Accumulation and Biological Activity of Oxidized Lipids in *Anabaena* PCC 7120

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Table of contents

List of abbreviations

λ	wave length
1D	one-dimension
2D	two-dimension
BHT	tert-Butylhydroxytoluol
BSA	bovine serum albumin
BSTFA	N,O-Bis-(trimethylsilyl)trifluoracetamide
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CI	chemical ionisation
DTT	dithiothreitol
DIEA	N,N-Diisopropylamine
DW/TG	dry weight / trockengewicht
EDTA	ethylene diamine tetraacetic acid
EI	electron ionisation
FW	fresh weight
GC-MS	gas chromatography – mass spectrometry
HETE	hydroxyeicosatetraenoic acid
HL	high light
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography-mass spectrometry
IAA	Iodoacetamide
IEF	isoelectric focusing
IPG	immobile pH gradient
IS	internal standard
JA	jasmonic acid
LDS	lithium dodecyl sulfate
LL	low light
m/z	mass charge ratio
MRM	multiple reaction monitoring
nano-LC-MS/MS	nano liquid chromatography tandem mass spectrometry
NH_2	aminopropyl
NCI	negative chemical ionisation

OD	optical density
OH-FA	hydroxy fatty acid
OPDA	12-oxo-phytodienoic acid
PAGE	polyacrylamide gel electrophoresis
PDA	
PFB-Br	pentafluorbenzylbromide
PG	prostaglandin
PP	phytoprostane
ROS	reactive oxygen species
RP	reverse phase
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription – polymerase chain reaction
SDS	sodium dodecyl sulfate
SiOH	silica
SPE	solid phase extraction
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TMS	trimethylsilyl
TPP	triphenylphosphine
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume pro volume
w/v	weight pro volume

I. INTRODUCTION

I.1 General introduction into the cyanobacteria

I.1.1 The appearance of cyanobacteria

The earth was formed about 4,500 billion years ago (Halliday, 2001) and the early life in it was dominated by microbes (Carroll, 2001) (Fig. I-1). Cyanobacteria, which were formerly termed 'blue green algae', belong to one of the oldest organism on the earth (Schopf and Packer, 1987). Earlier claims for the existence of cyanobacteria was 3,500 (Awramik, 1992; Schopf, 1993) and maybe early as 3,700 billion years ago (Des Marais, 2000; Kopp *et al.*, 2005). However, chemical evidence and recent fossil findings indicate that cyanobacteria existed only about 2,500 – 2,600 billion years ago (Olson and Blankenship, 2004).

I.1.2 Cyanobacteria as architects of the early earth atmosphere

Cyanobacteria are considered as a key to any understanding of earth's early biological and environmental history (Tomitani *et al.*, 2006). Photosynthesis was well established on the earth at least 3,500 billion years ago, and it is widely believed that these ancient organisms had similar metabolic capacities to modern cyanobacteria (Blankenship, 1992). Transition from an anoxic to an oxic atmosphere occurred nearly 2,300 billion years ago (Farquhar *et al.*, 2000) and the atmosphere started to accumulate significant quantities of molecular oxygen for about 2,200 – 2,400 billion years ago (Farquhar *et al.*, 2000; Line, 2002; Catling and Zahnle, 2002). These facts are in agreement with an oxygen build-up model from Brock *et al.* (1994) (Fig. I-1). The point in time of the initial O_2 rise is relatively well established, but the question of what triggered it remains hotly debated (Kasting, 2001). The evolution of O_2-producing cyanobacteria that use water as terminal reductant transformed Earth's atmosphere to a suitable one for the evolution of aerobic metabolism and complex life forms (Dismukes *et al.*, 2001).

