCC (20.00 mmol) in anhyd. THF (80 mL) at 0 °C for 1 h under inert conditions. The resulting suspension was refrigerated overnight between 3-5 °C. The precipitated

solid (DCCU) was filtered off and the THF filtrate was evaporated under reduced pressure to afford *N*-hydroxysuccinimide ester of corresponding acids as a colorless oil after flash chromatography.

Yield: 81% (5-Hexynoic acid ester **145**).

R_f : 0.65 (Et₂O).

¹H-NMR (400 MHz, CDCl₃): δ 2.86 (s, 1H, 6-H), 2.80 (t, J = 7.4 Hz, 2H, 2-H₂), 2.37 (dt, J = 6.9, 2.7 Hz, 4H, 2'-H₂, 3'-H₂), 2.05 (t, J = 2.6 Hz, 2H, 4-H₂), 1.99 (quin, J = 7.1 Hz, 2H, 3-H₂).

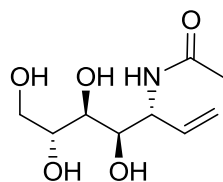
¹³C-NMR (100 MHz, CDCl₃): δ 168.42 (C-1', C-4'), 168.12(C-1), 82.37 (C-5), 69.78 (C-6), 29.60 (C-2), 25.52 (C-2', C-3'), 23.27 (C-3), 17.52 (C-4).

10.2.5 General procedure (B) for “one-pot” synthesis of **122**, **123**, **124** and **125** with modified petasis coupling

A solution of aldose sugar **126** (D-arabinose) (10.00 mmol), Dimethoxybenzhydramine **142** (2.43 g, 10.00 mmol) and vinyl boronic acid dibutyl ester (3.68 g, 20.00 mmol) in aqueous ethanol (100 mL, EtOH/H₂O = 4:1) was stirred at 50 °C for 72 h. TFA (3 mL) was added and the mixture was stirred for further 16 h. The solvent was evaporated and MeOH (50 mL) was added to dissolve the residue. Sodium bicarbonate (1.68 g, 20.00 mmol) and Ac₂O (1.27 g, 12.53 mmol) (for the preparation of **144**, **145** and **146**, the activated ester of corresponding carboxylic acids **147**, **148** and **149** were added instead of Ac₂O) were added to the solution and stirred for 1 h at rt. The suspension was filtered and the filtrate was concentrated under reduced pressure. The crude

product was purified by silica gel flash chromatography (eluted with DCM and MeOH) to afford the product in 55-85% yield.

10.2.6 *N*-(1*R*,2*R*,3*S*,4*R*)-2,3,4,5-Tetrahydroxy-1-vinyl-pentyl)-acetamide **122**



Following the general procedure **B** D-arabinose **126** (200 mg, 1.33 mmol), vinylboronic acid dibutyl ester **151** (478 mg, 2.63 mmol), amine **152** (315 mg, 1.33 mmol) and acetic anhydride (37 mg, 1.52 mmol) were treated. The acetamide **122** (176 mg, 800 μ mol) was obtained as light brown oil after purification by silica gel column.

Yield: 81%.

R_f : 0.42 (DCM/MeOH, 5:1).

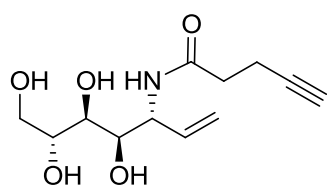
$[\alpha]_D^{20}$: +15.7 (c 1, CH₃OH).

¹H-NMR (400 MHz, CD₃OD): δ 6.05 (ddd, J = 17.2, 10.5, 5.9 Hz, 1H, 1''-H), 5.24 (dt, J = 17.2, 1.5 Hz, 1H, 2_a''-H₂), 5.13 (dt, J = 10.5, 1.5 Hz, 1H, 2_b''-H₂), 4.57-4.52 (m, 1H, 1-H), 3.82-3.58 (m, 5H, 5-H₂, 4-H, 3-H, 2-H), 2.02 (s, 3H, CH₃).

¹³C-NMR (100 MHz, CD₃OD): δ 173.36 (CO), 136.91 (C-1'), 116.82 (C-2'), 72.52, 72.06, 71.34 (C-4, C-3, C-2), 64.56 (C-5), 55.02 (C-1), 22.76 (CH₃).

MS (ESI): m/z [M+Na]⁺ calcd for [C₉H₁₇NO₉+Na]⁺ 219.12, found 219.12.

10.2.7 *N*-(1*R*,2*R*,3*S*,4*R*)-Pent-4-ynic acid(2,3,4,5-tetrahydroxy-1-vinylpentyl) amide **123**



Following the general procedure **B**, D-arabinose **126** (1.50 g, 10.00 mmol), vinylboronic acid dibutyl ester **151** (3.32 g, 18.03 mmol), amine **152** (1.92 g, 10.02 mmol) and 4-pentynoic acid ester **144** (2.00 g, 20.43 mmol) were treated. Light brown oil **123**

(2.20 g, 8.50 mmol) was obtained after the purification by silica column.

Yield: 85%.

R_f : 0.33 (DCM/MeOH, 7:1).

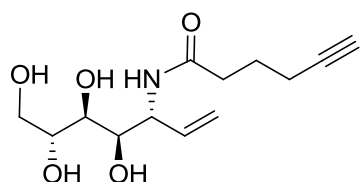
$[\alpha]_D^{20}$: +16.2 (c 1, CH₃OH).

¹H-NMR (400 MHz, CD₃OD): δ 6.05 (ddd, J = 17.3, 10.5, 5.7 Hz, 1H, 1''-H), 5.31 (td, J = 17.3, 1.5 Hz, 1H, 2_a''-H₂), 5.22 (td, J = 10.5, 1.5 Hz, 1H, 2_b''-H₂), 4.59-4.54 (m, 1H, 1-H), 3.81-3.60 (m, 5H, 5-H₂, 4-H, 3-H, 2-H), 2.53-2.48 (m, 4H, 2'-H₂, 3'-H₂), 2.32 (t, J = 2.4 Hz, 1H, 5'-H).

^{13}C -NMR (100 MHz, CD_3OD): δ 174.05 (C-1'), 136.65 (C-1''), 116.87 (C-2''), 83.54 (C-4'), 72.42, 72.07, 71.42 (C-4, C-3, C-2), 70.64 (C-5'), 64.84 (C-5), 54.94 (C-1), 36.04 (C-2'), 15.63 (C-3').

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{12}\text{H}_{19}\text{NO}_5+\text{Na}]^+$ 280.11, found 280.11.

10.2.8 *N*-(1*R*,2*R*,3*S*,4*R*)-Hex-5-ynic acid(2,3,4,5-tetrahydroxy-1-vinylpentyl) amide **124**



Following the general procedure **B** D-arabinose **126** (1.09 g, 5.73 mmol), vinylboronic acid dibutyl ester **151** (2.51 g, 11.46 mmol), amine **152** (1.39 g, 5.73 mmol) and activated ester of 5-hexynoic acid **145** (950 mg, 8.52 mmol) were treated in the solvent (50 mL). The crude product was purified by silica column to afford **124** (1.26 g, 4.29 mmol) as a thick light yellow oil, which turned to solid at 4 °C.

Yield: 75%.

R_f : 0.34 (DCM/MeOH, 7:1).

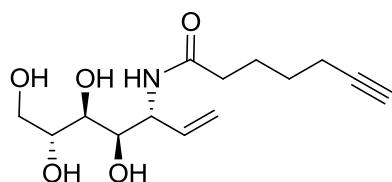
$[\alpha]_D^{20}$: +19.8 (c 1, CH_3OH).

^1H -NMR (400 MHz, CD_3OD): δ 6.05 (ddd, $J = 17.3, 10.5, 5.7$ Hz, 1H, 1''-H), 5.27 (td, $J = 17.3, 1.5$ Hz, 1H, 2_a''-H₂), 5.22 (td, $J = 10.5, 1.5$ Hz, 1H, 2_b''-H₂), 4.58-4.53 (m, 1H, 1-H), 3.84-3.50 (m, 5H, 5-H₂, 4-H, 3-H, 2-H), 2.50 (t, $J = 7.3$ Hz, 2H, 2'-H₂), 2.30-2.20 (m, 3H, 6'-H, 4'-H₂), 1.88-1.72 (m, 2H, 3'-H₂).

^{13}C -NMR (100 MHz, CD_3OD): δ 175.29 (C-1'), 137.04 (C-1''), 116.65 (C-2''), 84.12 (C-5'), 72.46, 72.11, 71.55 (C-4, C-3, C-2), 70.34 (C-6'), 64.94 (C-5), 55.11 (C-1), 35.89 (C-2'), 25.77 (C-3'), 18.58 (C-4').

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{13}\text{H}_{20}\text{NO}_5+\text{Na}]^+$ 294.13, found 294.13.

10.2.9 *N*-(1*R*,2*R*,3*S*,4*R*)-Hept-6-ynic acid(2,3,4,5-tetrahydroxy-1-vinylpentyl) amide **125**



D-Arabinose **126** (1.52 g, 10.00 mmol), vinylboronic acid dibutyl ester **151** (4.12 g, 18.04 mmol), amine **152** (1.92 g, 10.00 mmol) and activated ester of 6-heptynoic acid **146** (2.01 g, 15.82 mmol) were treated in the solvent (100 mL) in accordance with the general procedure **B**. The product was purified by flash chromatography to afford the compound **125** (2.11 g, 7.40 mmol) as a white powder.

Yield: 75%.

R_f : 0.37 (DCM/MeOH, 7:1).

$[\alpha]_D^{20}$: +17.5 (c 1, CH₃OH).

¹H-NMR (300 MHz, CD₃OD): δ 6.04 (ddd, J = 17.3, 10.5, 5.7 Hz, 1H, 1''-H), 5.26 (dt, J = 17.3, 1.5 Hz, 1H, 2_a''-H₂), 5.22 (dt, J = 10.5, 5.7 Hz, 1H, 2_b''-H₂), 4.57-4.53 (m, 1H, 1-H), 3.83-3.57 (m, 5H, 2-H, 3-H, 4-H, 5-H₂), 2.30 (t, J = 7.3 Hz, 2H, 2'-H₂), 2.26-2.18 (m, 3H, 5'-H₂, 7'-H), 1.81-1.72 (m, 2H, 3'-H₂), 1.85-1.79 (m, 2H, 4'-H₂).

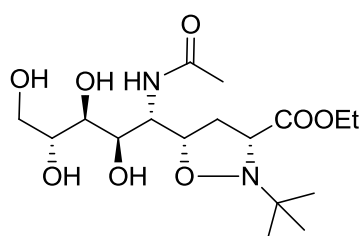
¹³C-NMR (75 MHz, CD₃OD): δ 176.02 (C-1'), 136.87 (C-1''), 116.95 (C-2''), 84.61 (C-6'), 72.68, 72.22, 71.57 (C-4, C-3, C-2), 69.79 (C-7'), 64.65 (C-5), 54.95 (C-1), 36.60 (C-2'), 29.19 (C-3'), 26.06 (C-4'), 19.00 (C-5').

MS (ESI): m/z [M+Na]⁺ calcd for [C₁₄H₂₃NO₅+Na]⁺ 308.15, found 308.15.

10.2.10 General procedure (C) for 1,3-dipolar cycloaddition of nitron 142

Polyhydroxy olefin (**122**, **123**, **124** and **125**) (1.00 mmol) and nitron **142** (346 mg, 2.01 mmol) in dioxane (20 mL) were stirred at 30 °C for 14 days. After the complete conversion of the starting material, monitored by TLC, the solvent was removed through evaporation under reduced pressure and the residue was purified by silica gel chromatography (MeOH/DCM 1:10 to 1:5) to yields isoxazolidines 70 to 85%.

10.2.11 (1S)-1-(Acetylamino)-1-[(3R,5S)-2-*tert*-butyl-3-(ethoxycarbonyl)isoxazolidin-5-yl]-1-deoxy-D-arabinitol **118**



General procedure **C** was followed for the treatment of polyhydroxy olefin **122** (109 mg, 490 μ mol) and nitron **142** (173 mg, 1.00 mmol). The crude product was purified by flash chromatography, eluting with DCM/MeOH (8:1 to 5:1) to afford the isoxazolidine **118** (171 mg, 433 μ mol) as a colorless oil.

Yield: 87%.

R_f : 0.41 (DCM/MeOH 10:1).

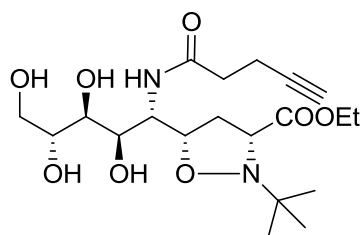
$[\alpha]_D^{20}$: +9.6 (c 1, CH₃OH).

¹H-NMR (300 MHz, CD₃OD): δ 7.70 (d, J = 7.8 Hz, 1H, NH), 4.69 (t, J = 7.1 Hz, 1H, 1-H), 4.16-4.06 (m, 2H, CH_{2,ester}), 3.88 (td, J = 7.9, 4.9 Hz, 2H, 5'-H, 3'-H), 3.78 (dd, J = 10.4, 1.5 Hz, 2H, 3-H, 2-H), 3.67 (dd, J = 10.3, 0.7, 1H, 4-H), 3.56 (dd, J = 11.1, 3.4 Hz, 1H, 5_a-H₂), 3.41-3.38 (m, 1H, 5_b-H₂), 2.62 (ddd, J = 12.3, 8.4, 7.1 Hz, 1H, 4_a'-H₂), 2.12-2.08 (m, 1H, 4_b'-H₂), 2.04 (s, 3H, CH_{3,amide}), 1.19 (t, J = 7.1 Hz, 3H, CH_{3,ester}), 1.04 (s, 9H, 3 \times CH_{3,tert-butyl}).

^{13}C -NMR (75 MHz, CD_3OD): δ 175.14 (CO_{ester}), 174.36 (NHCO), 77.32 ($\text{C-5}'$), 72.11, 71.48 (C-4 , C-3), 70.47 (C-2), 64.87 ($\text{C-3}'$), 62.54 (C-5), 62.19 ($\text{OCH}_{2,\text{ester}}$), 61.22 ($\text{C}_{\text{qua},\text{tert-butyl}}$), 53.94 (C-1), 38.96 ($\text{C-4}'$), 25.93 (OCH_3), 22.85 ($3 \times \text{CH}_{3,\text{tert-butyl}}$), 14.38 ($\text{CH}_{3,\text{ester}}$).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_8+\text{Na}]^+$ 415.21, found 415.21.

10.2.12 (1S)-1-[(3R,5S)-2-*tert*-Butyl-3-(ethoxycarbonyl)isoxazolidin-5-yl]-1-deoxy-1-(pent-4-ynoylamino)-D-arabinitol **119**



Synthesis of **119** was achieved through general procedure **C**, using polyhydroxy olefin **123** (2.00 g, 7.78 mmol) and nitrone **142** (2.69 g, 15.54 mmol). The crude product was purified by flash chromatography to afford the desired product **119** (2.93 g, 6.80 mmol) as a light yellow oil.

Yield: 87%.

R_f : 0.45 (DCM/MeOH, 7:1).

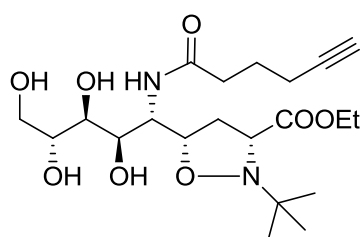
$[\alpha]_{\text{D}}^{20}$: +5.4 (c 1, CH_3OH).

^1H -NMR (400 MHz, CD_3OD): δ 4.76 (ddd, $J = 8.4, 6.8, 1.4$ Hz, 1H, 1-H), 4.25-4.18 (m, 2H, $\text{CH}_{2,\text{ester}}$), 3.98, 3.95 (2t, $J = 8.5$ Hz, 2H, 3'-H, 5'-H), 3.92 (dd, $J = 10.3, 1.5$ Hz, 1H, 3-H), 3.89 (dd, $J = 10.3, 0.7$ Hz, 1H, 2-H), 3.81 (dd, $J = 11.1, 3.4$ Hz, 1H, 5_a-H₂), 3.73-3.68 (m, 1H, 4-H), 3.60 (dd, $J = 11.1, 5.9$ Hz, 1H, 5_b-H₂), 3.52-3.48 (m, 1H, 4_a'-H₂), 3.33 (td, $J = 3.3, 1.6$ Hz, 1H, 4_b'-H₂), 2.56-2.53 (m, 2H, 3''-H₂), 1.99 (s, 1H, 5''-H), 1.29 (t, $J = 7.1$ Hz, 2H, 2''-H₂), 1.20 (t, $J = 7.0$ Hz, 3H, $\text{CH}_{3,\text{ester}}$), 1.16 (s, 9H, $3 \times \text{CH}_{3,\text{tert-butyl}}$).

^{13}C -NMR (100 MHz, CD_3OD): δ 175.73 (CO_{ester}), 174.6 ($\text{C-1}''$), 83.69 ($\text{C-4}''$), 77.32 ($\text{C-5}'$), 71.31 (C-4), 70.59 ($\text{C-5}''$), 66.88 (C-2), 65.3 (C-3), 64.81 ($\text{C-3}'$), 62.5 (C-5), 62.21 ($\text{CH}_{2,\text{ester}}$), 61.24 ($\text{C}_{\text{qua},\text{tert-butyl}}$), 53.7 (C-1), 38.94 ($\text{C-4}'$), 36.11 ($\text{C-2}''$), 20.28 ($3 \times \text{CH}_{3,\text{tert-butyl}}$), 15.82 ($\text{C-3}''$), 14.41 ($\text{CH}_{3,\text{ester}}$).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{20}\text{H}_{34}\text{N}_2\text{O}_8+\text{Na}]^+$ 453.22, found 453.22.

10.2.13 (1S)-1-[(3R,5S)-2-*tert*-Butyl-3-(ethoxycarbonyl)isoxazolidin-5-yl]-1-deoxy-1-(hex-5-ynoylamino)-D-arabinitol **120**



Polyhydroxy olefin **124** (1.53 g, 5.53 mmol) and nitrone **142** (2.01 g, 11.63 mmol) were treated according to the general procedure **C**. The crude product was purified by flash chromatography to afford the isoxazolidine **120** (2.06 g,

4.51 mmol) as a colorless oil.

Yield: 81%.

R_f : 0.42 (DCM/MeOH, 7:1).

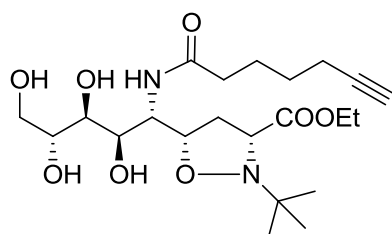
$[\alpha]_D^{20}$: +7.2 (c 1, CH₃OH).

¹H-NMR (400 MHz, CD₃OD): δ 4.63 (dt, J = 8.4, 1.6 Hz, 1H, 1-H), 4.11 (dq, J = 7.2, 1.5 Hz, 2H, OCH₂CH₃), 3.89 (t, J = 8.5 Hz, 1H, 5'-H), 3.86-3.47 (m, 5H, 5-H₂, 4-H, 3-H, 2-H), 3.30 (dd, J = 8.7, 0.8 Hz, 1H, 3'-H), 3.23-3.20 (m, 1H, 6''-H), 2.65-2.57 (m, 1H, 4_a'-H₂), 2.36 (t, J = 7.2 Hz, 2H, 4''-H₂), 2.18-2.06 (m, 3H, 4_b'-H₂, 2''-H₂), 1.79-1.71 (m, 2H, 3''-H₂), 1.14 (t, J = 7.1 Hz, 3H, OCH₂CH₃), 1.05 (s, 9H, 3 \times CH₃,*tert*-butyl).

¹³C-NMR (100 MHz, CD₃OD): δ 177.07 (CONH), 174.22 (CO_{ester}), 84.19 (C-5'), 77.34 (C-5), 72.15, 71.42, 70.63 (C-4, C-3, C-2), 70.35 (C-6''), 65.27 (C-5), 62.50 (CH₂,_{ester}), 62.23 (C-3'), 61.21 (C_{qua},*tert*-butyl), 53.71 (C-1), 39.07 (C-4'), 35.92 (C-2''), 25.91 (C-4''), 25.91 (CH₃,*tert*-butyl), 25.85 (C-3''), 14.45 (CH₃,_{ester}).

MS (ESI): m/z [M+Na]⁺ calcd for [C₂₁H₃₆N₂O₈+Na]⁺ 467.23, found 467.23.

10.2.14 (1S)-1-[(3R,5S)-2-*tert*-Butyl-3-(ethoxycarbonyl)isoxazolidin-5-yl]-1-deoxy-1-(hept-6-ynoylamino)-D-arabinitol **121**



Following the general procedure **C** polyhydroxy olefin **125** (1.01 g, 3.52 mmol) and nitrone **142** (1.21 g, 7.22 mmol) were treated in dioxane (70 mL). Flash chromatography afforded **121** (1.93 g, 4.19 mmol) as a light brown gel.

Yield: 79%.

R_f : 0.51 (DCM/MeOH, 7:1).

$[\alpha]_D^{20}$: +6.09 (c 1, CH₃OH).

¹H-NMR (300 MHz, CD₃OD): δ 4.74 (t, J = 8.3 Hz, 1H, 1-H), 4.20 (q, J = 7.1 Hz, 2H, CH₂,_{ester}), 4.01 (t, J = 8.5 Hz, 1H, 5'-H), 3.96-3.56 (m, 5H, 5-H₂, 4-H, 3-H, 2-H), 3.62 (d, J = 8.6 Hz, 1H, 3'-H), 3.35-3.30 (m, 1H, 7''-H), 2.77-2.66 (m, 1H, 4_a'-H₂), 2.38 (t, J = 7.3 Hz, 2H, 5''-H₂), 2.26-2.20 (m, 3H, 4_b'-H₂, 2''-H₂), 1.85-1.75 (m, 2H, 3''-H₂), 1.64-1.55 (m, 2H, 4''-H₂), 1.28 (t, J = 7.1 Hz, 3H, CH₃,_{ester}), 1.15 (s, 9H, 3 \times CH₃,*tert*-butyl).

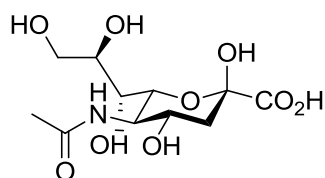
¹³C-NMR (75 MHz, CD₃OD): δ 177.63 (CONH), 174.29 (CO_{ester}), 84.69 (C-6''), 77.37 (C-5'), 72.17, 71.48, 70.67 (C-4, C-3, C-2), 69.82 (C-7''), 65.34 (C-5), 62.52 (CH₂,_{ester}), 62.23 (C-3'), 61.25 (C_{qua},*tert*-butyl), 53.71 (C-1), 39.11 (C-4'), 36.53 (C-2''), 29.15 (C-3''), 25.92 (C-5''), 19.89 (C-4''), 18.82 (3 \times CH₃,*tert*-butyl), 14.45 (CH₃,_{ester}).

MS (ESI): m/z $[M+Na]^+$ calcd for $[C_{22}H_{38}N_2O_8+Na]^+$ 481.25, found 481.25.

10.2.15 General procedure (D) for the base catalyzed ring opening of isoxazolidines

Isioxazolidine and MeONa were stirred overnight in anhyd. MeOH at room temperature. Water was added and the solution was stirred for further 24 h followed by neutralization with Amberlyst ion exchange resin. The solvent was removed under reduced pressure and the crude products were subjected to size exclusion chromatography with Biogel-2 to afford the products in the range of 40 to 50% yield.

10.2.16 *N*-Acetylneuraminic acid (Neu5Ac) **47**



General procedure **D** was followed for the treatment of isioxazolidine **118** (171 mg, 433 μ mol) in basic MeOH to afford Neu5Ac **47** (53 mg, 173 μ mol) as a white solid with α/β anomer ratio 1:9.

Yield: 40%.

R_f : 0.23 (DCM/MeOH, 5:2).

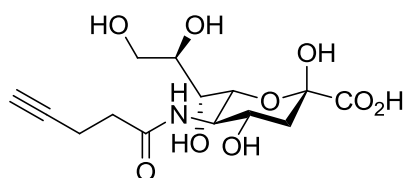
$[\alpha]_D^{20}$: -27.9 (c, 1.2 water) lit^[226], calculated -30.5 (c 1, CH₃OH).

¹H-NMR (300 MHz, CD₃OD): β -anomer: δ 3.99 (d, J = 10.2 Hz, 1H, 6-H), 3.98-3.92 (m, 1H, 4-H), 3.79 (t, J = 10.2 Hz, 1H, H-5), 3.71 (dd, J = 10.9, 2.4 Hz, 1H, 9-H_a), 3.64-3.59 (m, 1H, 8-H), 3.55 (dd, J = 10.9, 5.5 Hz, 1H, 9-H_b), 3.41 (d, J = 8.9 Hz, 1H, 7-H), 2.15 (dd, J = 12.8, 4.9 Hz, 1H, 3_{eq}-H₂), 2.03 (s, 3H, CH₃), 1.78 (dd, J = 12.8, 11.4 Hz, 1H, 3_{ax}-H₂).

¹³C-NMR (75 MHz, CD₃OD): δ 171.90 (C-1'), 163.39 (C-1), 96.64 (C-2), 71.65 (C-8, C-6), 70.11 (C-7), 67.77 (C-4), 64.77 (C-9), 54.79 (C-5), 40.60 (C-3), 22.21 (C-2').

MS (ESI): m/z $[M-H]^-$ calcd for $[C_{11}H_{19}NO_9-H]^-$ 308.09, found 308.11.

10.2.17 *N*-Pent-4-ynoylneuraminic acid (Neu5Pent) **115**



Following the general procedure **D** isioxazolidine **119** (2.53 g, 5.81 mmol) was treated with MeONa (0.05 M in MeOH) in anhyd. MeOH. The product was purified by size exclusion chromatography to afford **115** (846 mg, 2.44 mmol) as a light brown powder.

Yield: 42%.

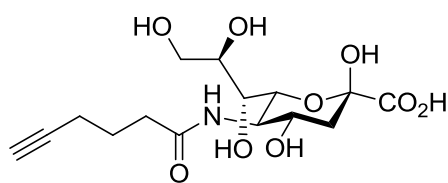
R_f : 0.28 (DCM/MeOH, 5:2).

$^1\text{H-NMR}$ (300 MHz, CD_3OD): β -anomer: δ 4.09-4.02 (m, 1H, 4-H), 4.01 (d, $J = 10.7$ Hz, 1H, 6-H), 3.85 (t, $J = 10.3$ Hz, 1H, 5-H), 3.80 (dd, $J = 11.5, 2.7$ Hz, 1H, 9_a-H_2), 3.89-3.74 (m, 1H, 8-H), 3.62 (dd, $J = 11.2, 5.6$, 1H, 9_b-H_2), 3.51 (d, $J = 9.3$, 1H, 7-H), 3.23-3.20 (m, 1H, $5'\text{-H}$), 2.26-2.20 (m, 2H, $2'\text{-H}_2$), 2.14 (dd, $J = 12.83, 4.87$, 1H, 3_{eq}-H_2), 1.86-1.80 (m, 3H, 3_{ax}-H_2 , $3'\text{-H}_2$).

$^{13}\text{C-NMR}$ (75 MHz, CD_3OD): δ 177.00 (C-1'), 173.49 (C-1), 96.49 (C-2), 84.05 (C-4'), 72.03 (C-8), 71.55 (C-6), 70.08 (C-7), 70.03 (C-5'), 67.63 (C-4), 64.68 (C-9), 53.94 (C-5), 40.94 (C-3), 35.67 (C-2'), 25.64 (C-3').

MS (ESI): m/z $[\text{M-H}]^-$ calcd for $[\text{C}_{14}\text{H}_{21}\text{NO}_9\text{-H}]^-$ 346.11, found 346.11.

10.2.18 *N*-Hex-5-ynoylneuraminic acid (Neu5Hex) **116**



Procedure **D** was adopted for the base catalyzed ring opening of isoxazolidine **120** (650 mg, 1.46 mmol). Size exclusion chromatography afforded Neu5Hex **116** (243 mg, 670 μmol) as a white solid.

Yield: 46%.

R_f : 0.29 (DCM/MeOH, 5:2).

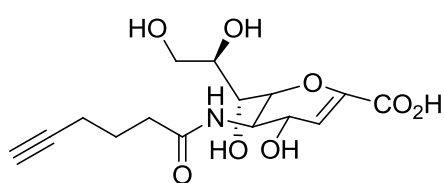
$[\alpha]_{\text{D}}^{20}$: -19.04 (c 1, H_2O).

$^1\text{H-NMR}$ (300 MHz, CD_3OD): β -anomer, δ 4.09-4.02 (m, 1H, 4-H), 4.01 (d, $J = 10.7$ Hz, 1H, 6-H), 3.84 (t, $J = 10.3$ Hz, 1H, 5-H), 3.80 (dd, $J = 11.5, 2.8$ Hz, 1H, 9_a-H_2), 3.89-3.74 (m, 1H, 8-H), 3.62 (dd, $J = 11.2, 5.6$, 1H, 9_b-H_2), 3.51 (d, $J = 9.3$, 1H, 7-H), 3.23-3.20 (m, 1H, $6'\text{-H}$), 2.40 (t, $J = 7.3$ Hz, 2H, $4'\text{-H}_2$), 2.26-2.20 (m, 2H, $2'\text{-H}_2$), 2.15 (dd, $J = 12.8, 4.9$ Hz, 1H, 3_{eq}-H_2), 1.86-1.80 (m, 3H, 3_{ax}-H_2 , $3'\text{-H}_2$).

$^{13}\text{C-NMR}$ (75 MHz, CD_3OD): δ 177.00 (C-1'), 173.49 (C-1), 96.49 (C-2), 84.05 (C-5'), 72.03 (C-8), 71.55 (C-6), 70.08 (C-7), 70.03 (C-6'), 67.63 (C-4), 64.68 (C-9), 53.94 (C-5), 40.94 (C-3), 35.67 (C-2'), 25.64 (C-3'), 18.49 (C-4').

MS (ESI): m/z $[\text{M-H}]^-$ calcd for $[\text{C}_{14}\text{H}_{21}\text{NO}_9\text{-H}]^-$ 360.13, found 360.18.

10.2.19 2-Deoxy-2,3-didehydro-*N*-Hex-5-ynoylneuraminic acid **168**



Synthesis of olefinic derivative of Neu5Hex **168** took place as a result of dehydration in the above reaction during the neutralization of the basic media with amberlyst resin. Size exclusion chromatography afforded **168** (102 mg,

272 μmol) as a white powder.

Yield: 21%.

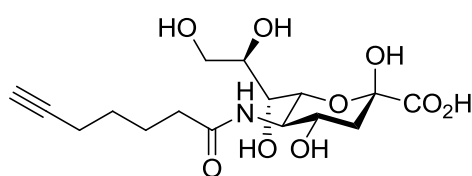
R_f : 0.29 (DCM/MeOH, 5:2).

$^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 5.67 (d, $J = 2.5$ Hz, 1H, 3-H), 4.34 (dd, $J = 10.4, 1.6$ Hz, 1H, 4-H), 4.01 (dd, $J = 10.4, 0.7$ Hz, 1H, 9_a-H), 3.80 (dd, $J = 10.8, 3.2$ Hz, 1H, 6-H), 3.74-3.68 (m, 2H, 7-H, 8-H), 3.65-3.59 (m, 1H, 5-H), 3.45 (dd, $J = 8.5, 0.8$ Hz, 1H, 9_b-H), 2.26-2.20 (m, 3H, 6'-H, 2'-H₂), 2.14 (td, $J = 7.1, 2.5$ Hz, 2H, 4'-H₂), 1.72 (quin, $J = 7.2$ Hz, 2H, 3'-H₂).

$^{13}\text{C-NMR}$ (75 MHz, CD_3OD): δ 175.82 (CONH), 173.70 (COOH), 132.91 (C-2), 110.22 (C-3), 84.25 (C-5'), 81.01 (C-4), 72.68 (C-8), 71.07 (C-6), 70.26 (C-7), 69.67 (C-6'), 65.07 (C-9), 52.82 (C-5), 35.82 (C-2'), 26.05 (C-3'), 18.79 (C-4').

MS (ESI): m/z $[\text{M-H}]^-$ calcd for $[\text{C}_{15}\text{H}_{20}\text{NO}_8\text{-H}]^-$ 342.12, found 342.13.

10.2.20 N-Hept-6-ynylneuraminic acid (Neu5Hep) 117



In accordance with the general procedure **D**, isoxazolidine **121** (500 mg, 1.39 mmol) was treated with MeONa (0.05 M in CH_3OH). Size exclusion chromatography afforded two fractions, the second of which (204 mg,

544 μmol) was a light brown powder assigned as **209**.

Yield: 42%.

R_f : 0.31 (DCM/MeOH, 5:2).

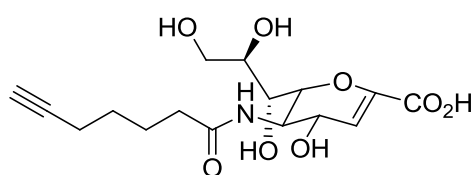
$[\alpha]_{\text{D}}^{20}$: -26.4 (c 1, CH_3OH).

$^1\text{H-NMR}$ (300 MHz, CD_3OD): β -anomer, δ 4.09-4.02 (m, 1H, 4-H), 4.02 (d, $J = 10.7$ Hz, 1H, 6-H), 3.85 (t, $J = 10.3$ Hz, 1H, 5-H), 3.80 (dd, $J = 11.5, 2.7$ Hz, 1H, 9_a-H₂), 3.89-3.74 (m, 1H, 8-H), 3.62 (dd, $J = 11.2, 5.6$ Hz, 1H, 9_b-H₂), 3.50 (d, $J = 9.3$ Hz, 1H, 7-H), 3.23-3.20 (m, 1H, 6'-H), 2.39 (t, $J = 7.3$ Hz, 2H, 4'-H₂), 2.26-2.20 (m, 2H, 2'-H₂), 2.14 (dd, $J = 12.8, 4.9$ Hz, 1H, 3_{eq}-H₂), 1.86-1.80 (m, 3H, 3_{ax}-H₂, 3'-H₂).

$^{13}\text{C-NMR}$ (75 MHz, CD_3OD): δ 177.00 (C-1'), 173.49 (C-1), 96.49 (C-2), 84.05 (C-5'), 72.03 (C-8), 71.55 (C-6), 70.08 (C-7), 70.03 (C-6'), 67.63 (C-4), 64.68 (C-9), 53.94 (C-5), 40.94 (C-3), 35.67 (C-2'), 25.64 (C-3'), 18.49 (C-4').

MS (ESI): m/z $[\text{M-H}]^-$ calcd for $[\text{C}_{16}\text{H}_{25}\text{NO}_9\text{-H}]^-$ 374.14, found 374.14.

10.2.21 2-Deoxy-2,3-didehydro-*N*-Hept-6-ynoylneuraminic acid **169**



Olefinic derivative of Neu5Hep **169** was obtained as a result of the dehydration of Neu5Hep **117** during the neutralization of the alkaline media with amberlyst resin.

The size exclusion chromatography of the above reaction mixture afforded **169** (110 mg, 308 μ mol) as a white powder.

Yield: 22%.

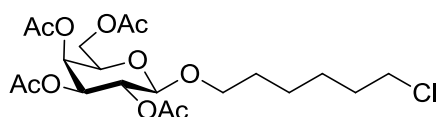
R_f : 0.27 (DCM/MeOH, 2:5).

$^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 5.65 (d, $J = 2.2$ Hz, 1H, 3-H), 4.33 (dd, $J = 10.4, 1.5$ Hz, 1H, 4-H), 4.01 (d, $J = 10.5$ Hz, 1H, 9_a-H), 3.81 (dd, $J = 10.8, 3.2$ Hz, 1H, 6-H), 3.7 (dd, $J = 8.6, 5.4$ Hz, 1H, 7-H), 3.64 (t, $J = 10.9$ Hz, 1H, 8-H), 3.59 (t, $J = 9.6$ Hz, 1H, 5-H), 3.44 (d, $J = 8.4$ Hz, 1H, 9_b-H₂), 2.21-2.18 (m, 3H, 2'-H₂, 7'-H₂), 2.13 (t, $J = 7.4$ Hz, 2H, 5'-H₂), 2.15-2.08 (m, 2H, 3'-H₂), 1.48 (quin, $J = 7.2$ Hz, 2H, 4'-H₂).

$^{13}\text{C-NMR}$ (75 MHz, CD_3OD): δ 176.31 (C-1), 173.72 (C-1'), 132.93 (C-2), 110.31 (C-3), 84.69 (C-6'), 80.99 (C-8), 72.67 (C-6), 71.07 (C-7), 69.8 (C-4), 69.68 (C-7'), 64.96 (C-9), 52.56 (C-5), 36.39 (C-2'), 28.99 (C-5'), 25.96 (C-3'), 18.56 (C-4').

10.3 Synthesis of Azide Derivatives

10.3.1 6-Chlorohexyl 2,3,4,6-tetra-*O*-acetyl- β -D-galactose **172**



A solution of penta acetyl galactopyranoside **170** (3.00 g, 7.68 mmol) and 6-chloro-1-hexanol **171** (1.05 g, 7.68 mmol) in dry DCM (100 mL) containing molecular sieves 4 Å (1.50 g) was cooled to 0 °C and $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (15

mL, 118.52 mmol) was added in 2 min. The mixture was stirred overnight at rt, neutralized with NEt_3 , washed with aqueous saturated NaHCO_3 , H_2O and brine, dried over Na_2SO_4 , filtered and concentrated. The residue was purified by silica gel chromatography ($\text{Et}_2\text{O}/\text{Pent}$, 1:1) to afford **172** (3.04 g, 6.53 mmol) as a slightly yellow syrup.

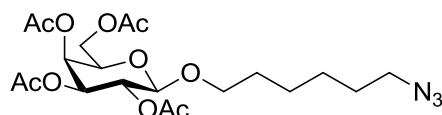
Yield: 85%.

R_f : 0.32 ($\text{Et}_2\text{O}/\text{Pent}$, 1:1).

10.3.2 General procedure (E) for the synthesis of azides

The solution of the chloride and NaN_3 (1.5 equiv.) in dry DMF was stirred at 80-100 °C overnight, filtered over hyflo, and concentrated with toluene. The residue was dissolved in DCM and washed with brine, dried over Na_2SO_4 and filtered. The crude product was purified by flash chromatography to afford the azide.

10.3.2.1 6-Azidohexyl-2,3,4,6-tetra *O*-acetyl- β -D-galactose **127**



General procedure **E** was followed for the treatment of galactopyranoside chloride **172** (2.15 g, 3.33 mmol) and NaN_3 (2.16 g, 33.23 mmol). Flash chromatography afforded the azide **127** (1.41 g, 2.99 mmol) as a colorless slurry.

Yield: 92%.

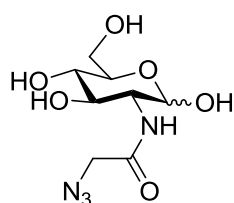
R_f : 0.25 ($\text{Et}_2\text{O}/\text{Pent}$, 1:1).

$[\alpha]_D^{20}$: -8.0 (c 1, CHCl_3).

^1H -NMR (400 MHz, CDCl_3): δ 5.32 (dd, $J = 3.4, 1.1$ Hz, 1H, 5'-H), 5.13 (dd, $J = 10.5, 7.9$ Hz, 1H, 2-H), 4.95 (dd, $J = 10.5, 3.4$ Hz, 1H, 3'-H), 4.39 (d, $J = 7.9$ Hz, 1H, 1'-H), 4.12-4.09 (m, 2H, 6'-H_a, 4'-H), 3.84-3.79 (m, 2H, 6_b'-H, 1_a-H₂), 3.43-3.37 (m, 1H, 1_b-H₂), 3.20 (t, $J = 6.9$ Hz, 2H, 6-H₂), 2.08 (s, 3H, CH₃), 1.98, 1.95 (2s, 6H, 2 \times CH₃), 1.92 (s, 3H, CH₃), 1.54-1.49 (m, 4H, 2-H₂, 5'-H₂), 1.33-1.27 (m, 4H, 3-H₂, 4-H₂).

^{13}C -NMR (100 MHz, CDCl_3): δ 170.32, 170.21, 170.11, 169.27 (4C, 4 \times CO_{ester}), 101.30 (C-1'), 70.90 (C-2'), 70.56 (C-4'), 69.89 (C-5'), 68.89 (C-3'), 67.02 (C-1), 61.22 (C-6'), 51.29 (C-6), 29.22, 28.71 (C-2, C-5), 26.35, 25.36 (C-3, C-4), 20.68, 20.60, 20.52 (4C, 4 \times CH₃).

10.3.3 *N*-Azidoacetylglucosamine **128**



MeONa (0.5 M in MeOH, 27 mL, 13.51 mmol) was added to the solution of glucosamine hydrochloride **225** (3.00 g, 13.53 mmol) in MeOH (60 mL). After 1 h of stirring at rt triethylamine (TEA) (2.1 mL, 14.01 mmol) and chloroacetic anhydride **256** (11.85 g, 69.04 mmol) were added to it and

stirred for additional 6 h. Removal of the solvent gave the chloride **257** as a crude oil, which was dissolved in DMF (30 mL). To this solution, NaN_3 (9.07 g, 139.55 mmol) was added and the reaction mixture was heated to 80 °C for 2 h. The residue obtained after the evaporation of the solvent was purified by silica gel chromatography eluting with $\text{H}_2\text{O}/\text{IPA}/\text{EtOAc}$ (1:3:6) and

1% NH_4OH solution (30%) to afford the azide **128** (1.29 g, 4.92 mmol) as a yellow oil with $\alpha:\beta$ ratio of 1:0.35.

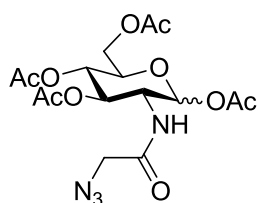
Yield: 36%.

R_f : 0.19 ($\text{H}_2\text{O}/\text{IPA}/\text{EtOAc}$, 1:3:6 with 1% NH_4OH solution (30%)).

^1H -NMR (400 MHz, CD_3OD): δ 5.13 (d, $J = 3.4$ Hz, 1H, 1- H_α), 4.66 (d, $J = 8.2$ Hz, 1H, 1- H_β), 3.96 (d, $J = 2.9$ Hz, 2H, 6- H_α), 3.92 (dd, $J = 10.5, 3.5$ Hz, 1H, 2- H_α), 3.78 (dd, $J = 11.5, 2.3$ Hz, 1H, 5- H_α), 3.78 (s, 2H, CH_2N_3), 3.44-3.34 (m, 1H, 3-H), 3.39 (dd, $J = 9.5, 8.9$ Hz, 1H, 4- H_α).

^{13}C -NMR (100 MHz, CD_3OD): δ 170.41 (CO_α), 96.70 (C-1 $_\beta$), 92.48 (C-1 $_\alpha$), 73.13, 72.71, 72.33 (C-3 $_\alpha$, C-4 $_\alpha$, C-5 $_\alpha$), 62.74 (C-6 $_\alpha$), 55.89 (C-2 $_\alpha$), 52.82 ($\text{C}_\alpha\text{H}_2\text{N}_3$).

10.3.4 1,3,4,6-Tetra-*O*-acetyl-*N*-azidoacetylglucosamine **129**



Azido glucosamine **128** (1.21 g, 4.63 mmol) was dissolved in a mixture of pyridine (60 mL) and Ac_2O (30 mL). After stirring for 12 h at rt the reaction mixture was poured into DCM (50 mL). The mixture was washed with 1 M HCl (2×20 mL), sat. Solution of NaHCO_3 (2×20 mL), H_2O (2×20 mL), brine (2×20 mL) and dried over anhyd. Na_2SO_4 . The crude product was concentrated under reduced pressure and purified by silica gel chromatography, eluting with EtOAc/Hex (1:1) to afford azide **128** (1.24 g, 2.88 mmol) as a white solid.

Yield: 62%.

R_f : 0.23 (EtOAc/Hex , 1:1).

^1H -NMR (400 MHz, CDCl_3): δ 6.47 (d, $J = 8.8$ Hz, 1H, NH), 6.24 (d, $J = 3.6$ Hz, 1H, 1- H_α), 5.84-5.82 (d, $J = 8.7$ Hz, 1H, 1- H_β), 5.31-5.36 (dd, $J = 10.9, 9.5$ Hz, 1H, 3- H_α), 5.22-5.28 (t, $J = 9.5$ Hz, 1H, 4- H_α), 4.51-4.48 (dd, $J = 10.8, 3.7$ Hz, 1H, 2-H), 4.14-4.04 (m, 3H, 5-H, 6-H), 3.97 (s, 2H, CH_2N_3), 2.12 (s, 6H, $2 \times \text{CH}_3$), 2.09 (s, 3H, CH_3), 2.09 (s, 3H, CH_3).

^{13}C -NMR (100 MHz, CDCl_3): δ 171.50, 170.62, 169.10, 168.63, 166.85 ($5 \times \text{CO}$), 92.19 (C-1 $_\beta$), 90.23 (C-1 $_\alpha$), 70.30 (C-5), 69.78 (C-4), 67.37 (C-3), 61.45 (C-6), 52.41 (C-2), 51.22 (CH_2N_3), 20.86, 20.65, 20.55, 20.52 ($4 \times \text{CH}_3$).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_{10}+\text{Na}]^+$ 453.1, found 453.1.

10.3.5 3-Azidopropan-1-amine **175**

$\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}_3$ The suspension of 1-bromo-3-aminopropane hydrobromide (3.25 g, 15.25 mmol) and NaN_3 (3.21 g, 50.00 mmol) in H_2O (25 mL) was refluxed for 24 h. The solvent was evaporated up to 2/3 under reduced pressure. The resulting solution was cooled to 0 °C in an ice bath, followed by the addition of Et_2O (50 mL) and finally KOH (4.00 g) maintaining the

temperature below 10 °C. The organic layer was separated and the aqueous layer was further extracted with Et₂O (2 × 30 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated to afford azide **175** as pale oil (1.45 g, 14.53 mmol).

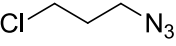
Yield: 96%.

R_f: 0.56 (Et₂O/Pent, 1:1 with 1% NEt₃).

¹H-NMR (400 MHz, CDCl₃): δ 3.35 (t, *J* = 6.7 Hz, 2H, 1-H₂), 2.78 (t, *J* = 6.8 Hz, 2H, 3-H₂), 1.70 (quin, *J* = 6.8 Hz, 2-H₂), 1.17 (br s, 2H, NH₂).

¹³C-NMR (100 MHz, CDCl₃): 49.31 (C-1), 39.49 (C-3), 32.64 (C-2).

10.3.6 1-Azido-3-chloropropane **178a**

 NaN₃ (1.72 g, 26.04 mmol) was added to the solution of 1-bromo-3-chloropropane (4.13 g, 26.04 mmol) in DMF (200 mL). After 24 h, Et₂O (200 mL) was added to the flask, washed the organic phase with water (4 × 100 mL) and dried over MgSO₄. The crude product was purified over silica gel column eluting with a gradient of Et₂O/Pent (1:1) to afford **178a** (2.75 g, 23.14 mmol) as a colorless oil.

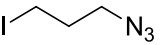
Yield: 88%.

R_f: 0.54 (Et₂O/Pent 1:1).

¹H-NMR (400 MHz, CDCl₃): δ 3.80 (t, *J* = 6.0 Hz, 2H, 1-H₂), 3.68 (t, *J* = 6.4 Hz, 2H, 3-H₂), 2.01 (quin, *J* = 6.1 Hz, 2H, 2-H₂).

¹³C-NMR (100 MHz, CDCl₃): δ 59.49 (C-1), 41.91 (C-3), 35.10 (C-2).

10.3.7 1-Azido-3-iodopropane **178**

 The solution of 1-azido-3-chloropropane **178a** (2.53 g, 21.05 mmol) and NaI (4.09 g, 27.34 mmol) in acetone (100 mL) was refluxed for 24 h followed by the partition of the solution in EtOAc (3 × 75 mL) and water. Combined organic layers were dried over MgSO₄ and concentrated to give a thick oily liquid. The crude product was purified by flash chromatography by eluting with a gradient of EtOAc/Hex (1:1) to afford **178** (3.33 g, 15.83 mmol) as a colorless thick oil.

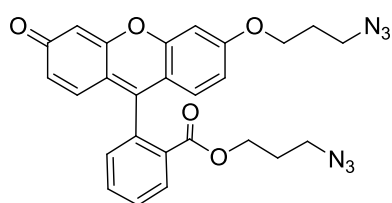
Yield: 75%.

R_f: 0.29 (Hex/EtOAc, 2:1).

¹H-NMR (300 MHz, CDCl₃): δ 3.37 (t, *J* = 6.4 Hz, 2H, 1-H₂), 3.18 (t, *J* = 6.6 Hz, 2H, 3-H₂), 1.98 (quin, *J* = 6.5 Hz, 2H, 2-H₂).

¹³C-NMR (75 MHz, CDCl₃): 51.41 (C-1), 32.28 (C-2), 2.29 (C-3).

10.3.8 3-Azidopropyl-2-[6-(3-azidopropoxy)-3-oxo-3H-xanthen-9-yl]benzoate **130**



Iodopropyl azide **178** (210 mg, 26.11 mmol) was added to the solution of sodium salt of fluorescein **177** (1.13 g, 2.32 mmol) in a mixture of distilled THF/MeOH (1:1, 25 mL) and the reaction mixture was stirred overnight. The crude mixture was

concentrated under reduced pressure, diluted with water and extracted with EtOAc (3×25 mL). The combined organic layers were dried, concentrated and purified by flash chromatography, eluting with a gradient of DCM/MeOH (30:1), which gave two pure fractions. The NMR and mass analysis of first fraction **130** showed double substitution on the fluorescein ring (550 mg, 1.13 mmol).

Yield: 55%.

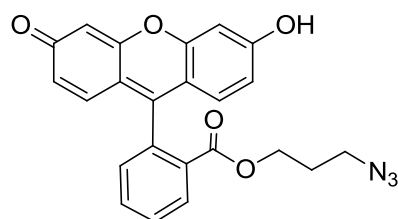
R_f : 0.34 (EtOAc).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.32 (dd, $J = 7.8, 1.0$ Hz, 1H, 6'-H), 7.86 (td, $J = 7.6, 1.2$ Hz, 1H, 4'-H), 7.79 (td, $J = 7.6, 1.3$ Hz, 1H, 5'-H), 7.44 (dd, $J = 7.3, 1.3$ Hz, 1H, 3'-H), 7.26 (d, $J = 2.3$ Hz, 1H, 5''-H), 7.08 (d, $J = 8.6$ Hz, 1H, 8''-H), 7.05 (d, $J = 9.6$ Hz, 1H, 1''-H), 6.97 (dd, $J = 9.1, 2.3$ Hz, 1H, 7''-H), 6.59 (dd, $J = 9.6, 2.1$ Hz, 1H, 2''-H), 6.51 (d, $J = 2.0$ Hz, 1H, 4''-H), 4.10 (t, $J = 5.9$ Hz, 2H, 3-H₂), 4.01 (dt, $J = 5.9, 1.1$ Hz, 2H, 1'''-H₂), 3.47 (t, $J = 6.5$ Hz, 2H, 1-H₂), 2.99 (t, $J = 6.7$ Hz, 2H, 3'''-H₂), 2.03 (quin, $J = 6.3$ Hz, 2H, 2-H₂), 1.55 (quin, $J = 6.5$ Hz, 2H, 2'''-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 187.13 (C-3'), 166.66 (CO_{ester}), 166.01 (C-6'), 161.27 (C-4a'), 156.20 (C-5a'), 155.73 (C-9a'), 134.99 (C-2'), 134.13, 132.51, 132.36, 131.72, 131.59, 131.33, 130.82 (C-3', C-4', C-5', C-6', C-1'', C-7'', C-8''), 129.51 (C-9'), 118.19, 116.21 (C-1', C-8a'), 115.90 (C-5''), 105.62 (C-2''), 102.11 (C-4''), 67.28 (C-1'''), 63.69 (C-3), 49.122, 48.94 (C-1, C-3'''), 29.48 (C-2'''), 28.82 (C-2).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{26}\text{H}_{22}\text{N}_6\text{O}_5+\text{Na}]^+$ 521.15, found 521.09.

10.3.9 3-Azidopropyl-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoate **179**



NMR and mass analysis of the second fraction **179** (224 mg, 530 μmol) of the above reaction showed that it was singly substituted. The azide linker was found to be substituted through ester linkage.

Yield: 23%.

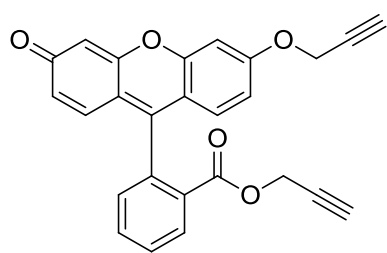
R_f : 0.31(EtOAc).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.17 (dd, $J = 7.8, 1.3$ Hz, 1H, 6'-H), 7.67 (dt, $J = 7.5, 1.4$ Hz, 1H, 4'-H), 7.61 (dt, $J = 7.6, 1.4$ Hz, 1H, 5'-H), 7.24 (dd, $J = 7.5, 1.1$ Hz, 1H, 3'-H), 6.89 (s, 1H, 5''-H), 6.87 (s, 1H, 8''-H), 6.80 (d, $J = 2.0$ Hz, 2H, 1''-H, 7''-H), 6.73 (dd, $J = 9.2, 2.1$ Hz, 2H, 2''-H, 4''-H), 3.97 (t, $J = 6.1$ Hz, 2H, 1-H), 2.96 (t, $J = 6.6$ Hz, 2H, 3-H), 1.50 (quin, $J = 6.4$ Hz, 2H, 2-H).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 175.77 (C-3'), 165.28 (CO_{ester}), 162.76 (C-6''), 157.83 (C-4_a''), 155.13 (C-5_a''), 155.11 (C-2'), 134.33 (C-9''), 132.64, 131.21 (C-2'', C-1'), 130.37 (C-9_a''), 130.27-129.82 (C-3', C-4', C-5', C-6', C-1'', C-8''), 122.21 (C-8_a''), 114.94 (C-7''), 104.01 (C-4'', C-5''), 62.43 (C-1), 47.73 (C-3), 27.71 (C-2).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{23}\text{H}_{17}\text{N}_3\text{O}_5+\text{Na}]^+$ 438.33, found 438.33.

10.3.10 Prop-2-yn-1-yl-2-[3-oxo-6-(prop-2-yn-1-yloxy)-3H-xanthen-9-yl]benzoate 179a



Propargyl bromide (1.22 g, 26.53 mmol) was added to the solution of fluorescein sodium salt (2.00 g, 5.31 mmol) in the mixture of distilled THF/MeOH (1:1, 30 mL) and stirred overnight. The crude mixture was concentrated, diluted with water and extracted with EtOAc (3×25 mL). The combined

organic layers were dried, concentrated and purified by flash chromatography to afford alkyne **179a** (1.85 g, 5.02 mmol) as a dark orange solid.

Yield: 94%.

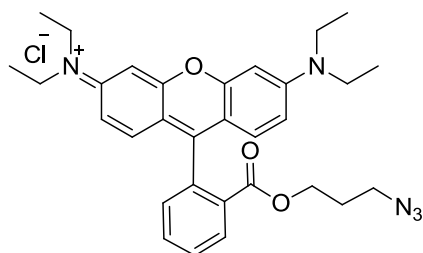
R_f : 0.37 (DCM/MeOH, 20:1).

$^1\text{H-NMR}$ (300 MHz, DMSO-d_6): δ 8.17 (dd, $J = 7.9, 1.3$ Hz, 1H, 6-H), 7.83 (td, $J = 7.5, 1.3$ Hz, 1H, 4-H), 7.74 (td, $J = 7.7, 1.3$ Hz, 1H, 5-H), 7.45 (dd, $J = 7.6, 1.1$ Hz, 1H, 3-H), 6.72 (dd, $J = 8.2, 1.2$ Hz, 2H, 8''-H, 5''-H), 6.61 (dd, $J = 5.1, 1.4$ Hz, 2H, 1''-H, 7''-H), 6.49 (s, 1H, 4''-H), 6.46 (d, $J = 2.1$ Hz, 1H, 2''-H), 4.71 (br s, 4H, 1'-H₂, 1'''-H₂), 3.16-3.21 (m, 2H, 3'''-H, 3'-H).

$^{13}\text{C-NMR}$ (75 MHz, DMSO-d_6): δ 175.57 (C-3'), 165.26 (CO_{ester}), 156.42 (C-6''), 154.06 (C-4_a''), 150.61 (C-5_a''), 148.62 (C-2), 134.23 (C-9''), 132.79 (C-2'', C-1), 130.57, 130.38 (C-9_a'', C-3, C-4), 129.61, 129.46 (C-5, C-6, C-1'', C-8''), 122.13 (C-8_a''), 112.77 (C-7''), 106.62 (C-4''), 103.18 (C-5''), 84.01 (2C, C-3', C-3''), 66.62 (2C, C-2', C-2''), 52.08 (C-1'), 38.52 (C-1'').

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{26}\text{H}_{16}\text{O}_5+\text{Na}]^+$ 431.09, found 430.99.

10.3.11 *N*-[9-{2-[(3-Azidopropoxy)carbonyl]phenyl}-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride **131**



TBAF (556 mg, 830 μmol , 2 equiv.) was added to the solution of rhodamine-B **180** (200 mg, 420 μmol) and azide **178** (1.75 g, 8.34 mmol, 20 equiv.) in THF (5 mL) at room temperature. After complete conversion of the educt into the product (18 h), the solvent was removed and the crude product

was purified by flash chromatography, eluting with DCM/MeOH (50:1) to afford **131** (185 mg, 310 μmol) as a violet powder.

Yield: 73%.

R_f : 0.24 (DCM/MeOH, 30:1).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 8.31 (dd, $J = 7.9, 0.9$ Hz, 1H, 6'''-H), 7.87 (td, $J = 7.5, 1.3$ Hz, 1H, 4'''-H), 7.82 (td, $J = 7.7, 1.3$ Hz, 1H, 5'''-H), 7.45 (dd, $J = 7.6, 0.9$ Hz, 1H, 3'''-H), 7.14 (d, $J = 4.7$ Hz, 1H, 1'-H), 7.11 (d, $J = 4.6$ Hz, 1H, 8'-H), 7.07 (d, $J = 2.6$ Hz, 1H, 2'-H), 7.04 (d, $J = 2.5$ Hz, 1H, 7'-H), 7.00-7.01 (2s, 2H, 4'-H, 5'-H), 4.04 (t, $J = 7.2$ Hz, 2H, 1''''-H₂), 3.56 (t, $J = 8.7$ Hz, 2H, 3''''-H₂), 1.98-1.94 (m, 4H, 1-H_{2a}, 1-H_{2b}), 1.49-1.45 (m, 4H, 1''-H_{2a}, 1''-H_{2b}), 1.63 (dt, $J = 7.1, 3.2$ Hz, 2H, 2''''-H₂), 1.56 (t, $J = 7.2$ Hz, 6H, 2-H_{3a}, 2-H_{3b}), 1.3 (t, $J = 7.4$ Hz, 6H, 2''-H_{3b}, 2''-H_{3b}).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 166.17 (CO_{ester}), 161.39 (C-10_a'), 159.86 (C-6'), 159.54 (C-4_a'), 159.17 (C-1''', C-9'), 157.04 (C-8_a', C-9_a'), 134.19 (C-4'''), 140 (C-2'''), 132.39, 132.32, 132.21 (C-6''', C-1', C-8'), 131.68, 131.57 (C-5''', C-3'''), 115.68 (C-2', C-7'), 114.76 (C-3'), 97.42, 97.29 (C-4', C-5'), 59.67 (t, $J = 2.8$ Hz, C-1'''), 49.05 (C-3'''), 46.96 (C-1_a'', C-1_b''), 24.9 (C-1_a, C-1_b), 20.68 (C-2'''), 13.99 (C-2_a'', C-2_b''), 12.92 (C-2_a, C-2_b).

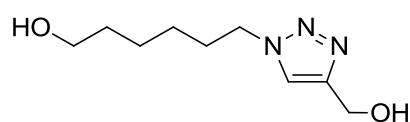
MS (ESI): m/z [M] calcd for $[\text{C}_{31}\text{H}_{38}\text{N}_5\text{O}_3]$ 526.30, found 526.29.

10.4 Synthesis of 1,2,3-Triazoles

10.4.1 General procedure (F) for the synthesis of 1,4-disubstituted 1,2,3-triazoles.

Azide and alkyne in equimolar quantities were suspended in a 1:1 mixture of water and *tert*-BuOH. Sodium ascorbate and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were added to the mixture. The heterogeneous mixture was stirred vigorously overnight till it cleared and TLC analysis indicated the complete consumption of the reactants. The solvent was removed under high vacuum and the crude product was purified by flash silica gel chromatography to afford the desired product.

10.4.1.1 6-[4-(Hydroxymethyl)-1*H*-1,2,3-triazole-1-yl]hexan-1-ol **183**



Following the general procedure **F**, azide **181** (143 mg, 1.01 mmol) and propargyl alcohol **182** (56 mg, 1.01 mmol) were treated to get triazole **183** as a clear slightly yellow oil

(159 mg, 800 μ mol).

Yield: 80%.

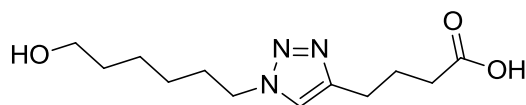
R_f : 0.37 (Et₂O/Pent, 1:1).

¹H-NMR (400 MHz, CD₃OD): δ 7.80 (s, 1H, 5'-H), 4.57 (s, 2H, 1''-H₂), 4.30 (t, J = 7.1 Hz, 2H, 6-H₂), 3.43 (t, J = 6.5 Hz, 2H, 1-H₂), 1.86 (quint, J = 7.2 Hz, 2H, 5-H₂), 1.46-1.39 (m, 2H, 2-H₂), 1.34-1.29 (m, 4H, 3-H₂, 4-H₂).

¹³C-NMR (100 MHz, CD₃OD): δ 149.06 (C-4'), 124.03 (C-5'), 62.74 (C-1), 56.50 (C-1'), 51.26 (C-6), 33.37 (C-2), 31.30 (C-5), 27.27, 26.33 (C-4, C-3).

MS (ESI): m/z [M+Na]⁺ calcd for [C₉H₁₇N₃O₂+Na]⁺ 222.13, found 222.13.

10.4.1.2 4-[1-(6-Hydroxyhexyl)-1*H*-1,2,3-triazole-4-yl]butanoic acid **184**



Following the general procedure **F**, azide **181** (127 mg, 1.01 mmol) was treated with 5-hexynoic acid **148** (217 mg, 850 μ mol). The crude product was purified by flash chromatography to afford **184** (159 mg, 800 μ mol) as a clear slightly yellow oil.

Yield: 85%.

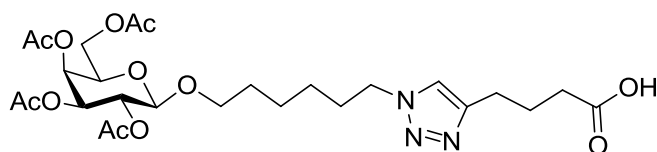
R_f : 0.42 (Et₂O/Pent, 1:1).

¹H-NMR (300 MHz, CD₃OD): δ 7.79 (s, 1H, 5'-H), 4.38 (t, J = 7.1 Hz, 2H, 1''-H₂), 3.55 (t, J = 6.4 Hz, 2H, 6''-H₂), 2.75 (t, J = 7.5 Hz, 2H, 4-H₂), 2.40-2.20 (m, 2H, 2-H₂), 2.00-1.86 (m, 4H, 2''-H₂, 3-H₂), 1.60-1.48 (m, 2H, 4''-H₂), 1.47-1.28 (m, 4H, 5''-H₂, 3''-H₂).

¹³C-NMR (75 MHz, CD₃OD): δ 180.34 (C-1), 148.61 (C-4'), 123.26 (C-5'), 62.67 (C-6'), 51.15 (C-1'), 36.25, 33.32, 31.22 (C-4, C-2, C-5'), 27.24, 26.58, 26.25, 25.84 (C-3, C-4'', C-3'', C-2'').

MS (ESI): m/z [M+Na]⁺ calcd for [C₁₂H₂₁N₃O₃+Na]⁺ 278.16, found 278.16.

10.4.1.3 4-{1-[6-(2,3,4,6-Tetraacetyl- β -D-galactosyl)-hexyl]-1*H*-1,2,3-triazole-4-yl} butanoic acid **185**



General procedure **F** was followed and azide **127** (457 mg, 1.01 mmol) was treated with 5-hexynoic acid **148**

(112 mg, 1.02 mmol). Flash chromatography of the crude product afforded **185** (440 mg, 750 μ mol) as a clear slightly yellow oil.

Yield: 75%.

R_f : 0.27 (Et₂O/Pent, 1:1).

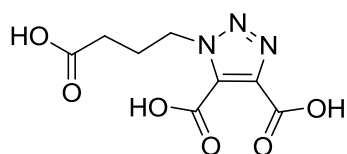
$[\alpha]_D^{20}$: -8.6 (c 1, CH₃OH).