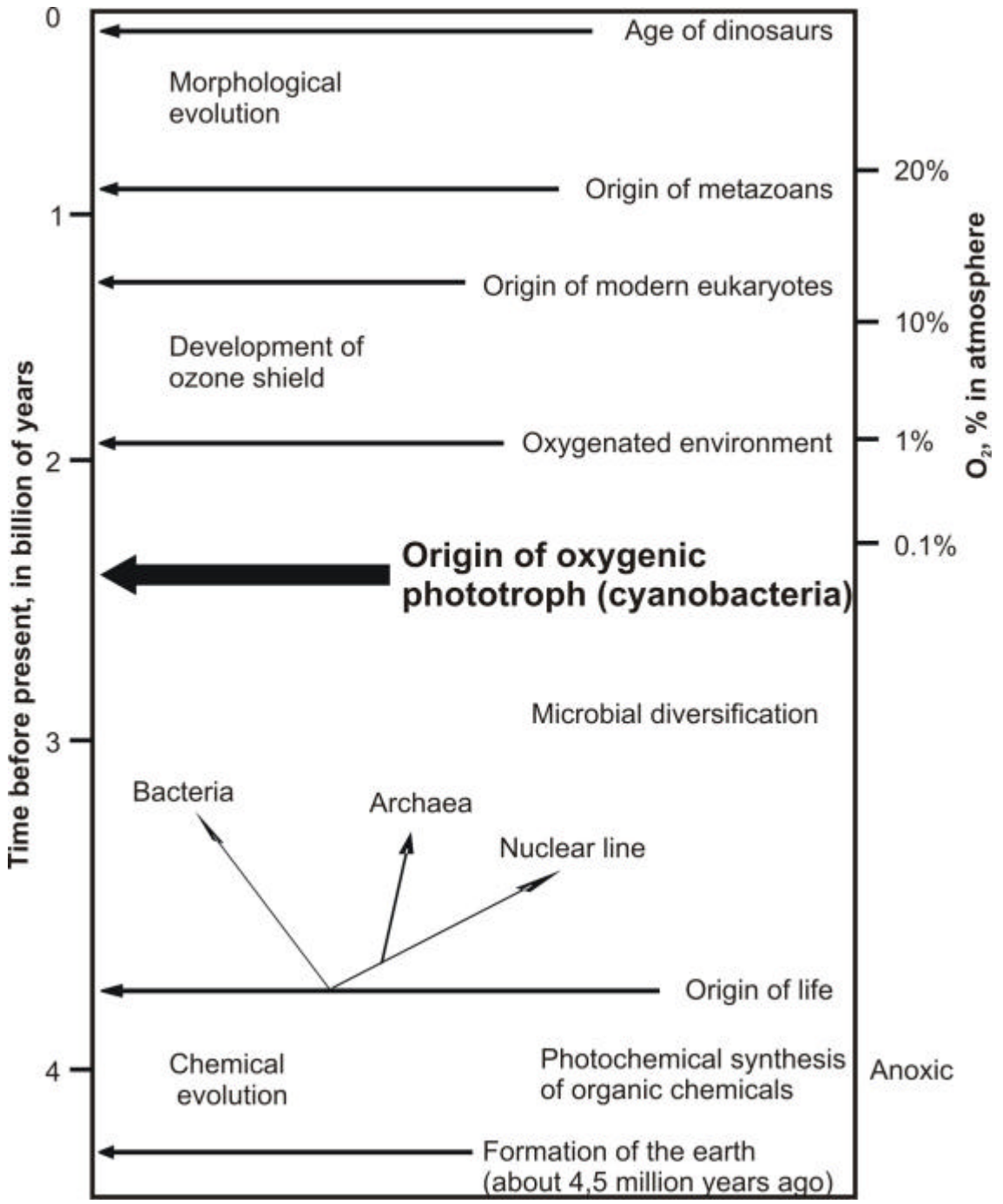


Figure 1. Major events in biological evolution. Time scale and % oxygen are estimated. The oxygenation of the atmosphere was slow and gradual process, occurring over a period of about a billion years (Figure taken from Brock *et al*., 1994).

The transition to an oxic atmosphere occurred when the net photosynthetic production of O_2 exceeded the input of reduced gases to the atmosphere (Kasting, 2001). In the face of rising O_2, which is usually considered the first bio-generated environmental pollutant to appear in large quantities on the planet, anaerobes died or restricted themselves to anoxic environments, whereas other organisms began using O_2 for metabolic transformations (Herkovits, 2006). Oxygen itself is not only toxic for anaerobes at the beginning of evolution

but also toxic for cyanobacteria themselves. Their adaptation capability to oxygenic atmosphere is quite remarkable because nitrogenase, the enzyme responsible for reducing N_2 is poisoned (Kasting and Siefert, 2002) or irreversibly inhibited by O_2 or reactive oxygen species (ROS) (Berman-Frank *et al.*, 2003). Thus, cyanobacteria have had to evolve complex mechanisms for protecting their nitrogenase (Kasting and Siefert, 2002). The filamentous *Anabaena* spp. fix nitrogen only in specialized cells called heterocysts (Wolk *et al.*, 1994; Adams, 2000) whereas other cyanobacteria fix nitrogen at night and photosynthesize by day (Kasting and Siefert, 2002).

I.1.3 Diversity, adaptation and ecological roles of cyanobacteria

Cyanobacteria are oxygenic photosynthetic bacteria that are widely distributed in aquatic and terrestrial environments, including such extreme habitats like hot springs, deserts, and pole regions (Whitton and Potts, 2000). Up to now, there are more than 2000 cyanobacterial species that have already been identified. Cyanobacteria are classified into five orders: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales (Van den Hoek *et al.*, 1995). Cyanobacteria are the largest group of gram-negative photosynthetic prokaryotes (Sinha *et al.*, 2001). Under environmental stress like cold, desiccation, nutrient deficiency, etc. they form akinetes to survive (Herdman, 1987; Van den Hoek *et al.*, 1995).