¹H-NMR (400 MHz, CD₃OD): δ 7.78 (s, 1H, 5'-H), 5.39 (dd, J = 3.2, 0.9 Hz, 1H, 5'''-H), 5.14 (dd, J = 10.4, 3.2 Hz, 1H, 3'''-H), 5.11 (dd, J = 10.4, 7.4 Hz, 1H, 2'''-H), 4.63 (d, J = 7.4 Hz, 1H, 1'''-H), 4.39 (t, J = 7.1 Hz, 2H, 1''-H₂), 4.17-4.09 (m, 3H, 6''-H₂, 1''-H), 3.85 (td, J = 9.7, 6.1 Hz, 1H, 6_a'''-H), 3.54 (td, J = 9.8, 6.3 Hz, 1H, 6_b'''-H), 2.77 (t, J = 7.5 Hz, 2H, 4-H₂), 2.36 (t, J = 7.4 Hz, 2H, 2-H₂), 2.16, 2.04, 2.03, 1.96 (4s, 12H, 4 x CH₃), 2.00-1.86 (m, 4H, 2''-H₂, 3-H₂), 1.62-1.55 (m, 2H, 4''-H₂), 1.45-1.32 (m, 4H, 5''-H₂, 3''-H₂).

¹³C-NMR (100 MHz, CD₃OD): δ 176.88 (C-1), 172.04, 171.99, 171.50, 171.32 (4 x COCH₃), 148.37 (C-4'), 123.33 (C-5'), 102.25 (C-1'''), 72.38, 71.76, 70.53, 68.90 (C-5''', C-4''', C-3''', C-2'''), 63.72 (C-6'''), 62.60 (C-6''), 51.16 (C-1''), 34.12, 31.18, 30.35 (C-4, C-2, C-5'), 27.06, 26.35, 25.88, 25.64 (C-3, C-4'', C-3'', C-2''), 20.88, 20.77, 20.59, 20.52 (4 x CH₃).

MS (ESI): m/z [M+Na]⁺ calcd for [C₂₅H₃₉N₃O₁₁+Na]⁺ 608.25, found 608.24.

10.4.2 1-(3-Carboxypropyl)-1*H*-1,2,3-triazole-4,5-dicarboxylic acid **195**



The solution of acetylene dicarboxylic acid **194** (197 mg, 1.73 mmol) and methyl- γ -azidobutyric acid **193** in H₂O (5 mL) was stirred for 12 h at rt. The solution was concentrated and purified with silica gel column chromatography, eluting with the gradient of

Hex/EtOAc (1:1) to afford **195** (352 mg, 1.54 mmol) as white powder.

Yield: 89%.

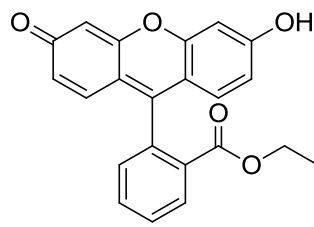
R_f : 0.18 (Hex/EtOAc, 1:1).

¹H-NMR (300 MHz, CD₃OD): δ 8.86 (br s, 3H, 3 x OH), 4.65 (t, J = 6.9 Hz, 2H, 1'-H₂), 2.25 (t, J = 7.7 Hz, 2H, 3'-H₂), 2.05 (quin, J = 11.2, 6.9 Hz, 2H, 2'-H₂).

¹³C-NMR (75 MHz, CD₃OD): δ 173.52 (C-4'), 161.81 (C-6_a), 159.47 (C-6_b), 139.83 (C-4), 131.81 (C-5), 49.82 (C-1'), 30.33 (C-3'), 24.92 (C-2').

MS (ESI): m/z $[M-H]^-$ calcd for $[C_8H_9N_3O_6-H]^-$ 242.09, found 242.02.

10.4.3 4-[1-(3-{[2-(6-Hydroxy-3-oxo-xanthen-9-yl)benzoyl]oxy}propyl)-1H-1,2,3-triazole-4-yl]butanoic acid **187**



Fluorescein azide **179** (55 mg, 132 μ mol) and 5-hexynoic acid **148** (15 mg, 141 μ mol) were suspended in a 3:1 mixture of *tert*-BuOH/H₂O (2 mL). The suspension was vigorously stirred for 10 min and the solution

of CuSO₄·5H₂O (0.5 M, 2.6 μ L, 0.132 μ mol) was added followed by the addition of the solution of ascorbic acid (0.5 M, 26.4 μ L, 1.32 μ mol). The suspension was stirred overnight and filtered on the completion of the reaction. The filtrate was concentrated and the crude product was purified by column chromatography, eluting with DCM/MeOH (20:1) to afford **187** (47 mg, 89 μ mol).

Yield: 68%.

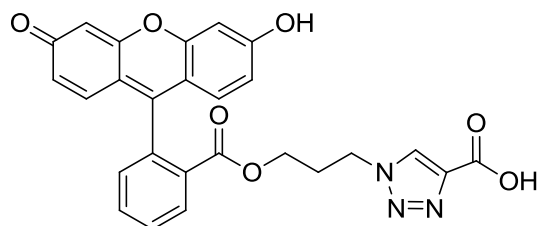
R_f: 0.48 (DCM/MeOH, 15:1).

¹H-NMR (300 MHz, DMSO-d₆): δ 8.17 (dd, J = 7.8, 1.2 Hz, 1H, 6'''-H), 8.16 (br s, 1H, 5'-H), 7.88 (dt, J = 7.5, 1.1 Hz, 1H, 4'''-H), 7.79 (t, J = 7.6 Hz, 1H, 5'''-H), 7.30 (d, J = 7.3 Hz, 1H, 3'''-H), 6.71, 6.68 (2s, 2H, 5''''-H, 8''''-H), 6.37 (dd, J = 9.3, 1.9 Hz, 2H, 1''''-H, 7''''-H), 6.31 (d, J = 1.7 Hz, 2H, 2''''-H, 4''''-H), 4.05 (t, J = 7.1 Hz, 2H, 3''-H₂), 3.96 (t, J = 6.0 Hz, 2H, 1''-H₂), 2.63 (t, J = 7.1 Hz, 2H, 2-H), 2.21-2.12 (m, 2H, 4-H), 1.84 (quin, J = 6.5 Hz, 4H, 3-H₂, 2'-H₂).

¹³C-NMR (75 MHz, DMSO-d₆): δ 186.87 (C-3'''), 178.48 (C-1), 165.33 (CO_{ester}), 165.09 (C-6'''), 156.73 (C-4_a'', C-5_a''), 151.25 (C-4'), 146.62 (C-2'''), 134.30 (C-9'''), 133.13 (C-2'''), 130.99, 130.85, 130.73 (C-1'', C-9_a'', C-3'''), 129.78 (C-4'', C-5'', C-6'', C-9_a''), 122.59 (C-8'', C-8_a''), 121.91 (C-5'), 109.72 (C-7'''), 103.36 (C-5''', C-4'''), 62.25 (C-3'), 46.61 (C-1'), 34.74 (C-2), 28.54 (C-3), 24.77, 24.47 (C-3, C-2').

MS (ESI): m/z $[M+Na]^+$ calcd for $[C_{29}H_{25}N_3O_7+Na]^+$ 550.15, found 550.06.

10.4.4 1-(3-[[2-(6-Hydroxy-3-oxo-xanthen-9-yl)benzoyl]oxy}propyl)-1H-1,2,3-triazole-4-carboxylic acid **188**



Fluorescein azide **179** (55 mg, 132 μmol) and propargylic acid (10 mg, 140 μmol) were suspended in a 3:1 mixture of *tert*-BuOH/H₂O (2 mL). The suspension was vigorously stirred for 10 min and the solution of CuSO₄·5H₂O (0.5 M, 2.6 μL , 1.3 μmol)

was added to the suspension followed by the addition of the solution of ascorbic acid (0.5 M, 26.4 μL , 13.2 μmol). The reaction mixture was stirred overnight and filtered. The crude product was purified by column chromatography, eluting with DCM/MeOH (20:1) to afford **188** (35 mg, 70 μmol) as a deep orange solid.

Yield: 55%.

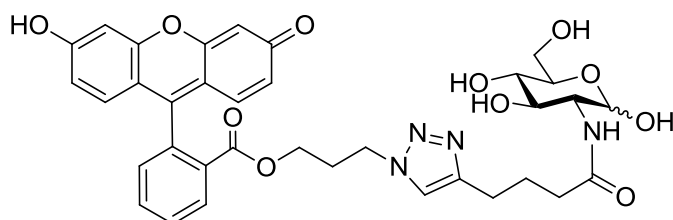
R_f: 0.45 (DCM/MeOH, 15:1).

¹H-NMR (300 MHz, DMSO-d₆): δ 8.07 (d, *J* = 7.8 Hz, 1H, 6''-H), 7.99 (br s, 1H, 5-H), 7.78 (t, *J* = 7.4 Hz, 1H, 4''-H), 7.77 (t, *J* = 7.6 Hz, 1H, 5''-H), 7.39 (d, *J* = 7.3 Hz, 1H, 3''-H), 6.62, 6.59 (2s, 2H, 5'''-H, 8'''-H), 6.31, 6.29 (2s, 2H, 1'''-H, 7'''-H), 6.21 (s, 2H, 2'''-H, 4'''-H), 4.08 (t, *J* = 7.3 Hz, 2H, 1'-H₂), 3.89 (t, *J* = 6.0 Hz, 2H, 3'-H₂), 1.79 (quin, *J* = 6.5 Hz, 2H, 2'-H₂).

¹³C-NMR (75 MHz, DMSO-d₆): δ 179.94 (C-3'''), 165.69 (CO_{acid}), 165.25 (CO_{ester}), 164.62 (C-6'''), 157.23 (C-4_a''', C-5_a'''), 150.94 (C-2'', C-4), 134.29 (C-9'''), 132.45 (C-2'''), 130.45, 130.42, 130.37, 130.29 (C-1'', C-9_a''', C-3'), 129.32, 129.06, 128.99 (C-4'', C-5'', C-6'), 126.32 (C-1'), 123.78 (C-8'''), 123.75 (C-8_a'''), 123.73 (C-5), 109.30 (C-7'''), 103.58 (C-4', C-5'''), 61.85 (C-3'), 48.56 (C-1'), 28.41 (C-2').

MS (ESI): *m/z* [M+Na]⁺ calcd for [C₂₆H₁₈N₃O₇Na+Na]⁺ 529.09, found 529.98.

10.4.5 3-[4-(4-Oxo-4-[2-amino-glucopyranosyl]butyl)-1H-1,2,3-triazole-1-yl]propyl-2-(6-hydroxy-3-oxo-xanthen-9-yl)benzoate **190**



DIPEA (27 mg, 210 μmol) was added to the solution of fluorescein azide **179** (25 mg, 66 μmol), *N*-hex-5-yn glucosamide **189** (19 mg, 70 μmol) and CuI (1.3 mg, 7 μmol) in MeOH (5 mL)

and the solution was stirred overnight at rt. The mixture was filtered over celite and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography, eluting with DCM/MeOH (10:1) to afford triazole **190** (40 mg, 60 μmol).

Yield: 85%.

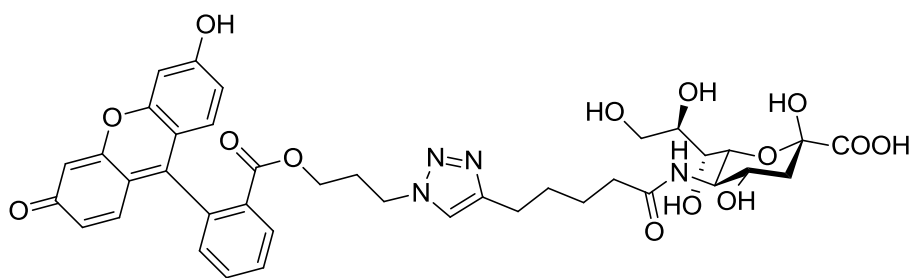
R_f : 0.31 (DCM/MeOH, 8:1).

$^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 8.20 (dd, $J = 7.8, 1.1$ Hz, 1H, 6-H), 7.81 (td, $J = 7.5, 1.2$ Hz, 1H, 4-H), 7.74 (td, $J = 7.6, 1.1$ Hz, 1H, 5-H), 7.46 (s, 1H, 5'''-H), 7.39 (d, $J = 7.5$ Hz, 1H, 3-H), 6.89 (s, 1H, 5'-H), 6.87 (s, 1H, 8'-H), 6.59 (s, 2H, 1'-H, 7'-H), 6.57 (s, 2H, 2'-H, 4'-H), 5.14 (d, $J = 3.3$ Hz, 1H, 1 $_{\alpha}$ ''''-H), 4.63 (d, $J = 8.3$ Hz, 1H, 1 $_{\beta}$ ''''-H), 4.04 (t, $J = 6.5$ Hz, 2H, 1''-H $_2$), 3.95 (t, $J = 5.6$ Hz, 2H, 3''-H $_2$), 3.88 (d, $J = 3.1$ Hz, 1H, 2''''-H), 3.88 (d, $J = 3.2$ Hz, 1H, 4''''-H), 3.85-3.82 (m, 1H, 5''''-H), 3.81 (d, $J = 3.2$ Hz, 1H, 6 $_{\alpha}$ ''''-H $_2$), 3.76 (t, $J = 5.2$ Hz, 1H, 3''''-H), 3.43-3.35 (m, 1H, 6 $_{\beta}$ ''''-H $_2$), 2.70 (t, $J = 7.2$ Hz, 2H, 3'''-H $_2$), 2.28 (t, $J = 7.2$ Hz, 2H, 1'''-H $_2$), 2.01-1.87 (m, 4H, 2'''-H $_2$, 2''-H $_2$).

$^{13}\text{C-NMR}$ (75 MHz, CD_3OD): δ 181.95 (C-3'), 175.97 (C-4'''), 167.18 (CO_{ester}), 159.86 (C-6'), 159.79 (C-4 $_{\alpha}$ '), 156.87 (C-5 $_{\alpha}$ ', C-2), 135.76 (C-9'), 133.82 (C-4''), 132.10, 131.70 (C-2', C-1), 131.50-130.86 (C-9 $_{\alpha}$ ', C-3, C-4, C-5, C-6, C-1'), 124.42 (C-8', C-8 $_{\alpha}$ '), 123.46 (C-5'''), 113.22 (C-7'), 104.78 (C-4', C-5'), 92.65 (C-1'''''), 73.08 (C-4'''''), 72.69 (C-5'''''), 72.60 (C-3'''''), 63.27 (C-2'''''), 62.85 (C-3''), 55.94 (C-6'''''), 49.87 (C-1'), 47.83 (C-3'''''), 36.43 (C-1'''''), 29.91 (C-2''), 26.59 (C-2''''').

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{35}\text{H}_{36}\text{N}_4\text{O}_{11}+\text{Na}]^+$ 711.01, found 711.01.

10.4.6 5-{5-[1-(3-{[2-(6-Hydroxy-3-oxo-xanthen-9-yl)benzoyl]oxy} propyl)-1H-1,2,3-triazole-4-yl]hexanoylamino}-3,5-di-deoxynonanoic acid **191**



CuI (1.3 mg, 70 μmol) and DIPEA (27 mg, 210 μmol) were added to the solution of fluorescein azide **179** (37 mg, 90 μmol) and

Neu5Hep **117** (25 mg, 70 μmol) in MeOH. The suspension was stirred overnight at rt followed by filtration and evaporation of the solvent. The crude product was purified by column chromatography, eluting with DCM/MeOH (10:1 to 5:1) to afford triazole **191** (42 mg, 530 μmol) as a deep orange powder.

Yield: 71%.

R_f : 0.19 (DCM/MeOH, 10:1).

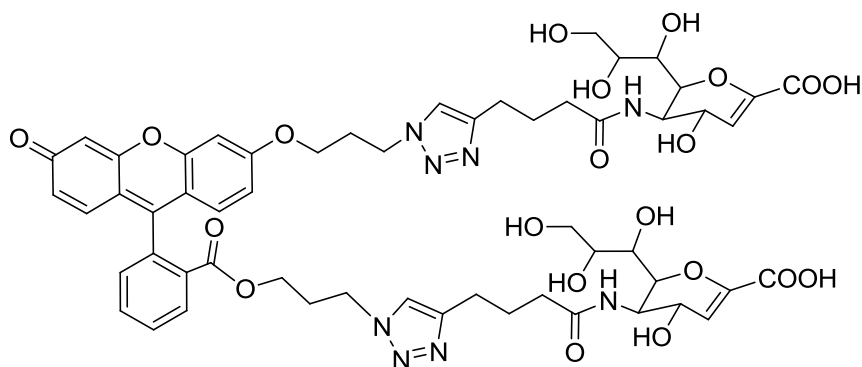
$^1\text{H-NMR}$ (300 MHz, DMSO-d_6): δ 8.12 (dd, $J = 7.9, 1.1$ Hz, 1H, 6''''-H), 7.83 (td, $J = 7.5, 1.1$ Hz, 1H, 4''''-H), 7.74 (tt, $J = 7.6, 1.5$ Hz, 1H, 5''''-H), 7.57 (d, $J = 12.8$ Hz, 1H, 5''-H), 7.43

(d, $J = 7.6$ Hz, 1H, 3''''-H), 6.70 (d, $J = 9.1$ Hz, 1H, 5''''-H), 6.68 (dd, $J = 9.1, 1.3$ Hz, 1H, 8''''-H), 6.43 (t, $J = 1.9$ Hz, 1H, 1''''-H), 6.41 (t, $J = 2.1$ Hz, 1H, 7''''-H), 6.40 (s, 1H, 2''''-H), 6.38 (s, 1H, 4''''-H), 4.17 (dd, $J = 10.3, 1.7$ Hz, 1H, 4-H), 4.07, 4.12 (t, $J = 6.5$ Hz, 1H, 3_a'''-H₂), 4.07 (t, $J = 6.8$ Hz, 1H, 3_b'''-H₂), 3.93-3.89 (m, 5H, 6-H, 5-H, 9_a-H, 1'''-H₂), 3.58 (dd, $J = 11.0, 3.6$ Hz, 1H, 9_b-H), 3.53-3.47 (m, 1H, 7-H), 3.36 (dd, $J = 11.0, 6.0$ Hz, 1H, 8-H), 2.57 (t, $J = 7.4$ Hz, 1H, 3-H_{ax}), 2.17 (t, $J = 7.4$ Hz, 1H, 3-H_{eq}), 2.00-1.83 (m, 4H, 2'-H₂, 5'-H₂), 1.78 (dd, $J = 15.1, 7.7$ Hz, 2H, 3'-H₂), 1.51 (dd, $J = 7.3, 3.2$ Hz, 2H, 2'''-H₂), 1.47-1.38 (m, 2H, 4'-H₂).

¹³C-NMR (75 MHz, DMSO-d₆): δ 189.88 (C-3'''''), 176.89 (C-1), 172.74, 172.66 (C-1', CO_{ester}), 156.72 (C-6'''''), 153.9 (C-5_a''''', C-4_a''''', C-2'''''), 146.43 (C-4'), 134.07 (C-9'''''), 132.85 (C-2'''''), 130.62, 130.55 (C-1''''', C-9_a'''''), 129.62, 129.57 (C-3''''', C-4''''', C-5''''', C-6''''', C-1'''''), 122.57 (C-8''''', C-8_a'''''), 121.73 (C-5'), 111.93 (C-7'''''), 103.22, 103.20 (C-4''''', C-5'''''), 89.79 (C-2), 72.04, 71.69 (C-8, C-6), 70.11 (C-7), 67.02 (C-4), 62.12 (C-9, C-3'''), 53.13 (C-5), 46.05, 45.94 (C-3, C-1'''), 28.5 (C-2'), 27.75 (C-5'), 24.72 (C-2'''), 24.394, 21.61 (C-3', C-4').

MS (ESI): m/z [M+Na]⁺ calcd for [C₃₉H₄₁N₄O₁₄Na+Na]⁺ 836.24, found 836.07.

10.4.7 2,6-Anhydro-5-({5-[1-(3-{[9-(2-{[3-(4-{4-[(3,5-dideoxynon-2-enonic acid-5-yl)amino]-4-oxobutyl}-1H-1,2,3-triazole-1-yl)propoxy]carbonyl} phenyl)-3-oxo-xanthen-6-yl]oxy}propyl)-1H-1,2,3-triazole-4-yl]butanoyl}amino)-3,5-dideoxynon-2-enonic acid **192**



CuI (1.3 mg, 70 μ mol) and DIPEA (27 mg, 210 μ mol) were added to the solution of fluorescein diazide **130** (37 mg, 90 μ mol) and Neu5Hex olefin **168** (25 mg, 70 μ mol) in

MeOH. The solution was stirred overnight at rt, filtered over celite and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography with a gradient of DCM/MeOH (10:1 to 5:1) to afford the triazole **192** (42 mg, 53 μ mol) as a dark orange solid.

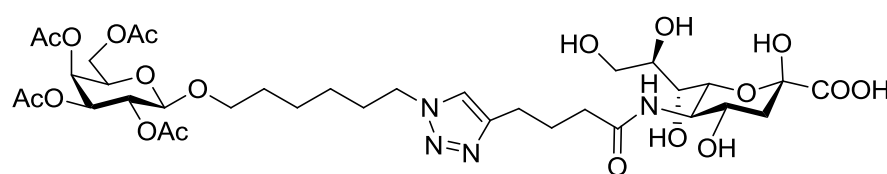
Yield: 54%.

R_f : 0.32 (DCM/MeOH, 5:1).

$^1\text{H-NMR}$ (600 MHz, DMSO-d_6): δ 8.21 (d, $J = 7.5$ Hz, 1H, 6''''-H), 7.89 (td, $J = 7.5$, 2.4 Hz, 1H, 4''''-H), 7.81 (t, $J = 7.3$ Hz, 1H, 5''''-H), 7.73-7.61 (m, 2H, 5''-H, 5''''''-H), 7.52 (d, $J = 7.5$ Hz, 1H, 3''''-H), 6.78 (s, 1H, 5''''''-H), 6.76 (s, 1H, 8''''''-H), 6.51-6.46 (m, 4H, 1''''''-H, 7''''''-H, 2''''''-H, 4''''''-H), 5.61 (d, $J = 1.6$ Hz, 2H, 3-H, 3''''''''-H), 4.53-4.42 (m, 4H, 1''''-H₂, 3''''''-H₂), 4.28 (d, $J = 9.9$ Hz, 1H, 4-H), 4.25 (d, $J = 8.1$ Hz, 1H, 4''''''''-H), 4.19 (t, $J = 6.7$ Hz, 2H, 3''''-H₂), 4.15 (t, $J = 6.9$ Hz, 1H, 1_a''''''-H₂), 4.03 (t, $J = 6.0$ Hz, 1H, 1_b''''''-H₂), 3.92 (dd, $J = 10.5$, 2.8 Hz, 2H, 9_a-H₂, 9_a''''''''-H₂), 3.66 (dd, $J = 10.9$, 3.3 Hz, 2H, 6-H, 6''''''''-H), 3.58-3.54 (m, 2H, 7-H, 7''''''''-H), 3.49-3.36 (m, 4H, 8-H, 8''''''''-H, 5-H, 5''''''''-H), 3.33 (d, $J = 8.5$ Hz, 1H, 9_b-H₂), 3.30 (d, $J = 8.6$ Hz, 1H, 9_b''''''''-H₂), 2.62 (t, $J = 7.5$ Hz, 2H, 1''''''''-H₂), 2.53 (t, $J = 7.4$ Hz, 2H, 4'-H₂), 2.31 (t, $J = 7.34$ Hz, 2H, 2'-H₂), 2.23 (td, $J = 10.6$, 2.3 Hz, 2H, 3''''''''-H₂), 2.14-2.03 (m, 4H, 2''''-H₂, 2''''''-H₂), 1.97-1.69 (m, 4H, 3'-H₂, 2''''''''-H₂).

$^{13}\text{C-NMR}$ (150 MHz, DMSO-d_6): δ 181.17 (C-3'''''), 176.35 (C-1, C-9'''''''), 172.23, 171.91, (C-1', C-4'''''''), 164.94 (CO_{ester}), 156.55 (C-6'''''), 151.07, 150.11 (C-4_a''''', C-5_a''''', C-2'''''), 147.61, 146.40, (C-4'', C-4'''''''), 134.15 (C-9'''''), 133.92, 133.81 (C-2, C-2'''''''), 132.94 (C-2'''''), 130.81, 130.78 (C-1''''', C-9_a'''''), 130.56, 129.7, 129.69, 129.51 (C-1''''', C-3''''', C-4''''', C-5''''', C-6'''''), 122.44 (C-8''''', C-7'''''), 121.72 (C-3, C-3'''''''), 112.25 (C-8_a'''''), 103.17 (C-4''''', C-5'''''), 79.34 (C-6, C-6'''''''), 71.27 (C-8, C-8'''''''), 69.58 (C-7, C-7'''''''), 67.73 (C-4, C-4'''''''), 63.64, (C-1''''', C-3'''''), 62.14 (C-9, C-9'''''''), 51.12, 50.74 (C-5, C-5'''''''), 49.97, 49.93 (C-1'', C-3'''''''), 40.21, 39.82 (C-2', C-3'''''''), 28.52, 28.48 (C-4', C-1'''''''), 27.83, 27.17 (C-2''', C-2'''''''), 25.12, 25.05 (C-3', C-2''''''').

10.4.8 3,5-Dideoxy-5-{[4-(1-{6-[(2,3,4,6-tetra-*O*-acetylhexopyranosyl)oxy] hexyl}-1*H*-1,2,3-triazole-4-yl)butanoyl]amino}non-2-ulopyranosonic acid **186**



Neu5Hex **116** (130 mg, 360 μmol) and acetylated glucose azide **127** (164 mg, 360 μmol) were suspended

in a 3:1 mixture of *tert*-BuOH and H_2O (20 mL). The solution was vigorously stirred for 10 min and the solution of sodium ascorbate (1 M, 36 μL) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1 M, 12 μL) were added. After overnight stirring the mixture was filtered over celite and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography, eluting with DCM/MeOH (10:1 to 7:1) to afford the triazole **186** (224 mg, 260 μmol) as a white powder.

Yield: 74%.

R_f : 0.23 (DCM/MeOH, 5:1).

$^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 8.53 (s, 1H, NH), 7.80 (s, 1H, 5''-H), 5.53 (d, $J = 3.3$ Hz, 2H, 4''''-H, 5''''-H), 5.29 (dd, $J = 10.3, 3.5$ Hz, 1H, 3''''-H), 5.13 (dd, $J = 10.2, 7.9$ Hz, 1H, 2''''-H), 4.62 (d, $J = 7.6$ Hz, 1H, 1''''-H), 4.47 (t, $J = 6.8$ Hz, 2H, 1'''-H₂), 4.12 (dd, $J = 6.4, 3.2$ Hz, 1H, 4-H), 4.09 (d, $J = 3.0$ Hz, 1H, 6-H), 4.03 (t, $J = 9.9$ Hz, 2H, 6'''-H₂), 3.96-3.87 (m, 2H, 8-H, 5-H), 3.85 (td, $J = 5.0, 1.8$ Hz, 1H, 6_a''''-H₂), 3.73-3.66 (m, 2H, 9_a-H₂, 7-H), 3.75-3.66 (m, 2H, 9_b-H₂, 6_b''''-H₂), 2.83 (t, $J = 7.6$ Hz, 2H, 4'-H₂), 2.51 (td, $J = 11.2, 1.0$ Hz, 1H, 2_a'-H₂), 2.41 (td, $J = 11.7, 1.5$ Hz, 2H, 3'-H₂), 2.33 (td, $J = 8.8, 1.7$ Hz, 1H, 2_b'-H₂), 2.17 (dd, $J = 8.8, 1.3$ Hz, 1H, 3_{eq}-H₂), 2.30, 2.13, 2.02 (4s, 12H, 4 \times CH₃), 2.05 (t, $J = 7.4$ Hz, 1H, 3_{ax}-H₂), 2.01-1.87 (m, 4H, 2'''-H₂, 5'''-H₂), 1.44-1.28 (m, 4H, 3'''-H₂, 4'''-H₂).

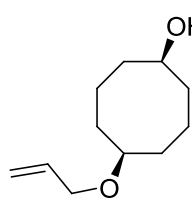
$^{13}\text{C-NMR}$ (75 MHz, CD_3OD): δ 177.07, 177.03 (C-1, C-1'), 172.11, 172.03, 171.54, 171.37 (4 \times COCH₃), 148.39 (C-4''), 123.51 (C-5''), 102.24 (C-1'''), 98.83 (C-2), 72.77, 72.34 (C-5''', C-8), 71.74, 70.77, 70.53 (C-4''', C-3''', C-6'''), 68.89, 68.23 (C-2''', C-6), 66.91 (C-4), 65.1, 64.47 (C-9, C-7), 62.59 (C-6''), 53.92 (C-5), 51.21 (C-1''), 36.31 (C-3), 31.24, 30.39 (C-2', C-4'), 27.14, 26.74, 26.41, 25.91, 25.59 (C-3', C-2'', C-3'', C-4'', C-5''), 20.81, 20.63, 20.55, 20.51 (4 \times CH₃).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{35}\text{H}_{54}\text{N}_4\text{O}_{19}+2\text{Na}]^+$ 880.32, found 880.12.

MS (ESI): m/z $[\text{M}-\text{H}]^-$ calcd for $[\text{C}_{35}\text{H}_{54}\text{N}_4\text{O}_{19}-\text{H}]^-$ 833.32, found 833.09.

10.5 Difluoro Cyclooctyne (DIFO I)

10.5.1 *cis*-5-(Allyloxy)cyclooctanol **197**



NaH (60% suspension in mineral oil, 3.06 g, 76.54 mmol) was added to the stirring solution of *cis*-1,5-cyclooctandiol **27** (9.73 g, 67.36 mmol) in DMF (150 mL) at 0 °C. The solution was stirred for 1 h and allyl bromide **196** (7.61 g, 67.36 mmol) was slowly added over a period of 1 h. After the complete addition of allyl alcohol the reaction mixture was allowed to warm to rt and stirred overnight. The solution was quenched with water (60 mL) and the aqueous phase was extracted with ether (5 \times 100 mL) and the combined organic layers were washed with water (3 \times 150 mL) and brine (60 mL), dried over MgSO_4 and evaporated. The crude product was purified through flash chromatography, eluting with Hex/ETBE to afford the desired product **197** (7.73 g, 42.01 mmol) as a colorless oil.

Yield: 62%.

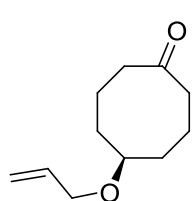
R_f : 0.4 (Hex/ETBE, 2:1).

$^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 5.83 (ddt, $J = 17.2, 10.4, 5.5$ Hz, 1H, 2'-H), 5.18 (dq, $J = 17.2, 1.7$ Hz, 1H, 3'-trans-H₂), 5.06 (dq, $J = 10.2, 1.5$ Hz, 1H, 3'-cis-H₂), 3.85 (dt, $J = 5.4, 1.4$ Hz, 2H, 1'-H₂), 3.77 (tt, $J = 13.4, 2.9$ Hz, 1H, 5-H), 3.31 (tt, $J = 13.1, 2.8$ Hz, 1H, 1-H), 1.79-1.84 (m, 2H, 2_a-H₂, 8_a-H₂), 1.77-1.71 (m, 4H, 3_a-H₂, 4_a-H₂, 6_a-H₂, 7_a-H₂), 1.61-1.49 (m, 4H, 2_b-H₂, 4_b-H₂, 6_b-H₂, 8_b-H₂), 1.42-1.33 (m, 2H, 3_b-H₂, 7_b-H₂).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3): δ 135.52 (C-2'), 116.43 (C-1'), 78.63 (C-3'), 71.75 (C-5), 69.21 (C-1), 36.44 (C-4, C-6), 32.82 (C-2, C-8), 20.67 (C-3, C-7).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{11}\text{H}_{20}\text{O}_2+\text{Na}]^+$ 207.03, found 207.03.

10.5.2 5-(Allyloxy)cyclooctanone **198**



Pyridinium chlorochromate (PCC, 11.05 g, 51.24 mmol) was added to the solution of alcohol **197** (6.74 g, 36.63 mmol) in anhyd. DCM (140 mL) at rt over 2 h, 1.45 g after every 15 min. After the complete addition of PCC, the solution was stirred for 30 min and concentrated. The crude product was purified by column chromatography, eluting with Hex/EtOAc (5:1) to afford **198** (6.34 g, 34.85 mmol) as a colorless thick oil.

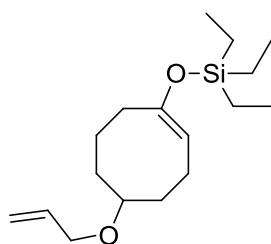
Yield: 95%.

R_f : 0.3 (Hex/EtOAc, 5:1).

$^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 5.83 (ddt, $J = 17.2, 10.4, 5.5$ Hz, 1H, 2'-H), 5.14 (dq, $J = 17.2, 1.7$ Hz, 1H, 3'-trans-H₂), 5.06 (dq, $J = 10.3, 1.5$ Hz, 1H, 3'-cis-H₂), 3.84 (dt, $J = 5.5, 1.4$ Hz, 2H, 1'-H₂), 3.12 (tt, $J = 12.9, 2.9$ Hz, 1H, 5-H), 2.49 (ddd, $J = 13.4, 9.4, 3.8$ Hz, 2H, 2_a-H₂, 8_a-H₂), 2.22 (ddd, $J = 13.5, 9.1, 3.3$ Hz, 2H, 2_b-H₂, 8_b-H₂), 2.04-1.92 (m, 2H, 3_a-H₂, 7_a-H₂), 1.86-1.56 (m, 6H, 3_b-H₂, 7_b-H₂, 4-H₂, 6-H₂).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3): δ 215.83 (C-1), 134.47 (C-3'), 116.12 (C-2'), 77.01 (C-1'), 68.92 (C-5), 41.74 (C-4, C-6), 33.11 (C-2, C-8), 22.33 (C-3, C-7).

10.5.3 {[5-(Allyloxy)cyclooct-1-en-1-yl]oxy}(triethyl)silane **199**



To a dried three neck flask containing magnetic stir bar and anhyd. THF (160 mL), lithium hexamethyldisilazide (LHMDS, 1 M in THF, 36 mL, 36.00 mmol) was added and cooled to -78°C . The solution of allyl cyclooctanone **198** (5.82 g, 32.23 mmol) in THF (5 mL), was slowly added over a period of 1 h. After 20 min of stirring, chlorotriethylsilane (6.2 mL, 5.57 g, 36.93 mmol) was added while maintaining the temperature at -78°C . After 10 min, the solution was warmed to rt by water bath and further stirred for 1 h. The solvent was evaporated

and the crude product was purified by flash chromatography, eluting with hexane to 25% Hex/EtOAc to afford the silane **199** (8.46 g, 28.54 mmol) as a colorless oil.

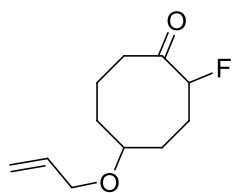
Yield: 92%.

R_f : 0.6 (Hex/EtOAc, 10:1).

$^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 5.83 (ddt, $J = 17.2, 10.4, 5.5$ Hz, 1H, $2'\text{-H}$), 5.25 (dq, $J = 17.1, 1.7$ Hz, 1H, $3'\text{-trans-H}_2$), 5.13 (dq, $J = 10.4, 1.5$ Hz, 1H, $3'\text{-cis-H}_2$), 4.74 (dd, $J = 9.2, 7.2$ Hz, 1H, 2-H), 3.96-3.91 (m, 2H, $1'\text{-H}_2$), 3.42-3.35 (m, 1H, 5-H), 1.51-2.18 (m, 8H, 3-H₂, 4-H₂, 6-H₂, 8-H₂), 1.31-1.21 (m, 2H, 7-H₂), 0.91 (dt, $J = 7.8, 0.7$ Hz, 9H, $3 \times \text{CH}_3$), 0.59 (dq, $J = 7.9, 0.7$ Hz, 6H, $3 \times \text{CH}_2\text{CH}_3$).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3): δ 152.75 (C-1), 135.52 (C-2'), 116.23 (C-3'), 104.26 (C-2), 79.92 (C-1'), 69.22 (C-5), 36.21 (C-4), 33.71 (C-8), 31.78 (C-6), 24.46 (C-3), 22.40 (C-7), 6.71 ($3 \times \text{CH}_2\text{CH}_3$), 5.01 (3C, $3 \times \text{CH}_2\text{CH}_3$).

10.5.4 5-(Allyloxy)-2-fluorocyclooctanone **200**



The solution of silylenolether **199** (8.46 g, 28.51 mmol) in DMF (35 mL) was slowly added via dropping funnel over 30 min to the stirring solution of selectfluor (12.13 g, 34.31 mmol) in DMF (30 mL) at 0 °C. After complete addition, the solution was warmed to rt, stirred for 30 min and quenched with

water (70 mL). The aqueous phase was extracted with Et_2O (4×80 mL) and the combined organic fractions were washed with water (3×80 mL), brine (50 mL), dried over MgSO_4 and evaporated under reduced pressure. The crude oily product was purified by flash chromatography (Hex/EtOAc, 10:1 to 5:1) to afford two diastereomers (*cis*-isomer **200a** 3.83 g, 19.02 mmol, *trans*-isomer **200b** 1.69 g, 8.41 mmol).

Yield: 66% (*cis*), 29% (*trans*).

R_f : *cis*-isomer, 0.38 (Hex/EtOAc, 9:1), *trans*-isomer 0.2 (Hex/EtOAc, 9:1).

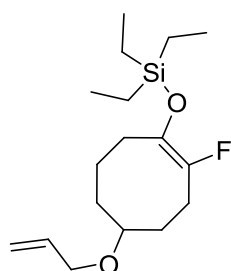
$^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 5.85 (ddt, $J = 17.2, 10.4, 5.6$ Hz, 1H, $2'\text{-H}$), 5.17 (dq, $J = 17.2, 1.7$ Hz, 1H, $3'\text{-trans-H}$), 5.08 (dq, $J = 10.3, 1.5$ Hz, 1H, $3'\text{-cis-H}_2$), 4.91 (ddd, $J = 48.3, 6.7, 3.6$ Hz, 1H, 2-H_{trans}), 3.84 (dq, $J = 5.6, 1.4$ Hz, 2H, $1'\text{-H}_2$), 3.35-3.29 (m, 1H, 5-H), 2.51-2.44 (m, 2H, 8-H₂), 2.42-2.34 (m, 1H, 3_a-H₂), 2.11-1.91 (m, 3H, 3_b-H₂, 7-H₂), 1.85-1.61 (m, 4H, 4-H₂, 6-H₂).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3): δ 213.37 (d, $J = 24.0$ Hz, C-1), 134.91 (C-2'), 116.76 (C-3'), 95.7 (d, $J = 186.4$ Hz, C-2), 76.59 (C-1'), 69.42 (C-5), 40.13 (d, $J = 2.3$ Hz, C-8), 33.39 (C-3), 30.71 (d, $J = 21.2$ Hz, C-4), 27.26 (d, $J = 3.0$ Hz, C-6), 20.19 (d, $J = 5.2$ Hz, C-7).

10.5.4.1 Conversion of *cis* to *trans* monofluorocyclooctanone

The solution of KHMDS in toluene (0.5 M, 318 μ L, 160 μ mol) was added to the stirred solution of *cis*-monofluoro allyl cyclooctanon (3.21 g, 16.11 mmol) in THF (40 mL) at 0 °C. The reaction mixture was stirred for 1 h and the solvent was evaporated to afford a crude product, which was purified by column chromatography, eluting with Hex/EtOAc (10:1 to 5:1). The column afforded the isolation of two diastereomers *cis/trans* (50:50).

10.5.5 [[5-(Allyloxy)-2-fluorocyclooct-1-en-1-yl]oxy](triethyl)silane



A solution of *trans*-monofluoro allyl ketone **200b** (3.55 g, 17.52 mmol) in THF (8 mL) was slowly added over a period of 2 h to the solution of KHMDS (0.5 M in toluene, 41.93 mL, 22.46 mmol) in THF (160 mL) at -78 °C and stirred for further 30 min. The mixture was allowed to warm to rt and further stirred for 1 h followed by the removal of the solvent. The thick oily crude product was purified by silica gel column, eluting with Hex/EtOAc (50:1 to 25:1) to afford two regioisomers, desired enolate (4.76 g, 15.13 mmol) in the form of clear oil and undesired isomer (550 mg, 1.73 mmol).

Yield: 86%.

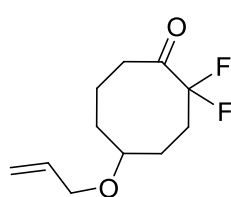
R_f : 0.69 (Hex/EtOAc, 9:1).

$^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 5.82 (ddt, $J = 17.2, 10.4, 5.5$ Hz, 1H, 2'-H), 5.19-5.08 (dq, $J = 17.2, 1.7$ Hz, 1H, 3'_{trans}-H₂), 5.07 (dq, $J = 10.4, 1.5$ Hz, 1H, 3'_{cis}-H₂), 3.91-3.81 (m, 2H, 1'-H₂), 3.39-3.32 (m, 1H, 5-H), 2.08-1.56 (m, 10H, 3-H₂, 4-H₂, 6-H₂, 7-H₂, 8-H₂), 0.91 (t, $J = 7.9$ Hz, 9H, 3 \times CH₃), 0.59 (q, $J = 7.9$ Hz, 6H, H-3 \times CH₂CH₃).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3): δ 148.49 (d, $J = 17.9$ Hz, C-1), 135.26 (d, $J = 8.4$ Hz, C-2'), 128.35 (C-2), 116.48 (d, $J = 17.5$ Hz, C-3'), 79.50 (d, $J = 67.1$ Hz, C-1'), 69.28 (d, $J = 12.8$ Hz, C-5), 33.56 (d, $J = 2.1$ Hz, C-8), 32.85 (C-4), 31.24 (d, $J = 1.9$ Hz, C-6), 24.50 (d, $J = 3.2$ Hz, C-3), 24.55 (d, $J = 27.1$ Hz, C-7), 6.62 (3C, 3 \times CH₂CH₃), 5.22 (3C, 3 \times CH₂CH₃).

$^{19}\text{F-NMR}$ (376 MHz, CDCl_3): δ -130.7 (dd, $J = 28.3, 20.2$ Hz, 1F, 2-F).

10.5.6 5-(Allyloxy)-2,2-difluorocyclooctanone **201**



The solution of fluoro silyl enol ether (5.31 g, 16.88 mmol) in DMF (25 mL) was slowly added over a period of 30 min to the stirring solution of selectfluor (7.74 g, 21.92 mmol) in THF (16 mL) at 0 °C. The solution was stirred at 0 °C for 30 min, allowed to warm to rt and stirred for further 2 h.

The reaction was quenched with water (25 mL) and the aqueous layer was extracted with Et₂O (4 × 50 mL). The combined organic layers were washed with water (3 × 50 mL), dried over MgSO₄ and filtered. The crude product was purified by silica gel column, eluting with Hex/EtOAc (10:1) to afford **201** (2.25 g, 10.34 mmol).

Yield: 75%.

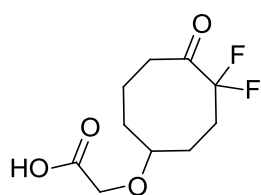
R_f: 0.28 (Hex/EtOAc, 1:1) with 1% AcOH.

¹H-NMR (300 MHz, CDCl₃): δ 5.69-5.96 (ddt, *J* = 17.2, 10.4, 5.5 Hz, 1H, 2'-H), 5.18 (dq, *J* = 17.2, 1.7 Hz, 1H, 3'_{trans}-H₂), 5.09 (dq, *J* = 10.4, 1.5 Hz, 1H, 3'_{cis}-H₂), 3.84 (td, *J* = 5.5, 1.4 Hz, 2H, 1'-H₂), 3.35-3.21 (m, 1H, 5-H), 2.81-2.39 (m, 2H, 8-H₂), 2.37-2.14 (m, 1H, 3_a-H₂), 2.13-1.92 (m, 2H, 4-H₂), 1.90-1.57 (m, 5H, 3_b-H₂, 6-H₂, 7-H₂).

¹³C-NMR (75 MHz, CDCl₃): δ 203.65 (C-1), 134.72 (d, *J* = 5.9 Hz, C-2'), 118.41 (C-2), 116.85 (C-3'), 75.82 (d, *J* = 7.1 Hz, C-1'), 69.46 (d, *J* = 5.3 Hz, C-5), 37.96 (C-8), 31.35 (C-3), 30.91 (C-6), 25.88 (C-4), 20.97 (C-7).

¹⁹F-NMR (376 MHz, CDCl₃): δ -106.2 (q, *J* = 230.3 Hz, 2F).

10.5.7 2-((4,4-Difluoro-5-oxocyclooctyl)oxy)acetic acid **202**



NaIO₄ (5.85 g, 27.52 mmol) and RuCl₃ (35 mg, 170 μmol) were added to a three neck flask containing the solution of allyl octanone **201** (1.43 g, 6.73 mmol) in CCl₄ (15 mL), MeCN (15 mL) and H₂O (19 mL) at 0 °C.

After 15 min the reaction mixture was allowed to warm to rt and stirred for further 2.5 h. The solution was diluted with 1 M HCl (60 mL) and brine (60 mL) the aqueous layer was extracted with DCM (7 × 60 mL), dried over MgSO₄ and evaporated. The oily crude product was purified by silica gel column, eluting with Hex/EtOAc (1:1) with 1% AcOH to afford **202** (1.56 g, 6.72 mmol) as a clear oil.

Yield: 98%.

R_f: 0.34 (Hex/EtOAc, 1:1).

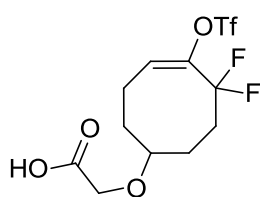
¹H-NMR (400 MHz, CD₃CN): δ 4.00 (s, 2H, 2-H₂), 3.40 (sep, *J* = 3.7 Hz, 1H, 1'-H), 2.78-2.71 (m, 1H, 6_a'-H₂), 2.61-2.52 (m, 1H, 6_b'-H₂), 2.43-2.28 (m, 1H, 3_a'-H₂), 2.20-2.08 (m, 1H, 3_b'-H₂), 1.94 (quin, *J* = 2.5 Hz, 2H, 7'-H₂), 2.07-1.64 (4 × m, 4H, 4'-H₂, 2'-H₂).

¹³C-NMR (100 MHz, CD₃CN): δ 204.16 (t, *J* = 27.4 Hz, C-5'), 172.07 (C-1), 118.37 (t, *J* = 251.3 Hz, C-4'), 78.60 (C-1'), 66.21 (C-2), 38.35 (C-8'), 31.51 (C-6'), 30.97 (t, *J* = 24.8 Hz, C-3'), 26.37 (t, *J* = 5.6 Hz, C-2'), 22.04 (C-7').

¹⁹F-NMR (376 MHz, CD₃CN): δ -105.7 (s, 2F).

MS (ESI): *m/z* [M+Na]⁺ calcd for [C₁₀H₁₄F₂O₄+Na]⁺ 259.07, found 259.07.

10.5.8 (E)-2-((6,6-Difluoro-5-(((trifluoromethyl)sulfonyl)oxy)cyclooct-4-en-1-yl)oxy)acetic acid **203**



The solution of ketone **202** (200 mg, 840 μ mol) in THF (2 mL) was slowly added over a time period of 20 min to the solution of KHMDS (0.5 M, 3.46 mL, 1.73 mmol) in THF (23 mL) at -78°C . The solution was stirred for 1 h at -78°C followed by the addition of the solution of Tf_2NPh (n-phenyl-bis(trifluoromethylsulfonamide) (618 mg, 1.75 mmol) in THF (4 mL) and the reaction was allowed to warm to rt. After 30 min, the reaction mixture was concentrated on rotavap and the crude product was dissolved in DCM (7 mL), 1 M HCl (7 mL) and brine (7 mL). The aqueous layer was extracted with DCM (5×5 mL), dried over MgSO_4 , filtered and evaporated reduced pressure. The crude product was purified by silica gel column chromatography, eluting with Hex/EtOAc (1:1) with 1% AcOH to afford **203** (180 mg, 490 μ mol) as a colorless thick oil.

Yield: 48%.

R_f : 0.32 (Hex/EtOAc 2:1 with 2% AcOH).

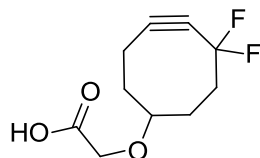
$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 6.10 (td, $J = 9.57, 3.04$ Hz, 1H, 4'-H), 4.05 (d, $J = 1.14$ Hz, 2H, 2-H₂), 3.62-3.51 (m, 1H, 1'-H), 2.73-2.44 (m, 2H, 3'-H₂), 2.32-2.14 (m, 2H, 6'-H₂), 2.02-1.84 (m, 4H, 2'-H₂, 8'-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 175.57 (C-1), 142.51 (t, $J = 29.3$, Hz, C-5'), 127.40 (C-6'), 118.70 (dd, $J = 282.3, 68.3$ Hz, C-4'), 77.20 (C-1'), 65.77 (C-2), 32.04 (t, $J = 25.9$ Hz, C-8'), 31.24 (C-2'), 26.00 (t, $J = 5.1$ Hz, C-3'), 19.20 (C-7').

$^{19}\text{F-NMR}$ (376 MHz, CDCl_3): δ -92.01(ddd, $J = 1399, 277.66, 26.60$ Hz, 2F, 6'-F₂), 74.3 (s, 3F, CF₃).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{11}\text{H}_{13}\text{F}_5\text{O}_6\text{S}+\text{Na}]^+$ 391.02, found 391.02.

10.5.9 2-((6,6-Difluorocyclooct-4-yn-1-yl)oxy)acetic acid **26**



The synthesis of DIFO was achieved in two steps.

Step 1: In first step LDA was prepared by slow addition of n-BuLi (2.5 M solution in hexane, 2.38 mL, 5.95 mmol) to the solution of DIPEA (1.0 mL, 7.13 mmol) in THF (8.6 mL) over a period of 45 min at -78°C . The solution was stirred at this temperature for 45 min and used in second step.

Step 2: In second step the LDA solution (0.5 M, 2.73 mL, 1.36 mmol), prepared above was slowly added to the stirred solution of the triflate **203** (200 mg, 540 μ mol) in fractions at -15°C . Each fraction equimolar to the triflate was added over a period of 30 min until 2.5 equiv. LDA was added. While addition the color of the solution was changed from colorless to light yellow

and finally amber. The reaction was quenched by saturated solution of NH_4Cl (1 mL) followed by the evaporation of the solvent. The residue was dissolved in DCM (10 mL), 1 M HCl (5 mL), brine (5 mL) and finally extracted with DCM (5×5 mL), dried over MgSO_4 and evaporated under reduced pressure. The purification was done by silica column, eluting with Hex/EtOAc (4:1 to 1:1) with 1% AcOH to afford DIFO **26** (very less traces).

Yield: ~ 1%.

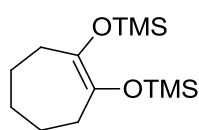
R_f : 0.34 (Hex/EtOAc 1:1 with 1% AcOH).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 3.95 (s, 2H, 2- H_2), 3.58-3.52 (m, 1H, 1'-H), 2.64-2.42 (m, 2H, 3'- H_2), 2.29-2.15 (m, 2H, 7'- H_2), 1.96-1.88 (m, 2H, 2'- H_2), 1.82-1.71 (m, 2H, 8'- H_2).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 174.76 (C-1), 118.28 (t, $J = 250.5$ Hz, C-6'), 114.66 (C-4'), 78.04 (C-5'), 77.23 (C-1'), 65.84 (C-2), 37.81 (C-3'), 32.09 (t, $J = 25.8$ Hz, C-2'), 25.66 (t, $J = 5.7$ Hz, C-8'), 20.71 (C-7').

10.6 Difluorocyclooctyne Benzoic Acid DIFO (II)

10.6.1 [Cyclohept-1-ene-1,2-diylbis(oxy)]bis(trimethylsilane).204



To a dried three neck flask containing magnetic stir bar, toluene (120 mL) and crushed borosilicate glass test tube, sodium metal (5.32 g, 231.02 mmol) cut into small pieces was added under nitrogen atmosphere. The reaction was set behind a blast shield of glass for safety. The solution was refluxed till the sodium melted and dispersed into fine sand, followed by the addition of trimethylsilyl chloride (29.2 mL, 231.00 mmol). A solution of diethyl pimelate **30** (12.0 mL, 55.14 mmol) in toluene (20 mL) was slowly added to the mixture over 3 h using the syringe pump and stirred overnight at reflux. The reaction mixture was cooled to rt and filtered through celite and washed the celite with toluene (3×50 mL). The solvent was evaporated and the crude product **204** (7.56 g, 27.79 mmol) was used in next step without any further purification.

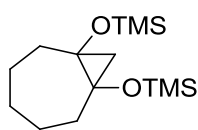
Yield: 50%.

R_f : 0.33 (Pent/DCM, 12:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 2.06-1.98 (m, 2H, 3_a- H_2 , 7_a- H_2), 1.84-1.64 (m, 2H, 3_b- H_2 , 7_b- H_2), 1.53-1.44 (m, 4H, 4- H_2 , 6- H_2), 1.38-1.33 (m, 2H, 5- H_2), 0.01 (s, 18H, 6 \times CH_3).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 129.16, 128.35 (C-1, C-2), 33.98 (C-5), 30.57 (C-3, C-7), 24.49 (C-4, C-6), 2.05 (6C, 6 \times CH_3).

10.6.2 [Bicyclo[5.1.0]octane-1,7-diylbis(oxy)]bis(trimethylsilane) **205**



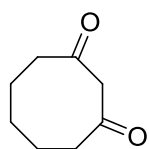
To a dried three neck flask containing a stir bar, CH_2I_2 (6.0 mL, 79.49 mmol), bis silyl enol ether **204** (7.32 g, 26.62 mmol) and dry toluene (40 mL) were added and the mixture was cooled to -10°C . Diethyl zinc solution (138 mL, 1.1 M in toluene, 151.81 mmol) was added over 30 min using a syringe pump. The reaction mixture was stirred overnight and slowly allowed to warm to rt, quenched with cold, saturated aqueous ammonium chloride solution (25 mL) and extracted with toluene (3×75 mL). The combined organic extracts were washed with saturated solution of ammonium chloride (100 mL), brine (100 mL) and dried over MgSO_4 , filtered through Celite and concentrated to yield desired crude product **205** (7.11 g, 24.82 mmol) that was carried out in next step without further purification.

Yield: 93%.

R_f : 0.33 (Pent/ Et_2O , 1:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 1.84-1.58 (m, 4H, 2- H_2 , 6- H_2), 1.59-1.44 (m, 2H, 4- H_2), 1.30-1.15 (m, 4H, 3- H_2 , 5- H_2), 0.89 (d, $J = 6.8$ Hz, 1H, 8 $_a$ - H_2), 0.73 (d, $J = 6.4$ Hz, 1H, 8 $_b$ - H_2), 0.17 (s, 18H, $3 \times \text{CH}_3$).

10.6.3 Cyclooctane-1,3-dione **206**



To a dried three neck flask, containing bicyclooctane **205** (7.32 g, 26.01 mmol) in dry DMF (24 mL), anhyd. FeCl_3 (16.64 g, 106.43 mmol) was added under nitrogen atmosphere at rt. The dark brown suspension was heated to 60°C and stirred overnight (16 h). The reaction mixture was cooled to rt, quenched in 10% aqueous HCl (400 mL) and extracted with CHCl_3 (4×400 mL). The combined organic layers were washed with 10% HCl (2×350 mL), brine (300 mL) and dried over MgSO_4 . Filtration and the removal of the solvent under vacuum afforded crude product (3.86 g). The product was taken up in Et_2O (250 mL) and extracted with 3% aqueous KOH solution (3×150 mL). The aqueous layer was acidified with conc. HCl, saturated with NaCl, and back-extracted with CHCl_3 (3×200 mL). Drying of the organic layer over MgSO_4 , followed by the filtration and removal of the solvent under reduced pressure afforded colorless oil (2.56 g). The crude product was purified by column chromatography, eluting with Et_2O /Pent (1:1) to afford **206** (2.25 g, 16.07 mmol) as a clear oil.

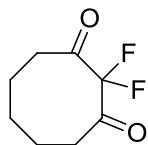
Yield: 64%.

R_f : 0.32 (DCM/Pent, 1:10).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 3.46 (s, 2H, 2- H_2), 2.45 (t, $J = 6.4$ Hz, 4H, 4- H_2 , 8- H_2), 1.79-1.73 (m, 4H, 5- H_2 , 7- H_2), 1.64-1.57 (m, 2H, 6- H_2).

^{13}C -NMR (100 MHz, CDCl_3): δ 206.17 (C-1, C-3), 58.92 (C-2), 43.95 (C-4, C-8), 27.31 (C-6), 24.11 (C-5, C-7).

10.6.4 2,2-Difluorocyclooctane-1,3-dione **215**



A dried round bottom flask containing a stir bar was charged with cyclooctane-1,3-dione **206** (1.00 g, 7.14 mmol) and MeCN (50 mL). Cs_2CO_3 (4.76 g, 14.55 mmol) was added to it and the reaction mixture was stirred at rt. After 30 min the mixture was cooled to 0 °C and Selectfluor (6.08 g, 17.05 mmol) was added, after which the mixture was stirred for an additional 30 min. The system was allowed to warm to rt, stirred for 2 h, concentrated under reduced pressure, diluted with 1 M HCl (50 mL) and extracted with diethyl ether (4×50 mL). The combined organic layers were washed with brine (2×25 mL), dried over MgSO_4 and filtered over celite. The crude product was purified by flash chromatography (6:1 hexanes/EtOAc) to afford **215** (1.03 g, 5.85 mmol) as a white solid.

Yield: 82%.

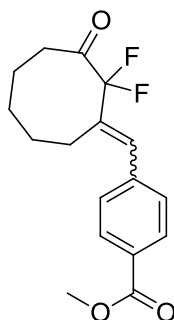
R_f : .038 (Hex/EtOAc, 4:1).

^1H -NMR (400 MHz, CDCl_3): δ 2.68-2.64 (m, 4H, 4- H_2 , 8- H_2), 1.84-1.78 (m, 4H, 5- H_2 , 7- H_2), 1.66-1.61 (m, 2H, 6- H_2).

^{13}C -NMR (100 MHz, CDCl_3): δ 197.67 (C-1, C-3), 109.35 (t, $J = 261.5$ Hz, C-2), 38.48 (C-4, C-8), 25.91 (C-6), 24.52 (C-5, C-7).

^{19}F -NMR (376 MHz, CDCl_3): δ -119.64 (t, $J = 1.8$ Hz, 2F).

10.6.5 Methyl-4-[(*E/Z*)-(2,2-difluoro-3-oxocyclooctylidene)methyl]benzoate **217**



To a dried three neck flask flushed with nitrogen gas, diketone **215** (1.00 g, 5.01 mmol), phasphonium bromide **216** (3.06 g, 6.21 mmol) and anhyd. THF (140 mL) were added. The system was cooled to 0 °C and DBU (0.893 mL) was added. The reaction mixture was stirred for 30 min at 0 °C and allowed to warm to rt, stirred for further 72 h, quenched with AcOH (1 mL) and diluted with MeOH (15 mL). The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (Hex/EtOAc, 6:1) to get **217** (1.50 g, 4.87 mmol) as a white solid.

Yield: 94%.

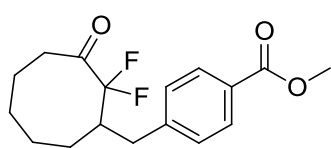
R_f : .053 (Hex/EtOAc, 4:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.04 (d, $J = 8.4$ Hz, 2H, 2-H, 6-H), 7.40 (d, $J = 8.2$ Hz, 2H, 3-H, 5-H), 7.25 (s, 1H, 1'-H), 3.95 (br s, 3H, OCH_3), 2.69 (tt, $J = 9.9, 1.5$ Hz, 2H, 8''-H₂), 2.52 (t, $J = 6.2$ Hz, 2H, 4''-H₂), 1.89-1.83 (m, 2H, 7''-H₂), 1.59-1.48 (m, 4H, 5''-H₂, 6''-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 201.99 (C-3''), 166.73 ($\text{CO}_{2,\text{ester}}$), 139.83 (C-1), 134.63 (t, $J = 19$ Hz, C-1'), 131.18 (t, $J = 10$ Hz, C-4), CH_3), 129.93 (C-1'), 129.71 (C-3, C-5), 128.91 (C-2, C-6), 115.13 (t, $J = 261$ Hz, C-2''), 52.33 (OCH_3), 37.51 (C-4'), 27.15 (C-8''), 26.01 (C-5''), 25.72 (C-7''), 25.29 (C-6'').

$^{19}\text{F-NMR}$ (376 MHz, CDCl_3): δ -112.25 (t, $J = 1.76$ Hz, 2F).

10.6.6 Methyl-4-[(2,2-difluoro-3-oxocyclooctyl)methyl]benzoate **218**



To a three neck flask, olefin **217** (1.25 g, 4.05 mmol) was dissolved in MeOH (50 mL) and the system was flushed with N_2 . Pd/C (105 mg, 200 μmol) was added and again flushed with N_2 followed by H_2 flush and the reaction mixture was stirred under H_2 atmosphere (using a balloon) for 24 h. The system was flushed thoroughly with N_2 , after which the reaction mixture was diluted with DCM (50 mL), filtered through celite and concentrated under reduced pressure. The crude product was purified by flash chromatography (Hex/EtOAc, 8:1) to afford **218** (1.20 g, 3.93 mmol) as a white solid.

Yield: 96%.

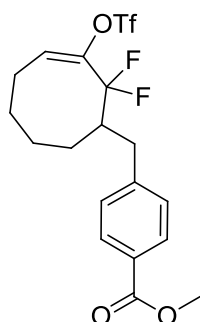
R_f : 0.6 (Hex/EtOAc, 4:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.97 (d, $J = 8.4$ Hz, 2H, 2-H, 6-H), 7.25 (d, $J = 8.1$ Hz, 2H, 3-H, 5-H), 3.91 (s, 3H, $\text{CH}_{3,\text{ester}}$), 3.29 (dd, $J = 13.6, 3.0$ Hz, 1H, 1''-H), 2.79-2.72 (m, 1H, 1_a'-H₂), 2.59-2.52 (m, 1H, 1_b'-H₂), 2.52 (dd, $J = 13.5, 10.7$ Hz, 1H, 4_a''-H₂), 2.44-2.29 (m, 1H, 4_b''-H₂), 2.13-1.83 (m, 2H, 5''-H₂), 1.62-1.42 (m, 4H, 6''-H₂, 7''-H₂), 1.38-1.29 (m, 1H, 8_a''-H₂), 1.22-1.12 (m, 1H, 8_b''-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 204.56 (dd, $J = 30.2, 24.3$ Hz, C-3''), 165.93 ($\text{CO}_{2,\text{ester}}$), 143.57 (C-4), 128.86 (C-3, C-5), 128.29 (C-2, C-6), 127.52 (C-1), 117.25 (t, $J = 250$ Hz, C-2''), 51.04 ($\text{CH}_{3,\text{ester}}$), 45.47 (t, $J = 21$ Hz, C-4'), 37.96 (C-1'), 32.66 (t, $J = 5$ Hz, C-5''), 26.08 (C-1'), 23.26 (d, $J = 7.1$ Hz, C-8''), 23.02 (d, $J = 3.2$ Hz, C-6''), 21.75 (C-7'').

$^{19}\text{F-NMR}$ (376 MHz, CDCl_3): δ -104.07 (d, $J = 245.5$ Hz, 1F), -123.83 (dd, $J = 246.0, 26.1$ Hz, 1F).

10.6.7 Methyl-4-[(2,2-difluoro-3-[(trifluoromethyl)sulfonyl]oxy)cyclooct-3-en-1-yl) methyl]benzoate **224**



To a flame dried three neck flask containing THF (55 mL), KHMDs (4.6 mL of a 0.5 M solution in toluene, 2.30 mmol) was added under inert conditions and the reaction mixture was cooled to -78°C with stirring. Ketone **218** (680 mg, 2.19 mmol) in THF (20 mL) was slowly added over 20 min via a syringe pump. After a stirring for additional 40 min, the solution of Tf_2NPh (823 mg, 2.33 mmol) in THF (20 mL) was slowly added via syringe pump. The system was warmed to rt, stirred for 21 h and finally quenched the reaction with MeOH (3 mL). The mixture was concentrated under vacuum and purified by flash chromatography (Hex/EtOAc, 8:1 and 1% Et_3N) to yield **224** (756 mg, 1.71 mmol) as a pale yellow oil.

Yield: 79%.

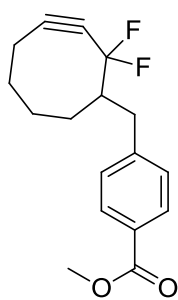
R_f : 0.13, (Toluene/Hex 2:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.98 (dt, $J = 8.4, 1.9$ Hz, 2H, 2-H, 6-H), 7.24 (d, $J = 8.5$ Hz, 2H, 3-H, 5-H), 6.09 (t, $J = 9.6$ Hz, 1H, 4''-H), 3.91 (s, 3H, CH_3), 3.28 (d, $J = 10.41$ Hz, 1H, 1''-H), 2.58-2.51 (m, 2H, 1'- H_2), 2.45-2.38 (m, 2H, 5''- H_2), 1.72-1.56 (m, 4H, 6''- H_2 , 7''- H_2), 1.55-1.37 (m, 2H, 8''- H_2).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 166.92 (CO_{ester}), 144.44 (C-3''), 142.66 (d, $J = 30.3$ Hz, C-4), 129.88 (C-2, C-6), 129.24 (C-3, C-5), 128.56 (C-1), 121.49 ($\text{C}_{\text{triflate}}$), 120.04 (C-2'), 116.86 (C-4'), 52.05 ($\text{CH}_{3,\text{ester}}$), 47.22 (dd, $J = 24, 21.7$ Hz, C-1'), 34.22 (dd, $J = 7.3, 2.7$ C-1'), 26.33 (C-5'), 25.54 (C-8'), 22.15 (C-6), 21.03 (C-7).

$^{19}\text{F-NMR}$ (376 MHz, CDCl_3): δ -104.07 (d, $J = 245.3$ Hz, 1F), -123.46 (dd, $J = 245.5, 23.4$ Hz, 1F).