Cyanobacteria represent important components of both aquatic and terrestrial ecosystems and possess a central position in the nutrient cycling due to their unique capacity to fix atmospheric nitrogen into NH^{4+}, a form through which nitrogen enters into the food chain. N_2-fixing cyanobacteria form a prominent component of microbial populations in wetland soils, especially in rice paddy fields, where they significantly contribute as a natural bio-fertilizer (Sinha and Haeder, 1998; Sinha *et al.*, 1998). For example, cyanobacteria represent only about 1% of marine biomass but they are the main organisms responsible for nitrogen fixation in the ocean (Tyrrell, 1999) and serve as global nitrogen budget (Capone *et al.*, 1997; Karl *et al.*, 2002). Cyanobacteria also play a vital role in the maintenance of soil fertility (Roger and Watanabe, 1986). Plant – cyanobacterial symbiosis was considered as a potential bionitrogen fertilizer for paddy rice (Sinha and Haeder, 1998). Cyanobacteria occur as free-living nitrogen fixers (*Anabaena, Calothrix, Nostoc*) or symbiotic nitrogen fixers (*Gumera – Nostoc*, *Azolla – Anabaena*). Especial symbiotic associations display a powerful nitrogen fixation, reaching up to 0.5 kg nitrogen per hectare in the rice field in the case of *Azolla – Anabaena* symbiosis (Taiz and Zeiger, 2006).

Cyanobacteria are not only playing an important role in natural ecosystems but they also display a great potential in medical research. Cyanovirin-N, a protein extracted from *Nostoc ellipsosporum* is the first cyanobacterial compound with anti-microbial activity against HIV (Boyd *et al.*, 1997).

I.1.4 Toxicity from cyanobacteria

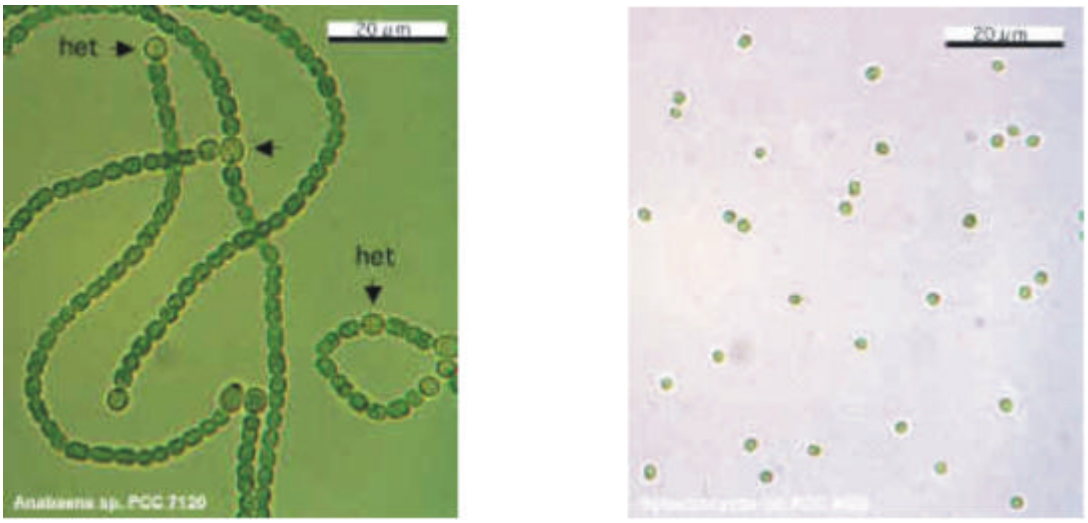
Although they have a great contribution to life on the earth (primary producer for oxygen and nitrogen), toxic cyanobacterial secondary metabolites are a source of nuisane for many other living organisms including humans (Duy *et al.*, 2000). During cyanobacterial blooming, cyanobacterial toxin concentrations are relatively high and, in some cases, become toxic for humans and animals. Cyanobacterial toxins are usually divided into two groups: hepatotoxins and neurotoxins, basing on their target organs of toxicity, liver and nervous system, respectively (Harada, 2004; Dittmann and Wiegand, 2006).

I.1.5 Some cyanobacterial species are biological model organisms for scientific research

Several different species of cyanobacteria have been used in scientific research. From those, *Synechocystis sp.* PCC 6803 and *Anabaena sp.* PCC 7120 are most widely used as biological model organisms.