10.6.8 Methyl-4-[(2,2-difluorocyclooct-3-yn-1-yl)methyl]benzoate **225**



In a dried three neck flask vinyl triflate **224** (100 mg, 230 μmol) was dissolved in THF (6 mL) and the mixture was cooled to -20°C . In a separate dried three neck flask, LDA solution (0.19 M) was prepared by adding $n\text{-BuLi}$ (846 μL of a 2.5 M solution in hexane, 2.11 mmol) dropwise to a solution of DIPA (359 μL , 2.54 mmol) in THF (9.3 mL) while stirring at -78°C . A portion of the LDA solution (1.9 mL, 370 μmol) was added dropwise to the first mixture over 1 h using a syringe pump at -20°C . The reaction mixture was warmed to rt over 20 min and quenched with MeOH (1 mL). The mixture was concentrated under reduced pressure and

purified by flash chromatography (0-3% EtOAc in 2:1 Hex/toluene) to yield **225** (52 mg, 180 μ mol) as a white solid.

Yield: 77%.

R_f : 0.30 (Hex/Toluene 1:2).

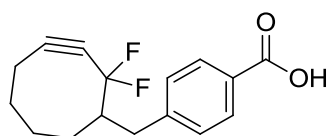
$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.89 (dt, $J = 8.3, 1.8$ Hz, 2H, 2-H, 6-H), 7.18 (d, $J = 8.1$ Hz, 2H, 3-H, 5-H), 3.83 (s, 3H, $\text{CH}_{3,\text{ester}}$), 3.07 (d, $J = 11.0$ Hz, 1H, $1_a'\text{-H}_2$), 2.46-2.39 (m, 2H, $1_b'\text{-H}_2$, $1''\text{-H}$), 2.27-2.21 (m, 2H, $5''\text{-H}_2$), 1.98-1.81 (m, 2H, $7''\text{-H}_2$), 1.71-1.63 (m, 2H, $6''\text{-H}_2$), 1.47-1.38 (m, 1H, $8_a''\text{-H}_2$), 1.11-1.02 (m, 1H, $8_b''\text{-H}_2$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 166.99 (CO_{ester}), 145.31 (C-4), 129.82 (C-2, C-6), 129.17 (C-3, C-5), 128.29 (C-1), 119.31 (t, $J = 239.3$ Hz, C-2'), 109.71 (t, $J = 11.2$ Hz, C-4'), 85.01 (t, $J = 47.4, 42.1$ Hz, C-3'), 57.98 (t, $J = 24.3$ Hz, C-1'), 52.02 ($\text{CH}_{3,\text{ester}}$), 34.33 (dd, $J = 5.1, 1.2$ Hz, C-1'), 32.45 (d, $J = 2.1$ Hz, C-8'), 30.71 (d, $J = 5.3$ Hz, C-5'), 27.85 (C-7'), 20.25 (C-6').

$^{19}\text{F-NMR}$ (376 MHz, CDCl_3): δ -96.16 (d, $J = 270.2$ Hz, 1F), -123.77 (dd, $J = 245.5, 23.4$ Hz, 1F).

MS (APCI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{17}\text{H}_{18}\text{O}_2\text{F}_2+\text{Na}]^+$ 315.12, found 315.12.

10.6.9 4-[(2,2-Difluorocyclooct-3-yn-1-yl)methyl]benzoic acid **28**



In a round-bottom flask cyclooctyne methyl ester **225** (520 mg, 180 μ mol) was dissolved in MeOH (1 mL), water (1 mL) and dioxane (4 mL) followed by the addition of LiOH (86 mg, 3.61 mmol). The reaction mixture was heated to 55 $^{\circ}\text{C}$ for 3 h, cooled to rt and diluted with 1 M HCl until the pH of the solution was <2. The solution was extracted with DCM (4 \times 5 mL). The combined organic layers were washed (1:1, 1 M HCl/brine, 5.0 mL), dried over MgSO_4 , filtered through a glass frit and concentrated under reduced pressure. The crude product was purified by flash chromatography (Hex/EtOAc, 8:1 with 1% AcOH) to afford DIFO **28** (25 mg, 89 μ mol) as a white solid.

Yield: 49%.

R_f : 0.45 (Hex/EtOAc, 4:1 with 1% AcOH).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): 7.96 (dt, $J = 8.4, 1.9$ Hz, 2H, 2-H, 6-H), 7.32 (d, $J = 8.1$ Hz, 2H, 3-H, 5-H), 3.11 (d, $J = 10.8$ Hz, 1H, $1''\text{-H}$), 2.61-2.49 (m, 2H, $1'\text{-H}_2$), 2.43-2.25 (m, 2H, $5''\text{-H}_2$), 2.06-1.89 (m, 2H, $6''\text{-H}_2$), 1.86-1.69 (m, 2H, $7''\text{-H}_2$), 1.54-1.45 (m, 1H, $8_a''\text{-H}_2$), 1.22-1.14 (m, 1H, $8_b''\text{-H}_2$).

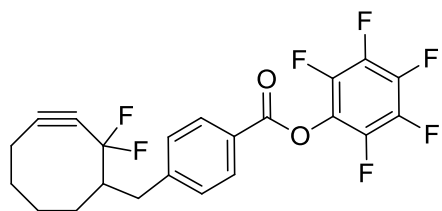
$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 169.8 (CO_{ester}), 146.69 (C-4), 131.02 (C-3, C-5), 130.30 (C-2, C-6), 130.06 (C-1), 120.82 (dd, $J = 240.2, 236.0$ Hz, C-2'), 111.07 (t, $J = 11.3$ Hz, C-4'), 85.86

(dd, $J = 47.2, 42.0$ Hz, C-3'') 59.22 (t, $J = 24.2$ Hz, C-1'), 35 (dd, $J = 5.1, 1.2$ Hz, C-1'), 33.60 (d, $J = 2.3$ Hz, C-5''), 31.92 (d, $J = 4.1$ Hz, C-8'') 28.88 (C-6''), 20.62 (C-7').

^{19}F -NMR (376 MHz, CD_3OD): δ -95.16 (d, $J = 271.3$ Hz, 1F), -102.07 (d, $J = 270.1$ Hz, 1F).

MS (APCI): m/z $[\text{M}-\text{H}]^-$ calcd for $[\text{C}_{16}\text{H}_{16}\text{F}_2\text{O}_2-\text{H}]^-$ 207.03, found 207.03.

10.6.10 2,3,4,5,6-Pentafluorocyclohexyl-4-[(2,2-difluorocyclooct-3-yn-1-yl)methyl]benzoate **227**

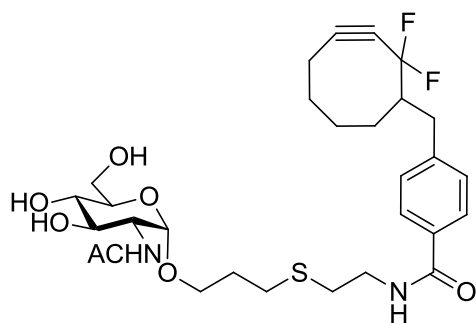


In a dried three neck flask, DIFO **28** (25 mg, 890 μmol) and DIPEA (12 mg, 92 μmol) were dissolved in DCM (5 mL) under nitrogen atmosphere. The solution was cooled to 0 $^\circ\text{C}$ along with continuous stirring, followed by slow addition of pentafluorophenyl trifluoroacetate **226** (290 mg, 1.12 mmol) and the solution was allowed to warm to rt. After 2 h, the solvent was evaporated and the residue was subjected to the flash chromatography, eluting with Hex/EtOAc (1:1) to afford **227** (30 mg, 67 μmol) as a white powder.

Yield: 75%.

R_f : 0.56 (Hex/EtOAc, 5:1).

10.6.11 *N*-{2-[(3-[(2-(Acetylamino)-2-deoxyhexopyranosyl]oxy)propyl)sulfanyl]ethyl}-4-[(2,2-difluorocyclooct-3-yn-1-yl)methyl]benzamide **229**



In a dried three neck flask, the activated ester of DIFO **227** (10 mg, 22 μmol) was dissolved in anhyd. DMF (3 mL) under inert conditions. GlcNAc cystamine **228** (7 mg, 25 μmol) was added to it followed by the addition of DIPEA (4.24 μL , 3.22 mg, 25 μmol) and stirred overnight. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography twice, eluting with DCM/MeOH (10:1 to 6:1) to afford **229** (9 mg, 15 μmol).

Yield: 72%.

R_f : 0.23 (DCM/MeOH, 10:1).

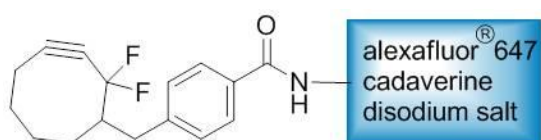
^1H -NMR (400 MHz, CD_3OD): δ 7.77 (d, $J = 8.3$ Hz, 2H, 2-H, 6-H), 7.32 (d, $J = 8.1$ Hz, 2H, 3-H, 5-H), 4.80 (d, $J = 3.6$ Hz, 1H, 1''''''-H_a), 3.89 (dd, $J = 10.7, 3.6$ Hz, 1H, 2''''''-H), 3.82-3.78 (m, 2H, 3''''''-H, 5''''''-H), 3.71-3.65 (m, 2H, 1'''-H₂), 3.61-3.54 (m, 3H, 6''''''-H_a, 3''''''-H₂), 3.50 (dt, $J = 9.9, 5.8$ Hz, 1H, 6''''''-H_b), 3.39-3.33 (m, 1H, 4''''''-H), 2.74 (dt, $J = 10.4, 7.3$ Hz, 4H,

2'''-H₂, 1''''-H₂), 2.48-2.59 (m, 2H, 5''-H₂), 2.28-2.43 (m, 1H, 1_a'-H₂), 2.12-2.00 (m, 1H, 1_b'-H₂), 1.98 (s, 3H, COCH₃), 1.94-1.86 (m, 3H, 2''''-H₂, 1''-H), 1.83-1.79 (m, 1H, 6_a''-H₂), 1.79-1.69 (m, 1H, 6_b''-H₂), 1.57-1.45 (m, 2H, 8''-H₂), 1.36-1.28 (m, 1H, 7_a''-H₂), 1.22-1.12 (m, 1H, 7_b''-H₂).

¹³C-NMR (100 MHz, CD₃OD): δ 175.04, 171.43 (2 × CO), 146.59 (C-4), 135.02 (C-2), 131.77, 131.29 (C-3, C-5), 130.58, 129.94 (C-2, C-4), 119.51 (C-3''), 114.26 (C-3''), 99.98 (C-1'''''), 75.24, 74.16, 73.79 (C-3'', C-2''''', C-3''''', C-4''''', C-5'''''), 68.65 (C-3'''''), 64.11 (C-6'''''), 56.91 (C-1'), 42.13 (C-1''), 36.48, 36.42 (C-2''', C-1'''''), 34.96 (C-1'), 33.32, 33.27, 31.97, 30.68, 30.29 (C-5'', C-6'', C-7'', C-8'', C-2'''''), 24.01 (OCH₃).

MS (ESI): m/z [M+Na]⁺ calcd for [C₂₈H₃₇O₂F₂N₂S+Na]⁺ 567.23, found 567.23.

10.6.12 Alexafluor[®]-4-[(2,2-difluorocyclooct-3-yn-1-yl)methyl]benzamide 231



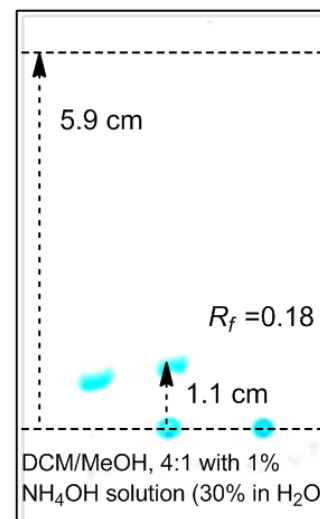
A dried three neck flask was charged with activated ester of DIFO **227** (5 mg, 11 μmol) and anhyd. DMF (2 mL) under inert conditions. Alexafluor

cadaverine disodium salt **230** (1 mg, 1 μmol) was added followed by DIPEA (1.7 μL, 1.3 mg, 10 μmol) and stirred overnight. The solvent was evaporated under vacuum and the residue was purified by flash chromatography twice, eluting with DCM/MeOH (10:1 to 6:1) with 1% NH₄OH solution (30%) to afford the conjugate of DIFO **231** (1 mg, 780 μmol) as a blue solid.

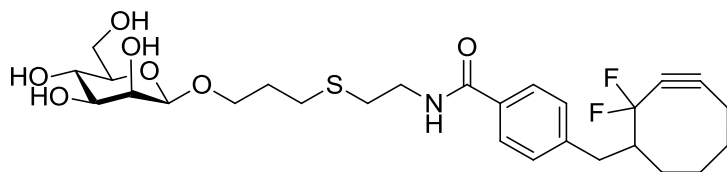
Yield: 78%.

R_f: 0.18 (DCM/MeOH, 3:1 with 1% NH₄OH solution (30%)).

Note: The amount (~ 1 mg) was too less for NMR analysis. Mass analysis by MALDI, ESI, EI and APCI did not work too. The only proof for the product is TLC, on which the spots of the educt and product elute differently. Product due to less polarity has more *R_f* value than Alexafluor[®] 647 cadaverine disodium salt, which is on the bottom in the shown TLC plate.



10.6.13 4-[(2,2-Difluorocyclooct-3-yn-1-yl)methyl]-N-(2-{[3-(hexopyranosyloxy)propyl]sulfanyl}ethyl)benzamide **233**



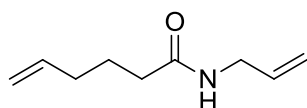
In a dried three neck flask, the activated ester of DIFO **227** (10 mg, 22 μ mol) was dissolved in anhyd. DMF (3 mL). Mannose cystamine **231** (8 mg, 25 μ mol) was added followed by DIPEA (4 μ L, 3.2 mg, 25 μ mol) and stirred overnight. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography twice, eluting with DCM/MeOH (8:1 to 5:1) to afford DIFO conjugate **233** (5 mg, 9 μ mol) as a white powder.

10.7 Azacyclooctynon

10.7.1 General procedure (G) for the synthesis of amides

EDCI (13.00 mmol) was added to a chilled solution of the acid (8.73 mmol), allylamine (8.72 mmol) and DMAP (2.27 mmol) in DCM (700 mL) at -7°C . The solution was stirred at 0°C for 1 h and then at rt for 5-12 h. The reaction mixture was washed with 0.1 N HCl (2×350 mL) and water (1×350 mL). The organic phase was dried over MgSO_4 , filtered, evaporated the solvent and purified by silica column to get the desired product.

10.7.1.1 N-(Prop-2-en-1-yl)hex-5-enamide **240**



According to the general procedure (G), EDCI **248** (2.49 g, 13.01 mmol) was treated with 5-hexenoic acid **238** (1.05 g, 8.72 mmol), allylamine **239** (500 mg, 8.72 mmol) and DMAP (277 mg, 2.27 mmol) in DCM (700 mL). Flash column chromatography of the crude product afforded **240** (1.31 g, 8.56 mmol) as a colorless thick oil.

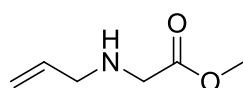
Yield: 89%.

R_f : 0.53 ($\text{Et}_2\text{O}/\text{Pent}$, 1:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 5.84-5.59 (m, 2H, 5-H, 2'-H), 4.92-4.87 (m, 4H, 6-H₂, 3'-H₂), 3.83-3.78 (m, 2H, 1'-H₂), 2.13 (t, $J = 7.8$ Hz, 2H, 2-H₂), 2.02 (tt, $J = 8.3, 1.3$ Hz, 2H, 4-H₂), 1.73-1.63 (m, 2H, 3-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 172.61 (C-1), 137.8 (C-5), 134.33 (C-2'), 116.25 (C-3'), 115.24 (C-6), 41.8 (C-1'), 35.81 (C-2), 33.13 (C-4), 24.70 (C-3).

10.7.2 Methyl(prop-2-en-1-ylamino)acetate **246**



Methyl-2-bromacetate **247** (7.00 g, 46.14 mmol) was slowly added to a vigorously stirred suspension of NaI (489 mg, 3.28 mmol) in allylamine **239** (13.11 g, 230.23 mmol). The mixture was stirred at 50 °C for 1.5 h, cooled to 5 °C and K₂CO₃ (13.00 g, 94.25 mmol) was added to it. The solution was kept at 5 °C for 72 h and water was added to dissolve K₂CO₃ taking care that pH should not fall below 10. The solution was extracted with Et₂O, dried over MgSO₄, filtered and concentrated in mild vacuum. The product was purified by flash chromatography, eluting with Et₂O/Pent (1:1) to afford **246** (3.74 g, 28.92 mmol) as a clear oil.

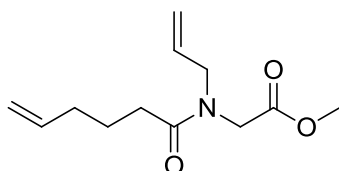
Yield: 63%.

R_f: 0.66 (Et₂O/Pent, 1:1).

¹H-NMR (400 MHz, CDCl₃): δ 5.82 (ddt, *J* = 17.2, 10.2, 1.7 Hz, 1H, 2'-H), 5.16 (qd, *J* = 17.1, 1.6 Hz, 1H, 3'_{trans}-H₂), 5.11 (qd, *J* = 10.1, 1.3 Hz, 1H, 3'_{cis}-H₂), 3.69 (s, 3H, CH₃), 3.38 (s, 2H, 2-H₂), 3.23 (td, *J* = 6.0, 1.4 Hz, 2H, 1'-H₂).

¹³C-NMR (100 MHz, CDCl₃): δ 172.85 (C-1), 136.04 (C-2'), 116.51 (C-3'), 51.78 (CH₃), 51.61 (C-2), 49.72 (C-1').

10.7.3 Methyl[hex-5-enoyl(prop-2-en-1-yl)amino]acetate **245**



General procedure **G** was followed for the synthesis of olefin **245**, using 5-hexenoic acid **238** (200 mg, 1.75 mmol), allylamino acetate **246** (226 mg, 1.75 mmol), DMAP (868 mg, 7.11 mmol) and EDCI **248** (679 mg, 3.55 mmol). The resulting colorless oil was flashed over silica gel column chromatography, eluting with Et₂O/Pent (1:1) to afford **245** (300 mg, 1.33 mmol) as a colorless oil.

Yield: 77%.

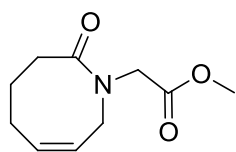
R_f: 0.47 (Et₂O/Pent, 1:1).

¹H-NMR (400 MHz, CDCl₃): δ 5.77-5.73 (m, 2H, 5'-H, 2''-H), 5.19-5.13 (m, 2H, 3''_{trans}-H₂, 6'_{trans} H₂), 4.99-4.94 (m, 2H, 3''_{cis}-H₂, 6'_{cis}-H₂), 4.03 (s, 2H, 2-H₂), 3.99-3.94 (m, 2H, 1''-H₂), 3.69 (s, 3H, CH₃), 2.35 (t, *J* = 7.5 Hz, 1H, 2_a'-H₂), 2.22 (t, *J* = 7.6 Hz, 1H, 2_b'-H₂), 2.08 (q, *J* = 7.1 Hz, 2H, 4'-H₂), 1.74 (quin, *J* = 7.4 Hz, 2H, 3'-H₂).

¹³C-NMR (100 MHz, CDCl₃): δ 173.51 (C-1'), 170.01 (C-1), 138.07 (C-5'), 132.67 (C-2''), 117.33 (C-6'), 115.20 (C-3''), 52.02 (CH₃), 51.31 (C-2), 47.08 (C-1'), 33.14 (C-2'), 31.81 (C-4'), 24.12 (C-3').

MS (ESI): *m/z* [M+H]⁺ calcd for [C₁₂H₂₂NO₃+H]⁺ 226.28, found 226.14.

10.7.4 Methyl(2-oxo-3,4,5,8-tetrahydroazocin-1(2H)-yl)acetate **244**



In a dry three neck flask, Methyl 2-(*N*-allylhex-5-enamido)acetate **245** (1.24 g, 5.36 mmol) was dissolved in dry DCM (250 mL) attaining the concentration of 0.02 M under nitrogen atmosphere followed by the addition of Grubb's catalyst (I) (450 mg, 540 μ mol) and the reaction mixture was refluxed overnight (12 h). After assuring the completion of the reaction (controlled by TLC), the solvent was evaporated under reduced pressure and the resulting dark brown residue was subjected to column chromatography, using Et₂O as eluent. The column was repeated thrice to get rid of the catalyst and collected **244** (1.04 g, 3.55 mmol) as a clear oil.

Yield: 71%.

R_f: 0.43 (Et₂O).

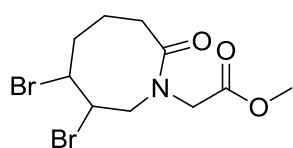
¹H-NMR (400 MHz, CDCl₃): δ 5.71-5.87 (tdd, *J* = 19.5, 7.9, 1.8 Hz, 1H, 6-H), 5.46-5.53 (tdd, *J* = 16.3, 4.6, 1.0 Hz, 1H, 7-H), 4.13 (s, 2H, CH_{2,acetate}), 3.96 (d, *J* = 4.6 Hz, 2H, 8-H₂), 3.66 (s, 3H, OCH₃), 2.53 (t, *J* = 6.5 Hz, 2H, 3-H₂), 2.19 (app. q, *J* = 7.9 Hz, 2H, 5-H₂), 1.77 (quin, *J* = 6.4 Hz, 2H, 4-H₂).

¹³C-NMR (100 MHz, CDCl₃): δ 175.22 (C-2), 170.02 (CO_{acetate}), 130.48 (C-6), 127.35 (C-7), 52.02 (CH_{2,acetate}), 50.96 (C-8), 49.08 (OCH₃), 32.72 (C-3), 26.09 (C-4), 23.98 (C-5).

MS (ESI): *m/z* [M+H]⁺ calcd for [C₁₀H₁₅NO₃+H]⁺ 198.24, found 198.12.

[M+Na]⁺ calcd for [C₁₀H₁₅NO₃+Na]⁺ 220.08, found 220.22.

10.7.5 Methyl (6,7-dibromo-2-oxoazocan-1-yl)acetate **243**



Cyclooctene **244** (600 mg, 3.04 mmol) was dissolved in anhyd. DCM (5 mL) followed by the addition of bromine (556 mg, 3.42 mmol) at -40 °C. The reaction mixture was stirred at this temperature for further 0.5 h. On the completion of the reaction, aqueous solution of Na₂S₂O₃ (0.1 N) was added till the brown color was diminished. The product was extracted with DCM, dried the combined fractions over MgSO₄ and evaporated the solvent under reduced pressure. The residue was subjected to column chromatography, eluting with Et₂O to afford bromide **243** (1.08 g, 2.91 mmol) as a light brown solid.

Yield: 95%.

R_f: 0.34 (Et₂O).

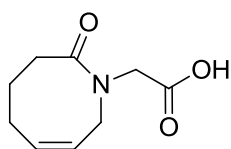
¹H-NMR (400 MHz, CDCl₃): δ 4.72 (dd, *J* = 8.6, 3.8 Hz, 1H, 6-H), 4.61 (app. quin, *J* = 3.4 Hz, 1H, 7-H), 4.22 (br s, 2H, CH_{2,acetate}), 3.70 (s, 3H, OCH₃), 2.76, 2.49 (2 br s, 2H, 8-H₂), 2.34 (t, *J* = 10.7 Hz, 1H, 5_a-H₂), 2.07-2.01 (m, 5H, 3-H₂, 4-H₂, 5_b-H₂).

^{13}C -NMR (100 MHz, CDCl_3): δ 174.31 (C-2), 169.61 ($\text{CO}_{\text{acetate}}$), 54.48 (C-6, C-7), 52.12 (CH_3), 51.84 ($\text{CH}_{2,\text{acetate}}$), 32.40 (C-8), 26.69 (C-5), 25.32 (C-3), 21.02 (C-4).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{10}\text{H}_{15}\text{Br}_2\text{NO}_3+\text{Na}]^+$ 380.03, found 379.93.

m/z $[\text{M}-\text{H}]^-$ calcd for $[\text{C}_{10}\text{H}_{15}\text{Br}_2\text{NO}_3-\text{H}]^-$ 358.07, found 357.95.

10.7.6 (2-Oxo-3,4,5,8-tetrahydroazocin-1(2H)-yl)acetic acid **254**



LDA (1 M) was prepared by the addition of *n*-BuLi (1 mL, 2 M solution in hexane) to the solution of DIPA (250 mg, 2.53 mmol) in dry THF (1 mL) at $-25\text{ }^\circ\text{C}$ in a dry three neck flask under nitrogen atmosphere. In a separate dried three neck flask dibromo cyclooctane **243** (100 mg, 280 μmol) was dissolved in dry THF. The LDA solution (1 mL, 1.00 mmol) was slowly added to the second flask at $-10\text{ }^\circ\text{C}$ and the mixture was allowed to warm to rt and stirred for 3 h. The reaction was quenched by the addition of ice water and concentrated under reduced pressure. The crude product was purified by column chromatography, eluting with EtOAc followed by the gradient of DCM/MeOH (20:1-10:1) to afford the acid **254** (32 mg, 170 μmol) as a colorless oil.

Yield: 62%.

R_f : 0.23 (EtOAc).

^1H -NMR (400 MHz, CD_3OD): δ 5.79-5.73 (m, 1H, 6-H), 5.54 (td, $J = 11.6, 4.7\text{ Hz}$, 1H, 7-H), 3.98 (d, $J = 4.0\text{ Hz}$, 2H, 8- H_2), 3.90 (s, 2H, $\text{CH}_{2,\text{acetate}}$), 2.50 (t, $J = 6.5\text{ Hz}$, 2H, 3- H_2), 2.13 (dd, $J = 12.4, 7.6\text{ Hz}$, 2H, 5- H_2), 1.73-1.68 (m, 2H, 4- H_2).

^{13}C -NMR (100 MHz, CD_3OD): δ 179.13 (C-2), 178.1 (CO_{acid}), 132.82 (C-6), 130.72 (C-7), 53.52 ($\text{CH}_{2,\text{acid}}$), 35.49 (C-8), 28.78 (C-3, C-5), 26.63 (C-4).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_9\text{H}_{13}\text{NO}_3+\text{Na}]^+$ 206.18, found 206.08.

m/z $[\text{M}-\text{H}]^-$ calcd for $[\text{C}_9\text{H}_{13}\text{NO}_3-\text{H}]^-$ 182.19, found 182.09.

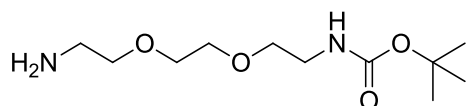
10.8 Dicarboxylic Acid and Aza Crown Ethers

10.8.1 General procedure (H) for the protection of diamines

The solution of Boc anhydride (0.1 equiv.) in dioxane was added dropwise to the solution of diamine dissolved in dioxane over 5 h. After additional stirring for 5-24 h, the solution was concentrated under reduced pressure. The resulting yellow oil was dissolved in water and extracted with DCM. The combined organic extracts were washed with brine, dried over anhyd. Na_2SO_4 and concentrated to afford yellow oil, which was subjected to silica gel column, eluting

with mixture of DCM and MeOH along with 1% NH_4OH solution (30%) to afford the target compounds.

10.8.1.1 *tert*-Butyl{2-[2-(2-aminoethoxy)ethoxy]ethyl}carbamate **266**



Following the general procedure (**H**) Boc anhydride **268** (2.23 g, 10.35 mmol) and ethoxy diamine **267** (9 mL, 60.73 mmol) were stirred overnight in dioxane

(43 mL). The resulting yellow oil was dissolved in water (20 mL) and extracted with DCM (4×25 mL). The crude product was purified by flash chromatography, eluting with DCM/MeOH (30:1) with 1% NH_4OH solution (30%) to afford **266** (2.21 g, 8.87 mmol) as a colorless oil.

Yield: 87%.

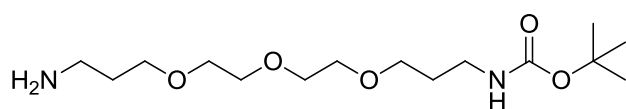
R_f : 1.5 (EtOAc with 1% NH_4OH solution (30%)).

^1H -NMR (400 MHz, CDCl_3): δ 5.18 (br s 1H, NH), 3.58 (s, 4H, $1''\text{-H}_2$, $2''\text{-H}_2$), 3.47, 3.52 (2t, $J = 5.2$ Hz, 4H, $2'\text{-H}_2$, $1'''\text{-H}_2$), 3.28 (dt, $J = 10.4$, 5.3 Hz, 2H, $1'\text{-H}_2$), 2.84 (t, $J = 4.9$ Hz, 2H, $2'''\text{-H}_2$), 1.87 (s, 2H, NH_2), 1.41 (s, 9H, $3 \times \text{CH}_3$).

^{13}C -NMR (100 MHz, CDCl_3): δ 156.09 (CO_{Boc}), 79.19 (C-1), 73.26 (C-1'''), 70.27, 70.23 (C-1'', C-2'', C-2'), 41.68 (C-1'), 40.39 (C-2'''), 28.46 (3C , $3 \times \text{CH}_3$).

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{11}\text{H}_{25}\text{N}_2\text{O}_4+\text{Na}]^+$ 249.18, found 249.18.

10.8.1.2 *tert*-Butyl(3-{2-[2-(3-aminopropoxy)ethoxy]ethoxy}propyl)carbamate **280**



General procedure (**H**) was followed for the treatment Boc anhydride **268** (2.23 g, 10.00 mmol) with bis(3-aminopropyl)

diethylene glycol **276** (9.00 g, 60.11 mmol) in dioxane (43 mL). After 12 h the resulting yellow suspension was dissolved in water (25 mL) and extracted with DCM (4×25 mL). The crude product was purified by flash chromatography, eluting with DCM/MeOH (30:1 to 20:1) with 1% NH_4OH solution (30%) to afford **280** (2.05 g, 6.23 mmol) as a colorless oil.

Yield: 62%.

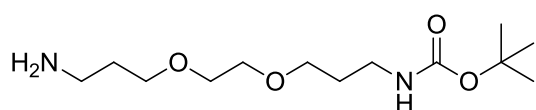
R_f : 0.19 (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

^1H -NMR (400 MHz, CDCl_3): δ 8.5 (s, 1H, NH), 3.64-3.71 (m, 8H, $1''\text{-H}_2$, $2''\text{-H}_2$, $1'''\text{-H}_2$, $2'''\text{-H}_2$), 3.63-3.59 (m, 2H, $3'\text{-H}_2$), 3.53 (t, $J = 6.2$ Hz, 2H, $1''''\text{-H}_2$), 3.13, 3.11 (2t, $J = 6.5$, 6.8 Hz, 4H, $1'\text{-H}_2$, $3''''\text{-H}_2$), 1.96 (quin, $J = 6.1$ Hz, 2H, $2''''\text{-H}_2$), 1.75 (quin, $J = 6.5$ Hz, 2H, $2'\text{-H}_2$), 1.61 (br s, 2H, NH_2), 1.45 (s, 9H, $3 \times \text{CH}_3$).

^{13}C -NMR (100 MHz, CDCl_3): δ 160.33 (CO_{Boc}), 81.73 (C-1), 73.52 (C-1'', C-2''), 73.19 (C-1''', C-2'''), 72.39 (C-3'), 71.84 (C-1''''), 42.08 (C-1'), 40.69 (C-3'''), 35.56 (C-2'), 32.89 (C-2'''), 30.79 (3C, $3 \times \text{CH}_3$).

MS (ESI): m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{15}\text{H}_{32}\text{N}_2\text{O}_5+\text{H}]^+$ 321.24, found 321.24.

10.8.1.3 *tert*-Butyl{3-[2-(3-aminopropoxy)ethoxy]propyl}carbamate **281**



Following the general procedure (**H**), 1,10-diamine **277** (5.00 g, 28.23 mmol) in dioxane (20 mL) was treated with the solution of Boc anhydride **268** (1.13 g, 5.14 mmol, 0.19 equiv.) in dioxane (8 mL). After concentration the residue was dissolved in H_2O (25 mL) and the aqueous layer was extracted with DCM (4×20 mL). The crude product was purified by column chromatography, eluting with DCM/MeOH (20:1 followed by 10:1) with 1% NH_4OH to afford **281** (700 mg, 2.53 mmol) as a colorless oil. Yield: 52%.

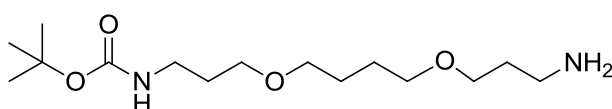
R_f : 0.31, (DCM/MeOH, 5:1 with 1 % NH_4OH .solution (30%)).

^1H -NMR (400 MHz, CDCl_3): δ 5.13 (br s, 1H-NH), 3.50 (s, 4H, 1''-H₂, 2''-H₂), 3.47 (t, $J = 6.0$ Hz, 4H, 3'-H₂, 1'''-H₂), 3.14 (dt, $J = 12.1, 5.9$ Hz, 2H, 1'-H₂), 2.76 (t, $J = 6.6$ Hz, 2H, 3'''-H₂), 2.45 (br s, 2H, NH₂), 1.69 (quin, $J = 6.2$ Hz, 4H, 2'-H₂, 2'''-H₂), 1.37 (s, 9H, $3 \times \text{CH}_3$).

^{13}C -NMR (100 MHz, CDCl_3): δ 158.46 (CO_{Boc}), 81.15 (C-1), 72.53 (C-1'), 72.45 (C-2'), 71.85 (C-3', C-1''), 41.79 (C-1'), 40.80 (C-3''), 35.06 (C-2'), 32.05 (C-2''), 30.81 (3C, $3 \times \text{CH}_3$).

MS (MALDI): m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{13}\text{H}_{28}\text{N}_2\text{O}_4+\text{H}]^+$ 276.21, found 276.21.

10.8.1.4 *tert*-Butyl{3-[4-(3-aminopropoxy)butoxy]propyl}carbamate **282**



General procedure (**H**) was followed for the treatment of 1,12-diaminododecane **278** (10.00 g, 49.02 mmol) in dioxane (20 mL) with the solution of Boc anhydride **268** (2.08 g, 9.43 mmol, 0.19 equiv.) in dioxane (10 mL). After 16 h, the solvent was evaporated, dissolved the residue in H_2O (30 mL) and extracted with DCM (4×50 mL). The crude product was purified by column chromatography, eluting with the gradient of DCM/MeOH (20:1 and 10:1) with 1% NH_4OH solution (30%) to afford **282** (1.45 g, 4.71 mmol) as a clear oil.

Yield: 51%.

R_f : 0.39, (DCM/MeOH, 5:1 with 1 % NH_4OH solution (30%)).

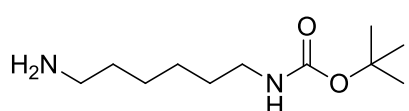
$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 5.04 (br s, 1H, NH), 3.43 (2t, $J = 6.1$ Hz, 4H, $3'\text{-H}_2$, $1'''\text{-H}_2$), 3.39-3.35 (m, 4H, $1''\text{-H}_2$, $4''\text{-H}_2$), 3.15 (dt, $J = 6.2$, 6.1 Hz, 2H, $1'\text{-H}_2$), 2.74 (t, $J = 6.8$ Hz, 2H, $3'''\text{-H}_2$), 1.62-1.73 (2quin, $J = 6.5$, 6.2 Hz, 4H, $2'\text{-H}_2$, $2'''\text{-H}_2$), 1.52-1.62 (quin, $J = 3.2$ Hz, 4H, $2''\text{-H}_2$, $3''\text{-H}_2$), 1.38 (s, 9H, $3 \times \text{CH}_3$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 156.02 (CO_{Boc}), 78.8 (C-1), 70.7 (C-4'), 70.66 (C-1'), 69.09 (C-3'), 68.88 (C-1''), 39.57 (C-1'), 38.66 (C-3''), 33.22 (C-2'), 29.68 (C-2''), 28.37 (3C, $3 \times \text{CH}_3$), 26.40 (C-2'', C-3').

MS (MALDI): m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{15}\text{H}_{32}\text{N}_2\text{O}_4+\text{H}]^+$ 305.243, found 305.29.

MS (MALDI): m/z $[\text{M}+\text{H}+\text{Ca}]^+$ calcd for $[\text{C}_{15}\text{H}_{32}\text{N}_2\text{O}_4+\text{H}+\text{Ca}]^+$ 345.21, found 345.29.

10.8.1.5 *tert*-Butyl(6-aminohexyl)carbamate **283**



Following the general procedure (**H**), the solution of 1,6-diaminohexane **279** (10.00 g, 86.25 mmol) in dioxane (40 mL) was treated with the solution of Boc anhydride **268** (3.73 g, 17.21 mmol, 0.13 equiv.) in 1,4-dioxane (20 mL). After 12 h the solvent was evaporated and the concentrate was dissolved in H_2O (30 mL) followed by extraction with DCM (4×50 mL). The crude product was purified by column chromatography, eluting with a gradient of DCM/MeOH (30:1 and 20:1) and 1% NH_4OH solution (30%) to afford **283** (2.49 g, 11.52 mmol) as a colorless oil.

Yield: 67%.

R_f : 0.27 (DCM/MeOH, 8:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, MeOD): δ 3.03 (t, $J = 7.0$ Hz, 2H, $1'\text{-H}_2$), 2.63 (t, $J = 6.9$ Hz, 2H, $6'\text{-H}_2$), 1.60-1.48 (m, 4H, $2'\text{-H}_2$, $5'\text{-H}_2$), 1.39 (s, 9H, $3 \times \text{CH}_3$), 1.25-1.21 (m, 4H, $3'\text{-H}_2$, $4'\text{-H}_2$).

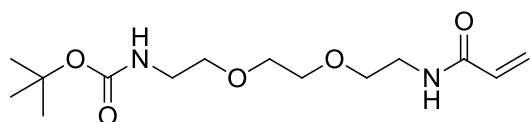
$^{13}\text{C-NMR}$ (100 MHz, MeOD): δ 155.98 (CO_{Boc}), 78.83 (C-1), 42.15 (C-1'), 40.55 (C-6'), 33.71 (C-2'), 30.01 (C-5'), 29.33 (C-3'), 29.19 (C-4'), 28.38 (3C, $3 \times \text{CH}_3$).

MS (MALDI): $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{11}\text{H}_{24}\text{N}_2\text{O}_2+\text{H}]^+$ 217.19, found 217.19.

10.8.2 General procedure (I) for the synthesis of acryloyl amides

Acryloyl chloride was slowly added to a cold (0°C) solution of mono-protected amine and DIPEA in DCM under nitrogen atmosphere. The reaction mixture was allowed to warm to rt and stirred for further 2-4 h. The solvent was evaporated under reduced pressure and the concentrate was subjected to silica gel column chromatography to afford the desired acryloyl amide.

10.8.2.1 *tert*-Butyl(2-{2-[2-(acryloylamino)ethoxy]ethoxy}ethyl)carbamate **264**



The synthesis of acryloyl carbamate **264** was achieved by following general procedure (I), using carbamate **266** (100 mg, 400 μ mol), DIPEA (103 mg, 850 μ mol)

and acryloyl chloride **289** (71 mg, 810 μ mol) in DCM (6 mL). The product was purified by column chromatography, eluting with DCM/MeOH (20:1) and 1% NH_4OH solution (30%) to afford the acryloyl carbamate **264** (217 mg, 390 μ mol) as a light yellow solid.

Yield: 93%.

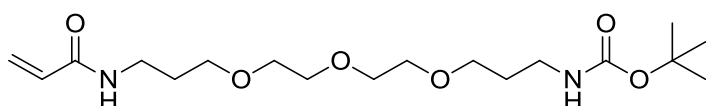
R_f : 0.23 (EtOAc).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 6.27 (dd, $J = 17.1, 9.2$ Hz, 1H, $3_{\text{trans}}^{\text{''''}}\text{-H}_2$), 6.21 (dd, $J = 17.2, 2.9$ Hz, 1H, $3_{\text{cis}}^{\text{''''}}\text{-H}_2$), 5.65 (dd, $J = 9.2, 2.8$ Hz, 1H, $2^{\text{''''}}\text{-H}$), 3.62 (s, 4H, $1^{\text{''}}\text{-H}_2, 2^{\text{''}}\text{-H}_2$), 3.58 (t, $J = 5.4$ Hz, 2H, $1^{\text{'''}}\text{-H}_2$), 3.51 (t, $J = 5.7$ Hz, 2H, $2^{\text{''}}\text{-H}_2$), 3.45 (t, $J = 5.5$ Hz, 2H, $2^{\text{'''}}\text{-H}_2$), 3.22 (t, $J = 5.6$ Hz, 2H, $1^{\text{'}}\text{-H}_2$), 1.44 (s, 9H, $3 \times \text{CH}_3$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 165.73 ($\text{C-1}^{\text{''''}}$), 156.03 (CO_{Boc}), 130.93 ($\text{C-2}^{\text{''''}}$), 126.13 ($\text{C-3}^{\text{''''}}$), 79.22 (C-1), 70.21 ($\text{C-2}^{\text{'}}$), 70.09 ($\text{C-1}^{\text{'}}$, $\text{C-1}^{\text{''}}$), 69.68 ($\text{C-2}^{\text{'}}$), 40.27 ($\text{C-1}^{\text{'}}$), 39.23 ($\text{C-2}^{\text{'}}$), 28.34 (3C , $3 \times \text{CH}_3$).

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_5+\text{Na}]^+$ 325.17, found 325.18.

10.8.2.2 *tert*-Butyl[3-(2-{2-[3-(acryloylamino)propoxy]ethoxy}ethoxy)propyl] carbamate **294**



Following the general procedure (I), acryloylchloride **289** (563 mg,

6.26 mmol) was treated with mono-protected amine **280** (1.00 g, 3.13 mmol) and DIPEA (808 mg, 6.26 mmol) in anhyd. DCM (45 mL). The crude product was purified by silica gel column chromatography, eluting with DCM/MeOH/ NH_4OH (10:1:0.1) to afford **294** (950 mg, 2.54 mmol) as a light brown solid.

Yield: 81%.

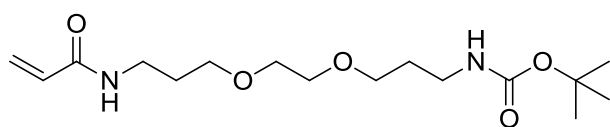
R_f : 0.32 (DCM/MeOH, 20:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 6.25 (dd, $J = 17.0, 1.7$ Hz, 1H, $3_{\text{cis}}^{\text{''''''}}\text{-H}_2$), 6.11 (dd, $J = 17.1, 10.2$ Hz, 1H, $3_{\text{trans}}^{\text{''''''}}\text{-H}_2$), 5.58 (dd, $J = 10.2, 1.7$ Hz, 1H, $2^{\text{''''''}}\text{-H}$), 3.64-3.54 (m, 8H, $1^{\text{''}}\text{-H}_2, 2^{\text{''}}\text{-H}_2, 1^{\text{'''}}\text{-H}_2, 2^{\text{'''}}\text{-H}_2$), 3.57 (t, $J = 5.6$ Hz, 2H, $1^{\text{''''}}\text{-H}_2$), 3.52 (t, $J = 6.0$ Hz, 2H, $3^{\text{'}}\text{-H}_2$), 3.45 (dt, $J = 12.3, 5.7$ Hz, 2H, $3^{\text{''''}}\text{-H}_2$), 3.21 (dt, $J = 12.2, 6.0$ Hz, 2H, $1^{\text{'}}\text{-H}_2$), 1.76 (quin, $J = 5.9$ Hz, 2H, $2^{\text{''''}}\text{-H}_2$), 1.72 (quin, $J = 6.3$ Hz, 2H, $2^{\text{'}}\text{-H}_2$), 1.43 (s, 9H, $3 \times \text{CH}_3$).

^{13}C -NMR (100 MHz, CDCl_3): δ 165.57 (C-1'''''), 156.07 (CO_{Boc}), 131.35 (C-2'''''), 125.61 (C-3'''''), 78.95 (C-1), 70.59-70.37 (C-2'', C-1'''), 70.17 (C-2'''), 70.03 (C-1''), 69.49 (C-1''', C-3'), 38.46 (C-3'''), 38.10 (C-1'), 29.68 (C-2'''), 28.70 (C-2'), 28.43 (3C, 3 \times CH_3).

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{18}\text{H}_{34}\text{N}_2\text{O}_6+\text{Na}]^+$ 397.23, found 397.23.

10.8.2.3 *tert*-Butyl(3-{2-[3-(acryloylamino)propoxy]ethoxy}propyl)carbamate **295**



General procedure (I) was followed for the treatment of acryloylchloride **289** (423 mg, 4.69 mmol), carbamate **281** (650 mg,

2.35 mmol) and DIPEA (607 mg, 4.77 mmol) in anhyd. DCM (35 mL). After 6 h the mixture was concentrated and purified by silica gel column chromatography, eluting with DCM/MeOH/ NH_4OH (10:1:0.1) to afford **295** (800 mg, 2.29 mmol) as a white solid.

Yield: 97%.

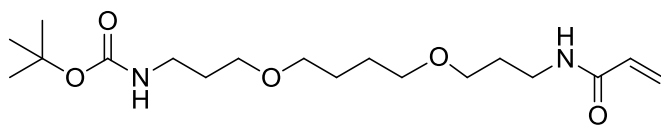
R_f : 0.47 (DCM/MeOH, 20:1 with 1% NH_4OH solution (30%)).

^1H -NMR (400 MHz, CD_3OD): δ 8.1 (br s, 2H, 2 \times NH), 6.26-6.18 (m, 2H, 3''''-H₂), 5.66 (dd, J = 8.2, 3.8 Hz, 1H, 2''''-H), 3.60 (s, 4H, 1''-H₂, 2''-H₂), 3.56-3.51 (m, 2H, 3'-H₂), 3.53 (t, J = 6.2 Hz, 2H, 1'''-H₂), 3.39-3.31 (m, 2H, 3'''-H₂), 3.14 (t, J = 6.8 Hz, 2H, 1'-H₂), 1.82 (quin, J = 6.4 Hz, 2H, 2'''-H₂), 1.74 (quin, J = 6.5 Hz, 2H, 2'-H₂), 1.45 (s, 9H, 3 \times CH_3).

^{13}C -NMR (100 MHz, CD_3OD): δ 169.75 (C-1'''''), 160.13 (CO_{Boc}), 133.89 (C-2'''''), 128.20 (C-3'''''), 81.54 (C-1), 72.97 (C-1'', C-2''), 71.64 (C-3', C-1'''), 40.45 (C-3'''), 39.59 (C-1'), 32.67 (C-2'''), 32.12 (C-2'), 30.56 (3C, 3 \times CH_3).

MS (MALDI): m/z $[\text{M}+\text{H}-\text{Boc}]^+$ calcd for $[\text{C}_{16}\text{H}_{30}\text{N}_2\text{O}_5+\text{H}-\text{Boc}]^+$ 231.17, found 231.25.

10.8.2.4 *tert*-Butyl(3-{4-[3-(acryloylamino)propoxy]butoxy}propyl)carbamate **296**



Following the general procedure (I), acryloylchloride **289** (814 mg, 9.04 mmol) was treated with carbamate **282** (1.41 g,

4.53 mmol) and DIPEA (1.16 g, 8.99 mmol) in anhyd. DCM (70 mL) at rt for 5 h. The crude product was purified by silica gel column chromatography, eluting with DCM/MeOH (10:1) with 1% NH_4OH solution (30%) to afford **296** (1.42 g, 3.91 mmol) as a clear oil.

Yield: 87%.

R_f : 0.28 (DCM/MeOH, 20:1 with 1% NH_4OH solution (30%)).

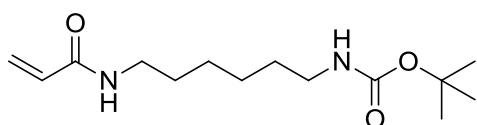
^1H -NMR (400 MHz, CD_3OD): δ 6.23 (d, J = 8.3 Hz, 1H, 3_{trans}''''-H₂), 6.22 (d, J = 3.6 Hz, 1H, 3_{cis}''''-H₂), 5.65 (dd, J = 8.3, 3.5 Hz, 1H, 2''''-H), 3.51-3.46 (m, 8H, 3'-H₂, 1''-H₂, 4''-H₂,

1''''-H₂), 3.35 (t, J = 6.6 Hz, 2H, 3'''-H₂), 3.14 (t, J = 6.8 Hz, 2H, 1'-H₂), 1.81 (quin, J = 6.6 Hz, 2H, 2'''-H₂), 1.73 (quin, J = 8.7 Hz, 2H, 2'-H₂), 1.68-1.60 (m, 4H, 2''-H₂, 3''-H₂), 1.45 (s, 9H, 3 × CH₃).

¹³C-NMR (100 MHz, CD₃OD): δ 169.05 (C-1''''), 159.40 (CO_{Boc}), 133.13 (C-2''''), 127.44 (C-3''''), 80.81 (C-1), 72.83 (C-4'), 72.71 (C-1'), 70.51 (C-3'), 70.42 (C-1'), 39.87 (C-3'), 38.88 (C-1'), 31.99 (C-2''), 31.49 (C-2'), 29.80 (3C, 3 × CH₃), 28.52 (2C, C-2'', C-3').

MS (ESI): m/z [M+H]⁺ calcd for [C₁₈H₃₄N₂O₅+H]⁺ 259.24, found 259.22.

10.8.2.5 *tert*-Butyl[6-(acryloylamino)hexyl]carbamate **297**



Following the general procedure (**I**), carbamate **283** (2.49 g, 11.51 mmol), acryloylchloride **289** (2.08 g, 23.00 mmol, 2 equiv.) and DIPEA (2.97 g, 23.02 mmol

2 equiv.) were treated in dry anhyd. DCM (150 mL). The crude product was purified by flash chromatography, eluting with DCM/MeOH (30:1) and 1% NH₄OH solution (30%) to afford **297** (2.95 g, 10.92 mmol) as a colorless thick oil, which was solidified at rt.

Yield: 95%.

R_f : 0.34 (DCM/MeOH, 25:1 with 1% NH₄OH solution (30%)).

¹H-NMR (400 MHz, CD₃OD): δ 6.23 (d, J = 8.4 Hz, 1H, 3_{trans}'-H₂), 6.22 (d, J = 3.5 Hz, 1H, 3_{cis}'-H₂), 5.65 (dd, J = 8.5, 3.6 Hz, 1H, 2''-H), 3.26 (t, J = 7.1 Hz, 2H, 6'-H₂), 3.04 (t, J = 7.0 Hz, 2H, 1'-H₂), 1.55 (quin, J = 7.0 Hz, 2H, 5'-H₂), 1.49 (quin, J = 6.7 Hz, 2H, 2'-H₂) 1.45 (s, 9H, 3 × CH₃), 1.39-1.34 (m, 4H, 3'-H₂, 4'-H₂).

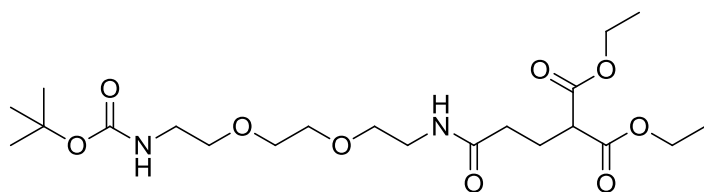
¹³C-NMR (100 MHz, CD₃OD): δ 169.66 (C-1'), 160.15 (CO_{Boc}), 133.75 (C-2'), 128.01 (C-3'), 81.37 (C-1), 42.84 (C-6'), 41.94 (C-1'), 32.48 (C-5'), 31.88 (C-2'), 30.42 (3C, 3 × CH₃), 29.26 (C-3'), 29.08 (C-4').

MS (ESI): m/z [M+Na]⁺ calcd for [C₁₄H₂₆N₂O₃+Na]⁺ 293.17, found 293.17.

10.8.3 General procedure (J) for domino Michael-addition-cyclization reaction

To a dried three neck flask sodium metal was added in alcohol (MeOH, EtOH) under nitrogen atmosphere to have sodium alkoxide solution. After the completion of the reaction, malonic ester was added followed by the addition of the solution of acryloyl carbamate in alcohol. The solution was stirred for 5-24 h and neutralized with glacial acetic acid. Light yellow semi solid obtained after the distillation of the solvent was dissolved in CHCl₃ and filtered. The filtrate was washed with brine and dried over MgSO₄, filtered and evaporated under reduced pressure. The viscous oil was purified by flash column chromatography to afford the pure product.

10.8.3.1 Dimethyl(3-{[2-(2-{2-[(*tert*-butoxycarbonyl)amino]ethoxy}ethoxy)ethyl] amino}-3-oxopropyl)malonate **263**



Following the general procedure (**J**), sodium metal (195 mg, 8.46 mmol) was treated with EtOH (12 mL) followed by the addition of diethyl malonate (1.42 g,

8.46 mmol) and the solution of acryloyl carbamate **264** (1.28 g, 4.23 mmol) in abs EtOH (12 mL). After 12 h, the crude product was purified by silica gel column chromatography, eluting with DCM/MeOH (12:1) and 1% NH₄OH solution (30%) to afford **263** (1.35 g, 3.22 mmol) as a light yellow thick oil.

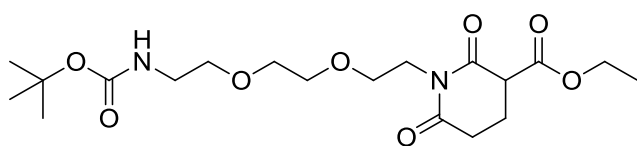
Yield: 75%.

R_f: 0.31 (DCM/MeOH, 10:1 with 1% NH₄OH solution (30%)).

¹H-NMR (400 MHz, CDCl₃): δ 4.06 (q, *J* = 7.1 Hz, 2H, CH_{2,ester}), 3.99 (q, *J* = 7.1 Hz, 2H, CH_{2,ester}), 3.51-4.46 (m, 5H, 1'''-H₂, 2'''-H₂, 2-H), 3.45-3.41 (m, 4H, 1''''-H₂, 2''-H₂), 3.35 (t, *J* = 5.4 Hz, 2H, 2''''-H₂), 3.16 (dt, *J* = 10.3, 5.1 Hz, 2H, 1''-H₂), 2.71 (dd, *J* = 8.3, 5.3 Hz, 1H, 2_a'-H₂), 2.59 (dd, *J* = 7.8, 5.3 Hz, 1H, 2_b'-H₂), 2.25-2.19 (m, 1H, 1_a'-H₂), 2.13-2.07 (m, 1H, 1_b'-H₂), 1.37 (s, 9H, 3 × CH_{3,Boc}), 1.24-1.19 (m, 6H, 2 × CH_{3,malonate}).

¹³C-NMR (100 MHz, CDCl₃): δ 168.83, 168.68 (2 × CO_{malonate}), 166.68 (C-3'), 156.09 (CO_{Boc}), 79.19 (C_{qua,Boc}), 70.38 (C-2'''), 70.31 (C-1'''), 69.99 (C-1'''), 67.64 (C-2''), 62.11 (CH₂CH_{3,malonate}), 61.54 (CH₂CH_{3,malonate}), 49.21 (C-2), 41.74 (C-1'), 38.76 (C-2'''), 30.47 (C-2'), 28.49 (3 × CH_{3,Boc}), 20.58 (C-1'), 14.12 (2 × CHCH_{3,malonate}).

10.8.3.2 Ethyl-1-(12,12-dimethyl-10-oxo-3,6,11-trioxa-9-azatridec-1-yl)-2,6-dioxopiperidine-3-carboxylate **260**



Following the general procedure (**J**) sodium metal (450 mg, 19.56 mmol) was treated with EtOH (10 mL) followed by the addition of

diethyl malonate (1.42 g, 8.46 mmol) and the solution of acryloyl carbamate **264** (1.01 g, 3.32 mmol) in abs EtOH (10 mL). After 12 h, the solvent was evaporated and thick oil was purified by silica gel column, eluting with DCM/MeOH (10:1) and 1% NH₄OH solution (30%). We obtained two fractions open-chain **263** (887 mg, 950 μmol) and cyclic ester **260** (395 mg, 1.92 mmol) as a light yellow thick oil.

Yield: acyclic (28%) and cyclic (57%).

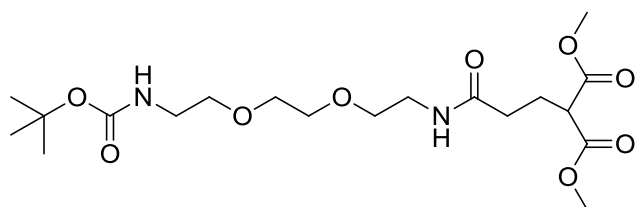
R_f: 0.19 cyclic (DCM/MeOH, 10:1 with 1% NH₄OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 4.20-4.12 (m, 2H, $\text{CH}_{2,\text{ester}}$), 3.95 (dt, $J = 6.0, 1.7$ Hz, 2H, $1'\text{-H}_2$), 3.56 (dd, $J = 7.5, 5.1$ Hz, 1H, 3-H), 3.53-3.46 (m, 6H, $4'\text{-H}_2, 5'\text{-H}_2, 7'\text{-H}_2$), 3.43 (t, $J = 5.2$ Hz, 2H, $2'\text{-H}_2$), 3.21 (2t, $J = 5.2$ Hz, 2H, $8'\text{-H}_2$), 2.67 (dd, $J = 8.2, 5.2$ Hz, 1H, $2_a'\text{-H}_2$), 2.60 (dd, $J = 7.8, 5.3$ Hz, 1H, $2_b'\text{-H}_2$), 2.25-2.16 (m, 1H, $1_a'\text{-H}_2$), 2.13-2.05 (m, 1H, $1_b'\text{-H}_2$), 1.35 (s, 9H, $3 \times \text{CH}_{3,\text{Boc}}$), 1.22 (t, $J = 7.2$ Hz, 3H, $\text{CH}_{2,\text{ester}}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 171.29 (C-6), 168.69 (C-2), 168.54 (CO_{ester}), 155.93 (C-10'), 78.99 (C-12'), 70.24 (C-4'), 70.13 (C-5'), 69.86 (C-7'), 67.47 (C-2'), 61.93 ($\text{CH}_{2,\text{ester}}$), 49.07 (C-3), 40.35 (C-8'), 38.61 (C-1'), 30.33 (C-5), 28.35 ($3\text{C}, 3 \times \text{CH}_{3,\text{Boc}}$), 20.44 (C-4), 14.00 ($\text{CH}_{3,\text{ester}}$).

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{19}\text{H}_{32}\text{N}_2\text{O}_8+\text{Na}]^+$ 439.21, found 439.21.

10.8.3.3 Dimethyl(3-([2-(2-{2-[(*tert*-butoxycarbonyl)amino]ethoxy)ethoxy]ethyl) amino}-3-oxopropyl)malonate **301**



Following the general procedure (**J**), sodium metal (194 mg, 8.46 mmol) was treated with MeOH (12 mL). Dimethyl malonate (1.12 g, 8.46 mmol) and finally acryloyl carbamate

264 (1.28 g, 4.23 mmol) in MeOH (12 mL) were added to the solution. The crude product was purified by column chromatography, eluting with DCM/MeOH (20:1 to 10:1) and 1% NH_4OH solution (30%) to afford **301** (1.08 g, 3.25 mmol) as a white solid.

Yield: 77%.

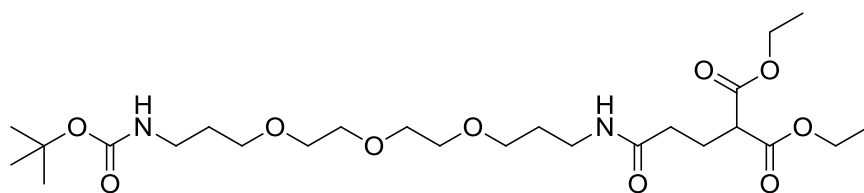
R_f : 0.27 (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 3.66 (s, 3H, $\text{OCH}_{3,\text{malonate}}$), 3.60 (s, 3H, $\text{OCH}_{3,\text{malonate}}$), 3.55 (br s, 4H, $1'''\text{-H}_2, 2'''\text{-H}_2$), 3.49, 3.48 (2t, $J = 5.2, 5.1$ Hz, 4H, $2''\text{-H}_2, 1'''\text{-H}_2$), 3.39 (t, $J = 5.5$ Hz, 2H, $2'''\text{-H}_2$), 3.30 (t, $J = 7.3$ Hz, 1H, 2-H), 3.25 (app t, $J = 4.8$ Hz, 2H, $1''\text{-H}_2$), 2.33 (t, $J = 8.0$ Hz, 2H, $2'\text{-H}_2$), 2.14 (td, $J = 7.4, 1.7$ Hz, 2H, $1'\text{-H}_2$), 1.37 (s, 9H, $3 \times \text{CH}_{3,\text{Boc}}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 173.06 (C-3'), 170.93 ($\text{CO}_{\text{malonate}}$), 167.86 ($\text{CO}_{\text{malonate}}$), 156.02 (CO_{Boc}), 79.09 ($\text{C}_{\text{qua},\text{Boc}}$), 70.19 (C-2'''), 70.14 (C-1'''), 70.09 (C-1'''), 69.47 (C-2''), 52.37 (C-2), 51.57 (OCH_3), 51.37 (OCH_3), 40.26 (C-1'), 39.42 (C-2'''), 31.12 (C-2'), 28.29 ($3 \times \text{CH}_{3,\text{Boc}}$), 24.62 (C-1').

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{19}\text{H}_{34}\text{N}_2\text{O}_9+\text{Na}]^+$ 457.21, found 457.21.

10.8.3.4 Diethyl[3-({3-[2-(2-{3-[(*tert*-butoxycarbonyl)amino]propoxy}ethoxy)ethoxy]propyl}amino)-3-oxo-propyl]malonate **303**



General procedure (**J**) was followed for the treatment of sodium metal (37 mg, 1.62 mmol) with abs EtOH

(5 mL), followed by the addition of diethyl malonate (256 mg, 1.63 mmol) and the solution of acryloyl carbamate **294** (374 mg, 800 μ mol) in abs EtOH (10 mL). After 12 h, the solvent was evaporated and thick oil was purified over silica gel column, eluting with DCM/MeOH (10:1) and 1% NH_4OH solution (30%), which afforded two fractions. The second fraction was acyclic moiety **303** (212 mg, 420 μ mol) in the form of light yellow thick oil.

Yield: 49%.

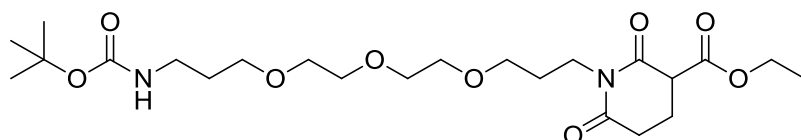
R_f : 0.37 (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 4.29-4.16 (q, $J = 7.1$ Hz, 2H, $\text{CH}_{2,\text{malonate}}$), 4.18 (q, $J = 7.0$ Hz, 2H, $\text{CH}_{2,\text{malonate}}$), 3.88 (t, $J = 7.0$ Hz, 2H, $3''''\text{-H}_2$), 3.47-3.62 (m, 13H, $3''\text{-H}_2$, $1'''\text{-H}_2$, $2'''\text{-H}_2$, $1''''\text{-H}_2$, $2''''\text{-H}_2$, $1''''' \text{-H}_2$, 2-H), 3.14 (t, $J = 6.8$ Hz, 2H, $1''\text{-H}_2$), 2.72 (t, $J = 6.7$, 2H, $2'\text{-H}_2$), 2.16 (q, $J = 6.3$, 2H, $1'\text{-H}_2$), 1.80 (quin, $J = 6.4$ Hz, 2H, $2''\text{-H}_2$), 1.70 (quin, $J = 6.4$, 2H, $2''''\text{-H}_2$), 1.45 (s, 9H, $3 \times \text{CH}_3,\text{Boc}$), 1.30 (dt, $J = 13.7, 7.1$ Hz, 6H, $2 \times \text{CH}_3,\text{malonate}$).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 175.48 (C-3'), 172.75, 172.44 ($2 \times \text{CO}_{\text{malonate}}$), 160.20 (CO_{Boc}), 81.65 ($\text{C}_{\text{qua},\text{Boc}}$), 73.35 (C-1'''), 73.31 (C-2'''), 73.01 (C-2''', C-1''), 72.05 (C-1'''), 71.66 (C-3'), 64.65, 64.63 (2C, $2 \times \text{CH}_{2,\text{malonate}}$), 51.91 (C-2), 40.51 (C-3'''), 40.40 (C-1''), 33.18 (C-2'''), 32.67 (C-2''), 30.80 (C-2'), 30.59 ($3 \times \text{CH}_3,\text{Boc}$), 23.26 (C-1'), 16.20 ($2 \times \text{CH}_3,\text{malonate}$).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{25}\text{H}_{46}\text{N}_2\text{O}_{10}+\text{Na}]^+$ 557.30, found 557.30.

10.8.3.5 Ethyl-1-(17,17-dimethyl-15-oxo-4,7,10,16-tetraoxa-14-azaoctadec-1-yl)-2,6-dioxopiperidine-3-carboxylate **304**



The first fraction isolated as a result of the column chromatography of the above

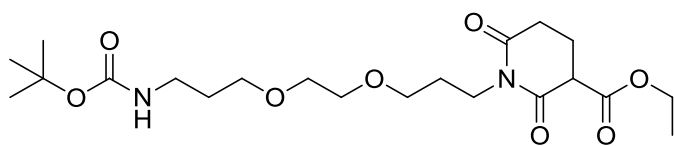
reaction mixture was a cyclic ester **304** (95 mg, 210 μ mol) in the form of a clear oil.

Yield: 22%.

R_f : 0.43 (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{23}\text{H}_{40}\text{N}_2\text{O}_9+\text{Na}]^+$ 511.56, found 511.26.

10.8.3.6 Ethyl-1-(14,14-dimethyl-12-oxo-4,7,13-trioxa-11-azapentadec-1-yl)-2,6-dioxopiperidine-3-carboxylate **306**



Following the general procedure (**J**), sodium metal (28 mg, 1.12 mmol, 3.2 equiv.) was treated with EtOH (5 mL)

followed by the addition of dimethyl malonate (132 mg, 1.01 mmol 3.2 equiv.) and acryloyl carbamate **295** (100 mg, 303 μ mol) in EtOH (5 mL). After 12 h, the solvent was evaporated and the crude product was purified by flash chromatography, eluting with EtOAc to afford **306** (120 mg, 270 μ mol) as a colorless oil.

Yield: 89%.

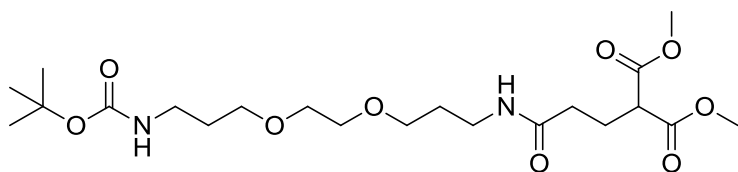
R_f : 0.29 (EtOAc).

$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 4.17-4.24 (m, 2H, $\text{CH}_{2,\text{ester}}$), 3.86 (t, $J = 7.0$ Hz, 2H, $1'-\text{H}_2$), 3.60 (dd, $J = 7.4, 5.3$ Hz, 1H, 3-H), 3.49-3.55 (m, 6H, $5'-\text{H}_2, 6'-\text{H}_2, 8'-\text{H}_2$), 3.47 (t, $J = 6.2$ Hz, 2H, $3'-\text{H}_2$), 3.18, 3.19 (2t, $J = 6.0, 6.0$ Hz, 2H, $10'-\text{H}_2$), 2.70 (dd, $J = 8.5, 5.3$ Hz, 1H, 5_a-H_2), 2.63 (dd, $J = 7.3, 5.4$ Hz, 1H, 5_b-H_2), 2.29-2.21 (m, 1H, 4_a-H_2), 2.16-2.08 (m, 1H, 4_b-H_2), 1.80 (quin, $J = 6.7$ Hz, 2H, $2'-\text{H}_2$), 1.73 (quin, $J = 6.3$ Hz, 2H, $9'-\text{H}_2$), 1.41 (s, 9H, $3 \times \text{CH}_3, \text{Boc}$), 1.27, (t, $J = 7.1$ Hz, 3H, $\text{CH}_3, \text{ester}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 171.42 (C-6), 168.78 (C-2), 168.76 (CO_{ester}), 156.11 (C-12'), 78.89 (C-14'), 70.31, 70.12 (C-5', C-6'), 69.62, 69.19 (C-3', C-8'), 62.07 ($\text{CH}_{2,\text{ester}}$), 49.23 (C-3), 38.61 (C-10'), 37.74 (C-1'), 30.49 (C-5), 29.73 (C-2'), 28.51 (3C, $3 \times \text{CH}_3$), 27.97 (C-9'), 20.62 (C-4'), 14.14 ($\text{CH}_3, \text{ester}$).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{21}\text{H}_{36}\text{N}_2\text{O}_8+\text{Na}]^+$ 467.24, found 467.24.

10.8.3.7 Dimethyl(3-{[3-(2-{3-[(*tert*-butoxycarbonyl)amino]propoxy)ethoxy]propyl}amino}-3-oxo-propyl)malonate **307**



Following the general procedure (**J**), acryloyl carbamate **295** (400 mg, 1.21 mmol) and dimethyl malonate (150 mg, 2.44 mmol) were treated in

the presence of MeONa in MeOH (5 mL). After 24 h the reaction mixture was saturated and the crude product was purified by flash chromatography, eluting with DCM/MeOH (20:1) and 1% NH_4OH solution (30%) to afford **307** (355 mg, 730 μ mol) as a colorless oil.

Yield: 59%.

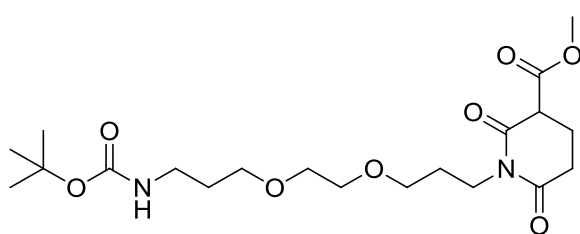
R_f : 0.37 (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): acyclic, δ 3.66 (s, 3H, $\text{CH}_{3,\text{malonate}}$), 3.60 (s, 3H, $\text{CH}_{3,\text{malonate}}$), 3.52 (s, 4H, $3''\text{-H}_2$, $1'''\text{-H}_2$), 3.49, 3.48 (2t, $J = 5.8, 6.0$ Hz, 4H, $2'''\text{-H}_2$, $1''''\text{-H}_2$), 3.31(dt, $J = 12.2, 6.2$ Hz, 2H, $3''''\text{-H}_2$), 3.24 (t, $J = 7.4$ Hz, 1H, 2-H), 3.15 (dt, $J = 12.4, 6.3$ Hz, 2H, $1''\text{-H}_2$), 2.32 (td, $J = 7.2, 3.0$ Hz, 2H, $2'\text{-H}_2$), 2.14 (t, $J = 7.68$ Hz, 2H, $1'\text{-H}_2$), 1.76-1.66 (m, 4H, $2''\text{-H}_2$, $2''''\text{-H}_2$), 1.37 (s, 9H, $3 \times \text{CH}_{3,\text{Boc}}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 173.01 (C-3'), 171.13 (C-1), 167.46 (C-3), 155.97 (CO_{Boc}), 78.88 ($\text{C}_{\text{qua,Boc}}$), 70.14, 70.02 (C-1''', C-2'''), 69.77, 69.37 (C-3'', C-1'''), 52.35 (C-2), 51.63, 51.57 ($2 \times \text{OCH}_{3,\text{malonate}}$), 38.35 (C-3'''), 37.98 (C-1'), 31.21 (C-2'), 29.60, 28.84 (C-2'', C-2'''), 28.35 ($3 \times \text{CH}_{3,\text{Boc}}$), 24.73 (C-1').

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{21}\text{H}_{38}\text{N}_2\text{O}_9+\text{Na}]^+$ 484.23, found 484.23.

10.8.3.8 Methyl-1-(14,14-dimethyl-12-oxo-4,7,13-trioxa-11-azapentadec-1-yl)-2,6-dioxopiperidine-3-carboxylate **308**



The NMR and mass analysis illustrated that the first fraction obtained as a result of the column chromatography of the above reaction mixture was a cyclic product **308** (50 mg, 116 μmol) obtained as a clear oil.

Yield: 26%.

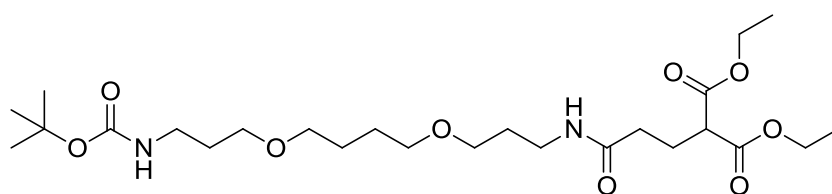
R_f : 0.42; (DCM/MeOH 20:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 3.89 (t, $J = 7.0$ Hz, 2H, $1'\text{-H}_2$), 3.78 (s, 3H, $\text{CH}_{3,\text{ester}}$), 3.64 (dd, $J = 7.6, 5.3$ Hz, 1H, 3-H), 3.58-3.51 (m, 6H, $5'\text{-H}_2$, $6'\text{-H}_2$, $8'\text{-H}_2$), 3.49 (t, $J = 6.2$ Hz, 2H, $3'\text{-H}_2$), 3.20 (dt, $J = 6.4, 6.0$ Hz, 2H, $10'\text{-H}_2$), 2.73 (dd, $J = 8.4, 5.2$ Hz, 1H, 5_a-H_2), 2.65 (dd, $J = 7.7, 5.3$ Hz, 1H, 5_b-H_2), 2.30-2.10 (m, 2H, 4-H₂), 1.82 (quin, $J = 6.6$ Hz, 2H, $2'\text{-H}_2$), 1.75 (quin, $J = 6.23$ Hz, 2H, $9'\text{-H}_2$), 1.42 (s, 9H, $3 \times \text{CH}_{3,\text{Boc}}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 171.43 (C-6), 169.3 (C-2), 168.71 (CO_{ester}), 156.19 (C-12'), 79.02 (C-14'), 70.39, 70.20 (C-5', C-6'), 69.70, 69.29 (C-3', C-8'), 53.04 ($\text{CH}_{3,\text{ester}}$), 49.18 (C-3), 38.69 (C-10'), 37.89 (C-1'), 30.60 (C-5), 29.81 (C-9'), 28.59 ($3\text{C}, 3 \times \text{CH}_{3,\text{Boc}}$), 28.04 (C-2'), 20.66 (C-4).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{20}\text{H}_{34}\text{N}_2\text{O}_8+\text{Na}]^+$ 453.22, found 453.22.

10.8.3.9 Diethyl(3-{[3-(4-{3-[(*tert*-butoxycarbonyl)amino]propoxy}butoxy)propyl]amino}-3-oxopropyl)malonate **309**



Following the general procedure (**J**), Na metal (170 mg, 8.42 mmol, 6 equiv.) was treated with

EtOH followed by the addition of diethyl malonate (672 mg, 4.23 mmol 3 equiv.) and acryloyl carbamate **296** (500 mg, 1.44 mmol). After overnight stirring, the reaction mixture was concentrated followed by workup. The crude product was purified by flash chromatography, eluting with DCM/MeOH (20:1) and 1% NH₄OH (30%) to afford **309** (450 mg, 870 μmol) along with the cyclic moiety **310** (136 mg, 290 μmol) as a clear thick oil.

Yield: 64% (acyclic) and 16% (cyclic).

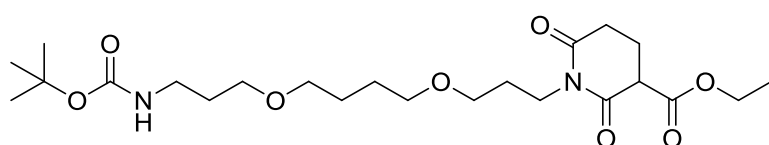
*R*_f: 0.23 acyclic (DCM/MeOH, 10:1) with 1% NH₄OH solution (30%).

¹H-NMR (400 MHz, CDCl₃): δ 4.15 (q, *J* = 7.1 Hz, 4H, 2 × CH_{2,malonate}), 3.83 (t, *J* = 7.0 Hz, 2H, 3''''-H₂), 3.35 (2t, *J* = 6.2, 6.2 Hz, 4H, 3''-H₂, 1'''-H₂), 3.31 (t, *J* = 7.6 Hz, 5H, 4'''-H₂, 1''''-H₂, 2-H), 3.14 (t, *J* = 5.84, Hz, 2H, 1''-H₂), 2.66 (dd, *J* = 8.5, 5.2 Hz, 1H, 2'-H_a), 2.59 (dd, *J* = 7.6, 5.3 Hz, 1H, 2'-H_b), 2.07-1.95 (m, 1H, 1'-H_a), 1.88 (ddt, *J* = 13.7, 8.4, 5.2 Hz, 1H, 1'-H_b), 1.72 (2quin, *J* = 6.6, 6.3 Hz, 4H, 2''-H₂, 2''''-H₂), 1.57-1.52 (m, 4H, 2'''-H₂, 3'''-H₂), 1.16 (s, 9H, 3 × CH_{3,Boc}), 1.02 (2t, *J* = 7.0 Hz, 6H, 2 × CH_{3,malonate}).

¹³C-NMR (100 MHz, CDCl₃): δ 171.38 (C-3'), 168.76 (2 × CO_{malonate}), 156.07 (CO_{Boc}), 78.88 (C_{qua,Boc}), 70.83 (C-3'), 70.61 (C-1'''), 68.63 (C-4'''), 62.05 (C-1''), 61.59-61.51 (2 × CH_{2,malonate}), 52.56 (C-2), 38.84 (C-3'''), 37.84 (C-1'), 30.47 (C-2'), 29.78 (C-1'), 28.09 (C-2'''), 28.48 (3 × CH_{3,Boc}), 26.49 (C-2'', C-3''), 20.6 (C-2''), 14.00 (2 × CH_{3,malonate}).

MS (MALDI): *m/z* [M+H]⁺ calcd for [C₁₈H₃₂N₂O₆+H]⁺ 373.23, found 373.25 (ring form without Boc).

10.8.3.10 Ethyl-1-(16,16-dimethyl-14-oxo-4,9,15-trioxa-13-azaheptadec-1-yl)-2,6-dioxopiperidine-3-carboxylate **310**



Following the general procedure (**J**), sodium metal (28 mg, 1.13 mmol, 3.2 equiv.) was treated with

EtOH (5 mL) followed by the addition of dimethyl malonate (132 mg, 1.10 mmol 3.2 equiv.) and acryloyl carbamate **296** (100 mg, 279 μmol) in EtOH (5 mL). After 12 h, the

solvent was evaporated and the crude product was purified by flash chromatography, eluting with EtOAc to afford **310** (110 mg, 233 μ mol) as a colorless oil.

Yield: 83%.

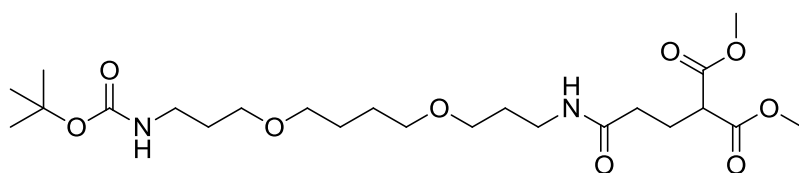
R_f : 0.32 (EtOAc).

$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 4.22-4.11 (m, 2H, $\text{CH}_{2,\text{ester}}$), 3.81 (t, $J = 7.1$ Hz, 2H, $1'-\text{H}_2$), 3.55 (dd, $J = 7.3, 5.4$ Hz, 1H, 3-H), 3.40 (t, $J = 6.0$ Hz, 2H, $10'-\text{H}_2$), 3.37-3.31 (m, 6H, $3'-\text{H}_2$, $5'-\text{H}_2$, $6'-\text{H}_2$), 3.13, 3.14 (2t, $J = 6.2, 6.0$ Hz, 2H, $12'-\text{H}_2$), 2.66 (dd, $J = 8.6, 5.3$ Hz, 1H, 5_a-H_2), 2.59 (dd, $J = 7.5, 5.3$ Hz, 1H, 5_b-H_2), 2.25-2.17 (m, 1H, 4_a-H_2), 2.12-2.04 (m, 1H, 4_b-H_2), 1.73 (quint, $J = 6.7$ Hz, 2H, $11'-\text{H}_2$), 1.67 (quint, $J = 6.3$ Hz, 2H, $2'-\text{H}_2$), 1.59-1.49 (m, 4H, $6'-\text{H}_2$, $7'-\text{H}_2$), 1.37 (s, 9H, $3 \times \text{CH}_{3,\text{Boc}}$), 1.23 (t, $J = 7.1$ Hz, 3H, $\text{CH}_{3,\text{ester}}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 171.39 (C-6), 168.78 (C-2), 168.71 (CO_{ester}), 156.10 (C-14'), 78.95 (C-16'), 70.88 (C-8'), 69.28 (C-5'), 68.67 (C-10'), 62.11 (C-3'), 61.52 ($\text{CH}_{2,\text{ester}}$), 49.27 (C-3), 38.90 (C-12'), 37.90 (C-1'), 30.51 (C-5), 29.82 (C-11'), 28.53 (3C, $3 \times \text{CH}_{3,\text{Boc}}$), 28.13 (C-2'), 26.55 (C-6'), 26.51 (C-7'), 20.66 (C-4), 14.17 ($\text{CH}_{3,\text{ester}}$).

MS (MALDI): m/z $[\text{M}+\text{H}-\text{Boc}]^+$ calcd for $[\text{C}_{23}\text{H}_{40}\text{N}_2\text{O}_8+\text{H}-\text{Boc}]^+$ 495.27, found 495.27.

10.8.3.11 Dimethyl(3-[[3-(4-{3-[(*tert*-butoxycarbonyl)amino]propoxy)butoxy]propyl]amino)-3-oxo-propyl]malonate **311**



Following the general procedure (**J**), sodium metal (200 mg, 8.41 mmol, 6 equiv.) was treated with MeOH (5 mL) followed by

the addition of dimethyl malonate (528 mg, 4.25 mmol 3 equiv.) and acryloyl carbamate **296** (500 mg, 1.43 mmol) in methanol (5 mL). After 18 h, the solvent was evaporated followed by workup. The crude product was purified by flash chromatography, eluting with DCM/MeOH (20:1) and 1% NH_4OH (30%) to afford **311** (176 mg, 360 μ mol) as a clear thick oil.

Yield: 25%.

R_f : 0.2 (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

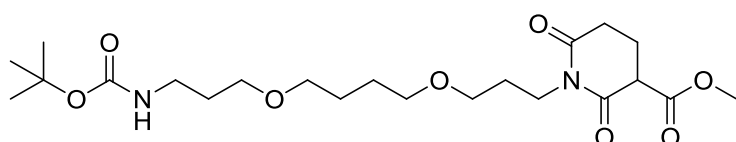
$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 3.83 (t, $J = 7.1$ Hz, 2H, $3''''-\text{H}_2$), 3.74, 3.68 (2s, 6H, $2 \times \text{CH}_{3,\text{malonate}}$), 3.41-3.36 (m, 9H, $3''-\text{H}_2$, $1'''-\text{H}_2$, $4'''-\text{H}_2$, $1''''-\text{H}_2$, 2-H), 3.15 (t, $J = 6.1$ Hz, 2H, $1'-\text{H}_2$), 2.69 (ddd, $J = 17.6, 8.4, 5.2$, 1H, $2_a'-\text{H}_2$), 2.57 (ddd, $J = 17.8, 7.6, 5.2$ Hz, 1H, $2_b'-\text{H}_2$), 2.13-2.06 (m, 2H, $1'-\text{H}_2$), 1.75-1.71 (m, 4H, $2''-\text{H}_2$, $2''''-\text{H}_2$), 1.59-1.48 (m, 4H, $2'''-\text{H}_2$, $3'''-\text{H}_2$), 1.38 (s, 9H, $3 \times \text{CH}_{3,\text{Boc}}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 171.30 (C-3'), 169.13, 168.55 ($2 \times \text{CO}_{\text{malonate}}$), 156.05 (CO_{Boc}), 78.87 ($\text{C}_{\text{qua},\text{Boc}}$), 70.72, 70.54 (C-3'', C-1'''), 69.08, 68.53 (C-1'', C-4''), 52.85, 52.49

(2 × CH_{3,maloante}), 48.99 (C-2), 38.69 (C-3'''), 37.82 (C-1''), 30.40 (C-2'), 29.65 (C-2'''), 28.38 (3 × CH_{3,Boc}), 27.96 (C-2''), 26.37, 26.34 (C-2'', C-3'''), 20.46 (C-1').

MS (MALDI): *m/z* [M+Na]⁺ calcd for [C₁₈H₃₄N₂O₇+H]⁺ 391.25, found 391.26 (without Boc).

10.8.3.12 Methyl-1-(16,16-dimethyl-14-oxo-4,9,15-trioxa-13-azaheptadec-1-yl)-2,6-dioxopiperidine-3-carboxylate **312**



The second fraction obtained as a result of the column chromatography of the reaction mixture of compound

311 afforded the cyclic moiety **312** (357 mg, 780 μmol) as a clear thick gel.

Yield: 55%.

R_f: 0.18 (DCM/MeOH, 10:1 with 1% NH₄OH solution (30%)).

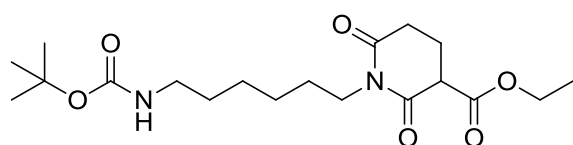
¹H-NMR (400 MHz, CDCl₃) δ 3.82 (t, *J* = 7.2 Hz, 2H, 1'-H₂), 3.74 (s, 3H, CH_{3,ester}), 3.60 (dd, *J* = 7.7, 5.3 Hz, 1H, 3-H), 3.43-3.33 (m, 8H, 10'-H₂, 3'-H₂, 5'-H₂, 8'-H₂), 3.14, 3.16 (2t, *J* = 6.2, 6.0 Hz, 2H, 12'-H₂), 2.69 (dd, *J* = 8.4, 5.3 Hz, 1H, 5_a-H₂), 2.61 (dd, *J* = 7.7, 5.2 Hz, 1H, 5_b-H₂), 1.28-2.19 (m, 1H, 4_a-H₂), 2.14-2.08 (m, 1H, 4_b-H₂), 1.74 (quin, *J* = 6.7 Hz, 2H, 11'-H₂), 1.69 (quin, *J* = 6.3 Hz, 2H, 2'-H₂), 1.58-1.54 (m, 4H, 6'-H₂, 7'-H₂), 1.38 (s, 9H, 3 × CH_{3,Boc}).

¹³C-NMR (100 MHz, CDCl₃): δ 171.30 (C-6), 169.13 (C-2), 168.55 (CO_{ester}), 156.05 (C-14'), 78.87 (C-16'), 70.72 (C-10'), 70.54 (C-3'), 69.08, 68.53 (C-5', C-8'), 52.85 (CH_{3,ester}), 48.99 (C-3), 38.69 (C-12'), 37.82 (C-1'), 30.40 (C-5), 29.65 (C-11'), 28.38 (3 × CH_{3,Boc}), 27.96 (C-2'), 26.37, 26.34 (C-6', C-7'), 20.46 (C-4).

MS (MALDI): *m/z* [M+Na]⁺ calcd for [C₂₂H₃₈N₂O₈+Na]⁺ 481.26, found 481.28 (with Boc).

MS (MALDI): *m/z* [M+H]⁺ calcd for [C₁₇H₃₀N₂O₆+H]⁺ 359.22, found 359.24 (without Boc).

10.8.3.13 Ethyl-1-(10,10-dimethyl-8-oxo-9-oxa-7-azaundecane-1-yl)-2,6-dioxopiperidine-3-carboxylate **314**



Following the general procedure (**J**), sodium (254 mg, 11.07 mmol) was treated with abs EtOH (5 mL) followed by the addition of diethylmalonate (1.22 g, 7.83 mmol) and the solution of acryloyl carbamate **297** (500 mg, 1.84 mmol) in abs EtOH (5 mL). The reaction was stirred overnight and purified the crude product by flash chromatography to afford an acyclic moiety (180 mg, 420 μmol) and cyclic product **314** (425 mg, 1.12 mmol) as a clear oil.

Yield: 61% cyclic and 23% acyclic.

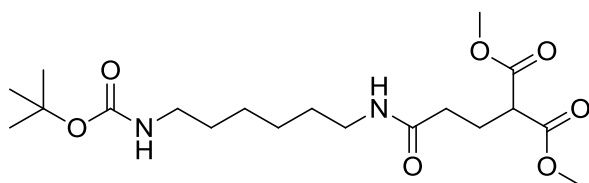
R_f: 0.51 cyclic, 0.39 open-chain (DCM/MeOH, 10:1 with 1% NH₄OH (30%)).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): Cyclic; δ 4.23 (q, $J = 7.1$ Hz, 2H, $\text{CH}_{2,\text{ester}}$), 3.75 (t, $J = 7.4$ Hz, 3H, 3-H, 1'-H₂), 3.02 (t, $J = 6.9$ Hz, 2H, 6'-H₂), 2.69 (t, $J = 6.6$ Hz, 2H, 5-H₂), 2.22-2.14 (m, 2H, 6-H₂), 1.47-1.42 (m, 4H, 2'-H₂, 5'-H₂), 1.37 (s, 9H, $3 \times \text{CH}_{3,\text{Boc}}$), 1.35-1.28 (m, 4H, 3'-H₂, 4'-H₂), 1.28 (t, $J = 7.1$ Hz, 3H, $\text{CH}_{3,\text{ester}}$).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 173.66 (CO_{ester}), 170.94, 170.66 (C-2, C-6), 158.52 (C-8'), 79.77 (C-10'), 62.83 ($\text{CH}_{2,\text{ester}}$), 50.13 (C-3), 41.22 (C-6'), 40.69 (C-1'), 31.32 (C-5), 30.81 (C-5'), 28.79 ($3 \times \text{CH}_{3,\text{Boc}}$), 28.72 (C-4'), 27.53, 27.46 (C-2', C-3'), 21.53 (C-4), 14.38 ($\text{CH}_{3,\text{ester}}$).

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{19}\text{H}_{32}\text{N}_2\text{O}_6+\text{Na}]^+$ 407.21, found 407.21.

10.8.3.14 Dimethyl[3-({6-[(*tert*-butoxycarbonyl)amino]hexyl}amino)-3-oxopropyl]malonate **315**



Following the general procedure (**J**), acryloyl carbamate **297** (500 mg, 1.84 mmol) dissolved in MeOH (5 mL) was treated with dimethyl malonate (1.12 g, 11.04 mmol) in MeOH (5 mL)

containing MeONa obtained by the addition of sodium metal (253 mg, 11.07 mmol). After 16 h, the mixture was concentrated and purified by flash chromatography, eluting with DCM/MeOH (15:1) and 1% NH_4OH solution (30%) to afford **315** (422 mg, 1.05 mmol) as a clear oil.

Yield: 57%.

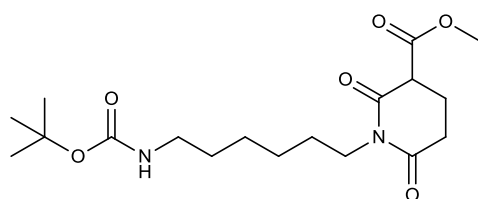
R_f : 0.54, (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 3.68, 3.66 (2s, 6H, $2 \times \text{OCH}_3$), 3.62-3.56 (m, 2H, 6''-H₂), 3.28 (t, $J = 7.3$ Hz, 1H, 2-H), 3.06 (dt, $J = 12.5, 6.1$ Hz, 2H, 1''-H₂), 2.67 (dd, 8.4, 5.3 Hz, 1H, 2'-H_a), 2.36 (dd, $J = 7.7, 5.1$ Hz, 1H, 2'-H_b), 2.18 (td, $J = 7.4, 3.5$ Hz, 2H, 1'-H₂), 1.47-1.38 (m, 4H, 5''-H₂, 2''-H₂), 1.36 (s, 9H, $3 \times \text{CH}_3$), 1.29-1.22 (m, 4H, 3''-H₂, 4''-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 171.34, 171.23 (2C, $2 \times \text{CO}_{\text{malonate}}$), 169.53 (C-3'), 156.08 (CO_{Boc}), 79.05 (C-1'''), 52.58, 52.53 ($2 \times \text{CH}_{3,\text{malonate}}$), 50.58 (C-2), 40.19, 39.21 (2C, C-6'', C-1'), 33.51 (C-2'), 29.97, 29.41 (2C, C-2'', C-5''), 28.41 (3C, $3 \times \text{CH}_{3,\text{Boc}}$), 26.21, 26.08 (C-3'', C-4''), 24.61 (C-1').

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{19}\text{H}_{34}\text{N}_2\text{O}_7+\text{Na}]^+$ 425.23, found 425.24.

10.8.3.15 Methyl-1-(10,10-dimethyl-8-oxo-9-oxa-7-azaundecane-1-yl)-2,6-dioxopiperidine-3-carboxylate. **316**



Column chromatography of the above reaction mixture afforded the cyclic molecule **316** (171 mg, 460 μ mol) as a clear colorless oil due to the intramolecular aminolysis. Yield: 28%.

R_f : 0.61, (DCM/MeOH, 15:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, MeOD): δ 3.77 (s, 3H, CH_3 , ester), 3.79-3.72 (m, 3H, $1'\text{-H}_2$, 3-H), 3.02 (t, $J = 7.0$ Hz, 2H, $6'\text{-H}_2$), 2.71-2.68 (m, 2H, 5- H_2), 2.21-2.16 (m, 2H, 4- H_2), 1.49 (quint, $J = 7.5$ Hz, 2H, $2'\text{-H}_2$), 1.46 (quint, $J = 7.4$ Hz, 2H, $5'\text{-H}_2$), 1.43 (s, 9H, $3 \times \text{CH}_3$, Boc), 1.34-1.29 (m, 4H, $3'\text{-H}_2$, $4'\text{-H}_2$).

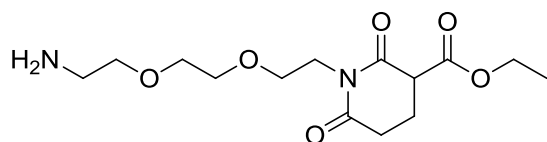
$^{13}\text{C-NMR}$ (100 MHz, MeOD): δ 173.56 (CO_{ester}), 171.06, 170.82 (C-2, C-6), 158.41 (C-8'), 79.71 (C-10'), 53.13 (CH_3 , ester), 49.92 (C-3), 41.19 (C-6'), 40.68 (C-1'), 31.35 (C-5), 30.77 (C-5'), 28.79 (3C, $3 \times \text{CH}_3$, Boc), 28.69 (C-2'), 27.49, 27.42 (C-3', C-4'), 21.49 (C-4).

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{18}\text{H}_{30}\text{N}_2\text{O}_6+\text{Na}]^+$ 393.26, found 393.20.

10.8.4 General procedure (K) for Boc deprotection

To a round bottom flask, carbamate was dissolved in DCM followed by the addition of TFA (10 to 20 equiv.) and the solution was stirred for 2-5 h till the completion of the reaction (monitored by TLC). The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography, eluting with mixture of DCM and MeOH.

10.8.4.1 Ethyl-1-{2-[2-(2-aminoethoxy)ethoxy]ethyl}-2,6-dioxopiperidine-3-carboxylate **259**



Following the general procedure (K) the mixture of acyclic oxoamine ester **263** and cyclic oxoamine ester **260** (1.31 g, 2.82 mmol) was treated with TFA **317** (3.13 g, 28.15 mmol) in DCM (30 mL).

The crude product was purified by column chromatography, eluting with a gradient of DCM/MeOH (30:1 to 20:1) and 1% NH_4OH solution (30%) to afford **259** (790 mg, 2.53 mmol) as a clear oil.

Yield: 89%.

R_f : 0.28 (DCM/MeOH, 10:1 with 1% NH_4OH solution (30%)).

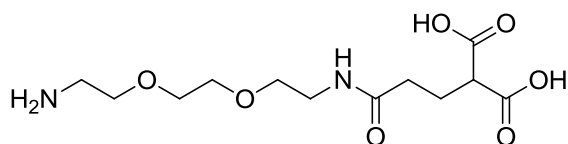
$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 4.17 (dq, $J = 7.1, 1.1$ Hz, 2H, $\text{CH}_{2,\text{ester}}$), 4.01 (t, $J = 5.5$ Hz, 2H, $1'-\text{H}_2$), 3.7 (t, $J = 4.8$ Hz, 2H, $2'-\text{H}_2$), 3.66 (dd, $J = 7.6, 5.4$ Hz, 1H, 3-H), 3.66-3.61 (m, 6H, $1''-\text{H}_2$, $2''-\text{H}_2$, $1'''-\text{H}_2$), 3.12 (t, $J = 6.8$ Hz, 2H, $2'''-\text{H}_2$), 2.74 (dd, $J = 8.0, 5.3$ Hz, 1H, 5_a-H_2), 2.66 (dd, $J = 7.6, 5.5$ Hz, 1H, 5_b-H_2), 2.31-2.22 (m, 1H, 4_a-H_2), 2.20-2.12 (m, 1H, 4_b-H_2), 1.22 (t, $J = 7.1$ Hz, 3H, $\text{CH}_{3,\text{ester}}$).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 172.00 (CO_{ester}), 169.26, 168.81 (C-2, C-6), 70.07, 69.84 (C-1'', C-2''), 67.96, 66.53 (C-2', C-1'''), 62.17 ($\text{CH}_{2,\text{ester}}$), 49.09 (C-3), 39.69, 38.96 (C-1', C-2'''), 30.30 (C-5), 20.41 (C-4), 13.96 ($\text{CH}_{3,\text{ester}}$).

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_6+\text{Na}]^+$ 339.15, found 339.07,

m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_6+\text{H}]^+$ 317.17, found 317.19.

10.8.4.2 [3-({2-[2-(2-Aminoethoxy)ethoxy]ethyl}amino)-3-oxopropyl]malonic acid **261**



Carbamate **262** (600 mg, 1.53 mmol) was treated with TFA (20 mL) in DCM (20 mL) in accordance with the general procedure (**K**). The crude product

was purified by silica column, eluting with DCM/MeOH (10:1) and 1% AcOH to afford **261** (345 mg, 1.13 mmol) as a white solid.

Yield: 72%.

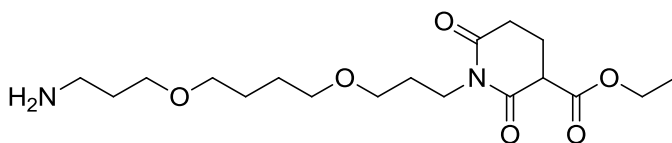
R_f : 0.25, DCM/MeOH (5:1), with 1% AcOH.

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.72 (t, $J = 5.1$ Hz, 2H, $1''''-\text{H}_2$), 3.68 (br s, 4H, $1'''-\text{H}_2$, $2'''-\text{H}_2$), 3.60 (t, $J = 5.5$ Hz, 2H, $2''-\text{H}_2$), 3.42 (t, $J = 5.9$ Hz, 3H, $2''''-\text{H}_2$, 2-H), 3.15 (t, $J = 4.9$ Hz, 2H, $1''-\text{H}_2$), 2.38 (t, $J = 7.7$ Hz, 2H, $2'-\text{H}_2$), 2.17-2.05 (m, 2H, $1'-\text{H}_2$).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 178.18 (C-3'), 174.83, 173.48 (2C, $2 \times \text{CO}_{\text{acid}}$), 73.16 (C-1'''), 73.13 (C-2'''), 72.29 (C-2''), 69.65 (C-1'''), 54.46 (C-2), 42.47 (C-2'''), 42.20 (C-1''), 33.96 (C-2'), 27.20 (C-1').

MS (MALDI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{12}\text{H}_{22}\text{N}_2\text{O}_7+\text{Na}]^+$ 329.11, found 329.02.

10.8.4.3 Ethyl-1-{3-[4-(3-aminopropoxy)butoxy]propyl}-2,6-dioxopiperidine-3-carboxylate **318**



Mixture of oxamines **309** and **310** (518 mg, 1.36 mmol) was treated with TFA (6 mL) in DCM (15 mL) at 0 °C following the general

procedure (**K**). After the completion of the reaction (3 h) the mixture was concentrated and purified by flash chromatography, eluting with DCM/MeOH (10:1 to 5:1) and 1% NH_4OH solution (30%) to afford **318** (476 mg, 1.28 mmol) as a clear oil.

Yield: 94%.

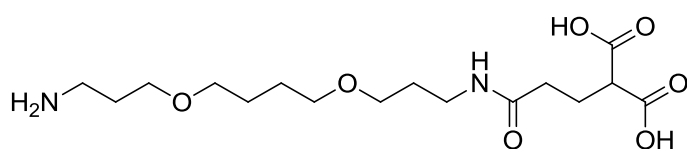
R_f : 0.23, DCM/MeOH (10:1) with 1% NH_4OH solution (30%).

^1H -NMR (400 MHz, CD_3OD): δ 4.25 (q, $J = 7.1$ Hz, 2H, $\text{CH}_{2,\text{ester}}$), 3.87 (t, $J = 7.1$ Hz, 2H, $1'-\text{H}_2$), 3.64 (dd, $J = 7.3, 5.2$ Hz, 1H, 3-H), 3.59 (t, $J = 5.7$ Hz, 2H, $3'-\text{H}_2$), 3.50 (t, $J = 6.2$ Hz, 2H, $1'''-\text{H}_2$), 3.43, 3.42 (2t, $J = 6.0$ Hz, 4H, $1''-\text{H}_2$, $4''-\text{H}_2$), 3.08 (t, $J = 7.0$ Hz, 2H, $3'''-\text{H}_2$), 2.72 (t, $J = 5.3$ Hz, 2H, 5- H_2), 2.25-2.19 (m, 2H, 4- H_2), 1.95 (quin, $J = 5.6$ Hz, 2H, $2'-\text{H}_2$), 1.78 (quin, $J = 6.3$ Hz, 2H, $2'''-\text{H}_2$), 1.65-1.61 (m, 4H, $2''-\text{H}_2$, $3''-\text{H}_2$), 1.30 (t, $J = 7.1$ Hz, 3H, $\text{CH}_{3,\text{ester}}$).

^{13}C -NMR (100 MHz, CD_3OD): δ 175.46 (CO_{ester}), 172.74 (C-2), 172.43 (C-6), 73.74, 73.41 (C-1'', C-4''), 71.37, 71.02 (C-3', C-1'''), 64.62 ($\text{CH}_{2,\text{ester}}$), 49.94 (C-3), 41.12, 40.33 (C-1', C-3'''), 33.13 (C-5), 30.83, 30.27 (C-2', C-2'''), 29.19, 29.15 (C-2'', C-3''), 23.22 (C-4), 16.16 ($\text{CH}_{2,\text{ester}}$).

MS (ESI): m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{18}\text{H}_{32}\text{N}_2\text{O}_6+\text{H}]^+$ 373.23, found 373.23.

10.8.4.4 [3-({3-[4-(3-aminopropoxy)butoxy]propyl}amino)-3-oxopropyl] malonic acid **320**



General procedure (**K**) was followed for the treatment of dicarboxylic carbamate **326** (462 mg, 430 μmol) with a mixture

of DCM (4 mL) and TFA (1.0 mL). After 5 h, the solvent was evaporated and the residue was purified by flash chromatography, eluting with DCM/MeOH (10:1) and 1% AcOH to afford **322** (141 mg, 390 μmol) as a colorless thick oil.

Yield: 90%.

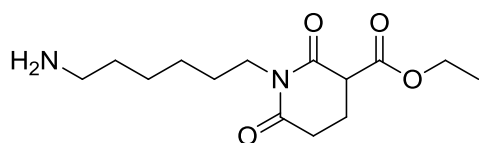
R_f : 0.21 (DCM/MeOH, 5:1 with 1% AcOH).

^1H -NMR (400 MHz, CD_3OD): δ 3.57 (t, $J = 5.5$ Hz, 2H, $3''''-\text{H}_2$), 3.49-3.41 (m, 6H, $1''''-\text{H}_2$, $4'''-\text{H}_2$, $1'''-\text{H}_2$), 3.37 (t, $J = 6.5$ Hz, 1H, 2-H), 3.24-3.31 (m, 2H, $3''-\text{H}_2$), 3.07 (t, $J = 6.5$ Hz, 2H, $1''-\text{H}_2$), 2.27-2.22 (m, 2H, $2'-\text{H}_2$), 2.10-2.06 (m, 2H, $1'-\text{H}_2$), 1.91-1.85 (m, 2H, $2''-\text{H}_2$), 1.79-1.71 (m, 2H, $2''''-\text{H}_2$), 1.69-1.63 (m, 4H, $2'''-\text{H}_2$, $3'''-\text{H}_2$).

^{13}C -NMR (100 MHz, CD_3OD): δ 178.47 (2C, $2 \times \text{CO}_{\text{acid}}$), 174.23 (C-3'), 71.91 (C-1'''), 71.73 (C-3''), 69.39 (C-4'''), 69.23 (C-1'''), 52.13 (C-2), 39.08 (C-3'''), 37.86 (C-1'), 30.41 (C-2'), 28.56, 28.28 (C-2'', C-2'''), 27.44, 27.42 (C-2''', C-3'''), 22.86 (C-1').

MS (MALDI): m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{16}\text{H}_{30}\text{N}_2\text{O}_7+\text{H}]^+$ 363.21, found 363.22.

10.8.4.5 Ethyl-1-(6-aminoheptyl)-2,6-dioxopiperidine-3-carboxylate **319**



The mixture of cyclic Boc diethylmalonate **314** and acyclic moiety **313** (650 mg, 1.54 mmol) was treated with TFA (5 mL) in DCM (20 mL) according to the general procedure (**K**). The crude product was purified by silica column, eluting with DCM/MeOH (10:1) and 1% NH_4OH solution (30%) to afford **319** (386 mg, 1.36 mmol) as a clear oil.

Yield: 88%.

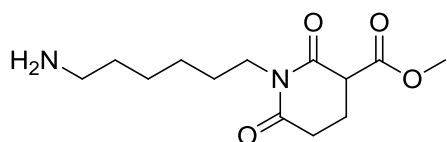
R_f : 0.43, (DCM/MeOH, 5:1 with 1% NH_4OH solution (30%)).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 4.24 (q, $J = 7.1$ Hz, 2H, $\text{CH}_{2,\text{ester}}$), 3.78 (t, $J = 6.2$ Hz, 2H, $1'-\text{H}_2$), 2.94 (t, $J = 7.5$ Hz, 2H, $6'-\text{H}_2$), 2.72 (t, $J = 6.2$ Hz, 2H, $5-\text{H}_2$), 2.20 (td, $J = 6.0, 1.2$ Hz, 2H, $4-\text{H}_2$), 1.67 (quin, $J = 7.0$ Hz, 2H, $2'-\text{H}_2$), 1.56 (quin $J = 7.3$ Hz, 2H, $5'-\text{H}_2$), 1.46-1.32 (m, 4H, $3'-\text{H}_2, 4'-\text{H}_2$), 1.30 (t, $J = 7.1$ Hz, 3H, $\text{CH}_{3,\text{ester}}$).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 175.16 (CO_{ester}), 172.47, 172.16 (C-2, C-6), 64.31 ($\text{CH}_{2,\text{ester}}$), 51.31 (C-3), 42.05 (C-1'), 41.95 (C-6'), 32.75 (C-5), 29.89, 29.81 (C-2', C-5'), 28.66, 28.36 (C-3', C-4'), 22.97 (C-4), 15.82 ($\text{CH}_{3,\text{ester}}$).

MS (ESI): m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_4+\text{H}]^+$ 285.18, found 285.18.

10.8.4.6 Methyl 1-(6-aminoheptyl)-2,6-dioxopiperidine-3-carboxylate **321**



Following the general procedure (**K**), cyclic methyl carbamate **316** (540 mg, 1.44 mmol) was treated with the mixture of DCM (10 mL) and TFA (5 mL). Flash column chromatography, eluting with DCM/MeOH (10:1) afforded (225 mg, 833 μmol) colorless thick oil.

Yield: 58%.

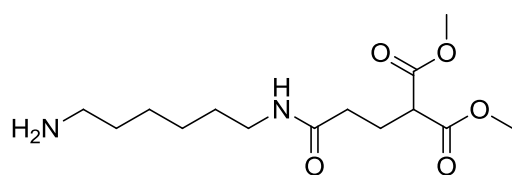
R_f : 0.64 (DCM/MeOH, 7:1).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.76 (s, 3H, $\text{CH}_{3,\text{ester}}$), 3.65 (dd, $J = 6.7, 2.9$ Hz, 1H, 3-H), 2.92 (t, $J = 7.7$ Hz, 2H, $13-\text{H}_2$), 2.70 (td, $J = 6.6, 1.3$ Hz, 2H, $8-\text{H}_2$), 2.18 (app. t, $J = 7.4$ Hz, 2H, $5-\text{H}_2$), 1.99-1.95 (m, 2H, $4-\text{H}_2$), 1.65 (quin, $J = 7.5$ Hz, 2H, $12-\text{H}_2$), 1.53 (quin, $J = 7.3$ Hz, 2H, $8-\text{H}_2$), 1.45-1.31 (m, 4H, $10-\text{H}_2, 11-\text{H}_2$).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 173.68 (CO_{ester}), 171.12, 170.94 (2C, C-2, C-6), 53.14 ($\text{CH}_{3,\text{ester}}$), 49.84 (C-3), 40.57, 40.48 (2C, C-13, C-8), 31.29 (C-5), 28.39, 28.29 (2C, C-9, C-12), 27.14, 26.86 (2C, C-10, C-11), 21.43 (C-4).

MS (MALDI): m/z $[\text{M}-\text{H}]^-$ calcd for $[\text{C}_{13}\text{H}_{22}\text{N}_2\text{O}_4-\text{H}]^-$ 269.15, found 268.96.

10.8.4.7 Dimethyl{3-[(6-aminohexyl)amino]-3-oxopropyl}malonate **322**



General procedure **K** was followed for deprotection of cyclic methyl carbamate **315** (500 mg, 1.44 mmol) in DCM (10 mL) and TFA (5 mL). Flash column chromatography, eluting with DCM/MeOH (10:1)

afforded (225 mg, 833 μ mol) colorless thick oil.

Yield: 40%.

R_f : 0.53 (DCM/MeOH, 7:1).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.69, 3.66 (2s, 6H, $2 \times \text{CH}_{3,\text{malonate}}$), 3.37 (dd, $J = 8.3, 6.6$ Hz, 1H, 2-H), 3.20 (td, $J = 6.9, 1.8$ Hz, 2H, $1''\text{-H}_2$), 2.93 (t, $J = 7.6$ Hz, 2H, $6''\text{-H}_2$), 2.35 (t, $J = 7.4$ Hz, 2H, $2'\text{-H}_2$), 2.24-2.04 (m, 2H, $1'\text{-H}_2$), 1.66 (quin, $J = 7.4$ Hz, 2H, $2''\text{-H}_2$), 1.54 (quin, $J = 6.9$ Hz, 2H, $5''\text{-H}_2$), 1.46-1.35 (m, 4H, 3-H₂, 4-H₂).

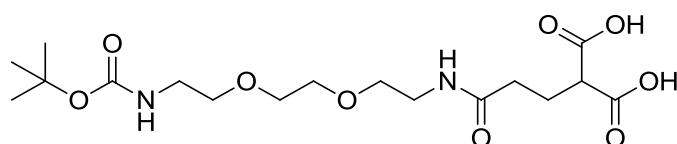
$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 174.74 (C-3'), 171.76, 170.82 (2C, $2 \times \text{CO}_{\text{malonate}}$), 52.81, 52.17 (2C, $2 \times \text{CH}_{3,\text{malonate}}$), 49.88 (C-2), 40.59 (C-1'), 40.24 (C-6'), 32.11 (C-2'), 29.95 (C-2''), 28.41 (C-5'), 27.13, 26.89 (2C, C-3'', C-4''), 25.17 (C-1').

MS (MALDI): m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_5+\text{H}]^+$ 303.18, found 303.18.

10.8.5 General procedure (L) for the hydrolysis of esters

LiOH was added to the solution of the ester in water/dioxane (1:4) in a round bottom flask fitted with reflux condenser and stirred at 55 $^\circ\text{C}$ till the completion of the reaction (3-4 h). The reaction mixture was allowed to cool to rt and neutralized with 1 N HCl. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography, eluting with a mixture of DCM and MeOH to afford the desired dicarboxylic acid.

10.8.5.1 (3-[[2-(2-{2-[(*tert*-Butoxycarbonyl)amino]ethoxy}ethoxy)ethyl]amino]-3-oxopropyl)malonic acid **262**



Following the general procedure (**L**), carbamate ester **301** (700 mg, 1.61 mmol) and LiOH (680 mg, 38.13 mmol, 28 equiv.) were refluxed in the solvent (10 mL). After the completion of the reaction, the solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography, eluting with DCM/MeOH (8:1) and 1% AcOH to afford **262** (600 mg, 1.48 mmol) as a white solid.

Yield: 92%.

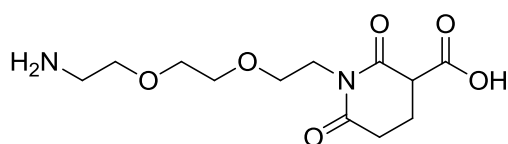
R_f : 0.19 (DCM/MeOH, 6:1 with 1% AcOH).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.63 (s, 4H, $1'''$ -H₂, $2'''$ -H₂), 3.58 (t, J = 5.3 Hz, 2H, $1''''$ -H₂), 3.53 (t, J = 5.5 Hz, 2H, $2'''$ -H₂), 3.44 (t, J = 5.3 Hz, 1H, 2-H), 3.41-3.35 (m, 3H, $2''''$ -H₂, 2-H), 3.24 (t, J = 5.5 Hz, 2H, $1''$ -H₂), 2.37 (t, J = 7.4 Hz, 2H, $2'$ -H₂), 2.24-2.04 (m, 2H, $1'$ -H₂), 1.45 (s, 9H, $3 \times \text{CH}_3$, Boc).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 178.39, 178.40 (2C, $2 \times \text{CO}_{\text{acid}}$), 173.87 (C-3'), 160.41 (C-1'''''), 82.15 (C-1'''''), 73.30, 73.26 (C-1''', C-2'''), 73.05 (C-2'), 72.38 (C-1'''), 51.92 (C-2), 43.21 (C-2'''), 42.57 (C-1'), 34.31 (C-2'), 30.82 ($3 \times \text{CH}_3$, Boc), 27.59 (C-1').

MS (MALDI): m/z $[\text{M}+\text{Li}]^+$ calcd for $[\text{C}_{17}\text{H}_{30}\text{N}_2\text{O}_9+\text{Li}]^+$ 413.21, found 413.27.

10.8.5.2 1-{2-[2-(2-aminoethoxy)ethoxy]ethyl}-2,6-dioxopiperidine-3-carboxylic acid **258**



General procedure (**L**) was followed for the treatment of the cyclic amino ester **259** (270 mg, 840 μmol) and LiOH (205 mg, 8.42 mmol) in the solvent system (5 mL). On the completion of the reaction (2.5 h) workup was performed and the residue was purified by flash chromatography to afford **258** (207 mg, 720 μmol) as a clear gel.

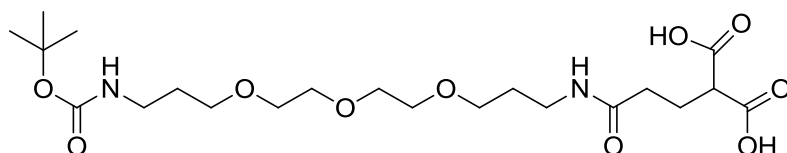
Yield: 85%.

R_f : 0.17 (DCM/MeOH, 10:1).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.78-3.74 (m, 2H, $1'$ -H₂), 3.69 (br s, 4H, $1''$ -H₂, $2''$ -H₂), 3.62 (t, J = 5.0 Hz, 2H, $1'$ -H₂), 3.46-3.40 (m, 3H, 3-H, $1'''$ -H₂), 3.17 (t, J = 6.8 Hz, 2H, $2'''$ -H₂), 2.38 (t, J = 7.1 Hz, 2H, 5-H₂), 2.14-2.11 (m, 2H, 4-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 176.64 ($3 \times \text{CO}$, C-2, C-6, CO_{acid}), 71.28 (C-1'', C-2''), 70.31 (C-2'), 67.72 (C-1'''), 49.53 (C-2), 40.7, 40.40 (C-1', C-1'''), 32.45 (C-5), 26.06 (C-4).

10.8.5.3 [3-({3-[2-(2-{3-[(*tert*-Butoxycarbonyl)amino]propoxy)ethoxy]ethyl}amino)-3-oxo-propyl]malonic acid **323**



General procedure (**L**) was followed for the hydrolysis of malonic ester carbamate **303** (270 mg, 510 μmol) with LiOH (239 mg, 9.96 mmol) in the solvent (5 mL). The crude product was flashed through silica gel column, eluting with DCM/MeOH (10:1) and 1% AcOH to afford **323** (224 mg, 470 μmol) as a clear thick oil.

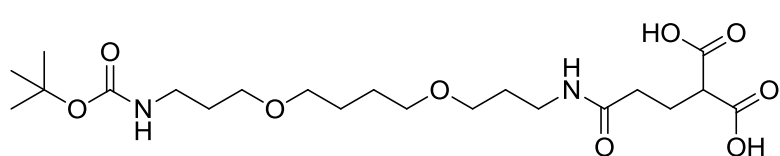
Yield: 92%.

R_f : 0.2 (DCM/MeOH, 6:1 with 1% AcOH).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.68-3.64 (m, 4H, $2'''$ -H₂, $1''''$ -H₂), 3.63-3.58 (m, 7H, $1'''$ -H₂, $2''''$ -H₂, $3''''$ -H₂, 2-H), 3.56 (t, J = 5.1 Hz, 2H, $3''$ -H₂), 3.53 (t, J = 5.1 Hz, 2H, $1''''$ -H₂), 3.14 (t, J = 6.8 Hz, 2H, $1''$ -H₂), 2.32 (t, J = 3.7 Hz, 2H, $2'$ -H₂), 2.11 (dt, J = 16.7, 7.5 Hz, 2H, $1'$ -H₂), 1.78 (quin, J = 6.0 Hz, 2H, $2''''$ -H₂), 1.73 (quin, J = 6.3 Hz, 2H, $2''$ -H₂), 1.45 (s, 9H, $3 \times \text{CH}_3$, Boc).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 176.38 (2C, $2 \times \text{CO}_2\text{H}$), 169.2 (C-3'), 158.23 (C-1'''''), 79.89 (C-1'''''), 71.52, 71.50 (C-2'', C-1'''), 71.24, 71.21 (C-1''', C-2'''), 69.88, 69.77 (C-3'', C-1'''), 54.5 (C-2), 38.71, 38.00 (C-1'', C-3'''), 32.33 (C-2'), 30.89, 30.21 (C-2'', C-2'''), 28.79 ($3 \times \text{CH}_3$, Boc), 27.2 (C-1').

10.8.5.4 (3-[[3-(4-{3-[(*tert*-Butoxycarbonyl)amino]propoxy}butoxy)propyl]amino]-3-oxopropyl)malonic acid **326**



Following the general procedure (**L**), the solution of LiOH (96 mg, 4.00 mmol) and ester **311**

(200 mg, 410 μmol) was refluxed in the solvent (4 mL) for 3 h. The workup was performed and the crude product was purified by flash chromatography, eluting with DCM/MeOH (10:1) and 1% AcOH to afford **326** (135 mg, 300 μmol) as a colorless oil.

Yield: 70%.

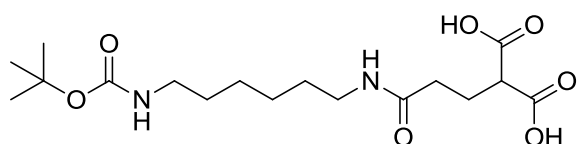
R_f : 0.45 (DCM/MeOH, 10:1 with 1% AcOH).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.50-3.42 (m, 8H, $3''$ -H₂, $1'''$ -H₂, $4'''$ -H₂, $1''''$ -H₂), 3.33-3.21 (m, 3H, $3''''$ -H₂, 2-H), 3.12 (t, J = 6.9 Hz, 2H, $1''$ -H₂), 2.32-2.29 (m, 2H, $2'$ -H₂), 2.20-2.04 (m, 2H, $1'$ -H₂), 1.78 (quin, J = 6.8 Hz, 2H, $2''''$ -H₂), 1.71 (quin, J = 6.0 Hz, 2H, $2''$ -H₂), 1.62 (dt, J = 5.9, 3.1 Hz, 4H, $2'''$ -H₂, $3'''$ -H₂), 1.43 (s, 9H, $3 \times \text{CH}_3$, Boc).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 177.25 (C-3'), 175.24, 173.78 (2C, $2 \times \text{CO}_{\text{acid}}$), 158.41 (C-1'''''), 79.82 (C-1'''''), 71.80, 71.74 (C-1''', C-4'''), 69.51, 69.33 (C-3'', C-1'''), 54.14 (C-2), 38.84, 38.11 (C-1'', C-3'''), 33.27 (C-2'), 30.97, 30.32 (C-2'', C-2'''), 28.79 ($3 \times \text{CH}_3$, Boc), 27.49 (C-2'', C-3'''), 27.16 (C-1').

MS (ESI): m/z $[\text{M}+\text{Na}]^+$ calcd for $[\text{C}_{21}\text{H}_{38}\text{N}_3\text{O}_9+\text{Na}]^+$ 485.24, found 485.24.

10.8.5.5 [3-({6-[(*tert*-Butoxycarbonyl)amino]hexyl}amino)-3-oxopropyl]malonic acid **325**



General procedure (**L**) was followed for the treatment of ester **322** (650 mg, 2.15 mmol) and LiOH (388 mg, 16.21 mmol, 10 equiv.) in the

solvent (15 mL). The crude product was purified by silica column, eluting with DCM/MeOH (10:1) and 1% AcOH to afford **325** (490 mg, 1.78 mmol) as a colorless oil.

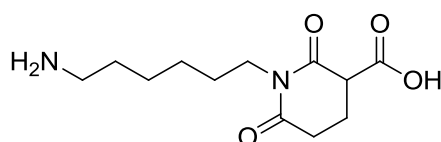
Yield: 83%.

R_f : 0.32 (DCM/MeOH, 7:1 with 1% AcOH).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.78-3.76 (m, 2H, 6''-H₂), 3.74-3.71 (m, 1H, 2-H), 3.04 (t, $J = 7.0$ Hz, 2H, 1''-H₂), 2.72-2.68 (m, 2H, 2'-H₂), 2.18 (dt, $J = 6.2, 5.81$ Hz, 2H, 1'-H₂), 1.56-1.48 (m, 4H, 2''-H₂, 5''-H₂), 1.45 (s, 9H, 3 \times CH₃,Boc), 1.32-1.29 (m, 4H, 4''-H₂, 5''-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 17.55 (C-3'), 171.06, 170.82 (2C, 2 \times CO_{acid}), 158.40 (C-1''), 79.71 (C-1'''), 53.13 (C-2), 41.19, 40.68 (C-1'', C-6'), 31.35, 30.77 (C-2'', C-5'), 28.79 (3 \times CH₃,Boc), 28.69 (C-2'), 27.49, 27.42 (C-3'', C-4'), 21.48 (C-1').

10.8.5.6 1-(6-aminohexyl)-2,6-dioxopiperidine-3-carboxylic acid **327**



General procedure (**L**) was followed for the treatment of ester **319** (350 mg, 1.23 mmol) and LiOH (600 mg, 25.02 mmol, 20 equiv.) in the solvent (12 mL). The crude

product was purified by silica column, eluting with DCM/MeOH (10:1) and 1% AcOH to afford **328** (280 mg, 1.08 mmol) as a colorless oil.

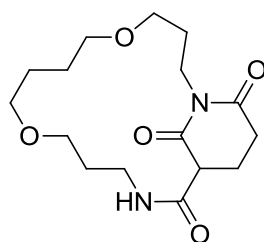
Yield: 87%.

R_f : 0.21 (DCM/MeOH 2:1).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.27-3.16 (m, 2H, 1'-H₂), 2.92 (t, $J = 6.5$ Hz, 2H, 6''-H₂), 2.28 (t, $J = 7.7$ Hz, 2H, 5-H₂), 2.16-2.02 (m, 2H, 4-H₂), 1.66 (quin, $J = 6.6$ Hz, 2H, 5'-H₂), 1.55 (quin, $J = 6.4$ Hz, 2H, 2'-H₂), 1.45-1.34 (m, 4H, 3'-H₂, 4'-H₂).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 178.44 (CO_{acid}), 176.85 (C-2), 173.79 (C-6), 49.49 (C-3'), 40.42 (C-6'), 39.87 (C-1'), 34.27 (C-5), 29.91 (C-2'), 28.29 (C-5'), 27.54 (C-4), 26.96 (C-3), 26.67 (C-4').

10.8.6 5,10-Dioxa-1,14-diazabicyclo[14.3.1]icosane-15,19,20-trione **328**



KOH (500 mg) was added to the solution of ester **318** (150 mg, 430 μmol) in EtOH/H₂O (10 mL) and stirred for 4 h. The crude product obtained after aqueous workup was purified by flash chromatography, eluting with DCM/MeOH (10:1) to afford **328** (100 mg, 300 μmol) as a clear thick oil.

Yield: 75%.

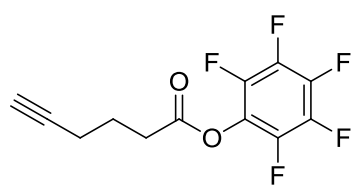
R_f : 0.26 (DCM/MeOH, 7:1).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.56 (t, $J = 5.8$ Hz, 2H, 4- H_2), 3.56-3.46 (m, 7H, 11- H_2 , 9- H_2 , 6- H_2 , 16-H), 3.29-3.27 (m, 2H, 13- H_2), 3.06 (t, $J = 7.0$ Hz, 2H, 2- H_2), 2.25 (t, $J = 7.6$ Hz, 2H, 18- H_2), 2.12-2.01 (m, 2H, 17- H_2), 1.92-1.89 (m, 2H, 3- H_2), 1.76 (quin, $J = 6.4$ Hz, 2H, 12- H_2), 1.65-1.62 (m, 4H, 7- H_2 , 8- H_2).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 179.58 (C-19), 177.35 (C-20), 173.97 (C-15), 71.93, 71.74 (C-9, C-6), 69.32 (C-4, C-11), 56.70 (C-16), 39.2 (C-2), 37.78 (C-13), 35.22 (C-18), 30.39 (C-12), 28.55, 28.24 (C-3, C-17), 27.42, 27.41 (C-7, C-8).

10.8.7 Conjugation of amines with hexynoic acid

10.8.7.1 Pentafluorophenyl hex-5-ynoate **331**



In a dried three neck flask, 5-hexynoic acid **148** (1.00 g, 8.92 mmol) and DIPEA (2.83 g, 3.8 mL, 21.72 mmol) were dissolved in DCM (100 mL) under nitrogen atmosphere. The solution was cooled to 0 °C with continuous stirring followed by slow addition of pentafluorophenyl trifluoroacetate **266** (2.85 g, 10.08 mmol) and the solution was allowed to warm to rt. After 2 h, the solvent was evaporated and the residue was subjected to flash chromatography, eluting with Et_2O /Pent (1:1) to afford **331** (1.45 g, 5.21 mmol) as a clear oil.

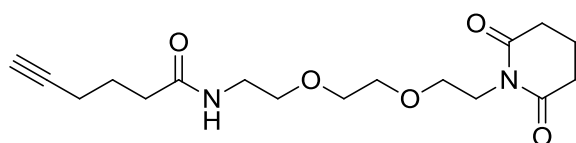
Yield: 58%.

R_f : 0.36 (Et_2O /Pent, 1:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 2.83 (t, $J = 7.4$ Hz, 2H, 2- H_2), 2.36 (td, $J = 6.9, 2.6$ Hz, 2H, 4- H_2), 2.02 (t, $J = 2.1$ Hz, 1H, 6-H), 1.98 (quin, $J = 7.1$ Hz, 2H, 3- H_2).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ 169 (C-1), 142.63 (C-1'), 140.91 (C-4'), 140.09 (C-2'), 139.32 (C-5'), 138.39 (C-3'), 136.85 (C-4'), 82.54 (C-5), 69.83 (C-6), 32.02 (C-2), 23.49 (C-4), 17.75 (C-3).

10.8.7.2 *N*-(2-{2-[2-(2,6-Dioxopiperidin-1-yl)ethoxy]ethoxy}ethyl)hex-5-ynamide **332**



In a dried three neck flask, activated ester of 5-hexynoic acid **331** (55 mg, 200 μmol) and amino dicarboxylic acid **261** (50 mg, 160 μmol) were dissolved in anhyd. DMF (3 mL) under inert atmosphere, followed by the addition of DIPEA (102 mg, 810 μmol) at rt. The solution was stirred overnight and the residue was purified by flash chromatography, eluting with DCM/MeOH (10:1) to afford **331** (30 mg, 750 μmol) as a clear oil.

Yield: 46%.

R_f : 0.35 (DCM/MeOH, 15:1).

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 4.01 (t, $J = 6.0$ Hz, 2H, $1'\text{-H}_2$), 3.61 (t, $J = 5.9$ Hz, 2H, $2'\text{-H}_2$), 3.60-3.58 (m, 2H, $1''\text{-H}_2$), 3.57-3.55 (m, 2H, $2''\text{-H}_2$), 3.52 (t, $J = 5.0$ Hz, 2H, $1'''\text{-H}_2$), 3.44 (t, $J = 5.5$ Hz, 2H, $2'''\text{-H}_2$), 2.66 (2t, $J = 6.4$ Hz, 4H, 3-H_2 , 5-H_2), 2.34 (t, $J = 7.4$ Hz, 2H, $2''''\text{-H}_2$), 2.25 (dt, $J = 10.3$, 2.6 Hz, 2H, $4''''\text{-H}_2$), 1.96 (t, $J = 2.6$ Hz, 1H, $6''''\text{-H}$), 1.94 (quin, $J = 6.8$ Hz, 2H, 4-H_2), 1.86 (quin, $J = 7.2$ Hz, 2H, $3''''\text{-H}_2$).

$^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 175.47 (CO_{amide}), 174.97, 174.96 (C-2, C-6), 84.21 (C-6'''), 71.27, 71.15 (C-1'', C-2'), 70.56 (C-2'), 70.19 (C-5'''), 68.84 (C-1'''), 40.34 (C-1'), 39.28 (C-2''), 35.81 (C-2'''), 33.53, 33.52 (C-3, C-5), 25.94 (C-3'''), 18.63 (C-4'''), 18.07 (C-4).

MS (ESI): m/z $[\text{M-H}]^-$ calcd for $[\text{C}_{17}\text{H}_{26}\text{N}_2\text{O}_5\text{-H}]^-$, 337.18, found 337.18.

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