Synechocystis sp. PCC 6803 is a unicellular cyanobacterium in fresh water, which is not capable of nitrogen fixation (Fig. I-2). It has been extensively used as a powerful model to study the molecular mechanisms of heat-induced damage, stress responses (Balogi *et al.*, 2005) and photosynthesis (Matsuda *et al.*, 2005). Its genome was completely sequenced in 1996 ("http://www.kazusa.or.jp/cyanobase/Synechocystis/index.html").

Anabaena sp. PCC 7120 is a filamentous, nitrogen fixing cyanobacterium, which occurs in rice paddy fields (Fig. I-2). It is generally used as a model organism to study nitrogen fixation, preferable in rice ecosystem. *Anabaena* develops special cells called heterocysts to fix atmospheric nitrogen. Heterocysts provide a suitable anaerobic environment for nitrogenase and a single heterocyst develop at intervals of about 10 – 15 vegetable cells (Adams, 2000). Its genome was completely sequenced in 2001 ("http://www.kazusa.or.jp/cyanobase/Anabaena/index.html").



Anabaena sp. PCC 7120 *Synechocystis sp.* PCC 6803

Figure I2. *Anabaena* PCC 7120 with heterocyst (het) (left) and *Synechocystis* PCC 6803 (right). (Source: http://www.ibvf.cartuja.csic.es/cultivos/main.html).

I.1.6 Cyanobacterial fatty acid profile and its meaning

Unlike other bacteria, membrane lipids of cyanobacteria contain polyunsaturated fatty acids (Brock *et al.*, 1994). Therefore, some authors used them for chemotaxonomic classification of cyanobacteria (Cohen *et al.*, 1995; Li *et al.*, 1998; Li and Watanabe, 2001; Gugger *et al.*, 2002). Polyunsaturated fatty acids are important for *Synechocystis* to grow in extreme environments, e.g. at low temperature (Gombos *et al.*, 1992, 1994) or under salt stress (Allakhverdiev *et al.*, 1999). The fatty acid composition of *Synechocystis* PCC 6803 is not affected by light intensity (Kis *et al.*, 1998). Interestingly, Gombos *et al.* (1997) demonstrated that *Synechococcus* PCC 7942 with a higher content of unsaturated fatty acids exhibited an enhanced tolerance to high light stress.

It is generally assumed that the chloroplasts of plants are derived from a cyanobacterial ancestor via a process called endosymbiosis (Delwiche and Palmer, 1997; Blankenship, 2002). Up to 75% of the genes are identical in the genome of plant chloroplasts and cyanobacteria (Raven and Allen, 2003). Both, the plant organelle and cyanobacteria contain a high ratio of polyunsaturated fatty acids in their membranes. Looking at the polyunsaturated fatty acids in chloroplast, one can see that α-linolenic acid is the most abundant (Taiz and Zeiger, 2006). Fatty acid composition in cyanobacteria varies from one species to another. However, the polyunsaturated fatty acid content is usually high, up to 58% of the total lipids (Kenyon, 1972). The main difference in polyunsaturated fatty acid profile between *Anabaena* and *Synechocystis* is that the former contains α-linolenic acid and the

latter possesses γ-linolenic acid but their relative amounts are almost equal, in a range of about 20% of total lipid in each species (Li and Watanabe, 2001).

I.2 Oxidative stress in cyanobacteria

All living organisms must acclimate to environmental stresses that can potentially damage cellular processes (Asada, 1994). Production of reactive oxygen species (ROS) is a typical characteristic for all aerobic organisms, however, photosynthesis is an additional, specific ROS generating process in "green" organisms (Perelman *et al.*, 2003). ROS are also produced in response to pathogen attack (Apel and Hirt, 2004). In the past, ROS or reactive oxygen intermediates were considered as toxic by-products of aerobic metabolism, but recent studies show that plants also actively produce ROS as signaling molecules to regulate processes such as programmed cell death, gene expression, abiotic stress responses, pathogen defense, and systemic acquired resistance (Mittler, 2002; Apel and Hirt, 2004). The most important ROS are singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) or nitric oxide (NO, also classified as reactive nitrogen species). In plants and animals, superoxide can be generated by NADPH oxidase through transferring electrons from NADPH inside the cell across the membrane to reduce molecular oxygen (Mittler, 2002). Superoxide can be metabolized by superoxide dismutase to hydrogen peroxide, which in turn in a substrate for catalases and peroxidases (Fig. I-3). The accumulation of ROS is assumed to be involved in the initiation of the hypersensitive response in plants when being infected by a pathogen. Among the ROS, the hydroxyl radical is the strongest oxidant and can initiate radical chain reactions in a variety of organic molecules, leading to lipid peroxidation, protein oxidation and inactivation, as well as oxidative nucleic acid degradation (Lamb and Dixon, 1997; Imlay, 2003) (Fig. I-3).

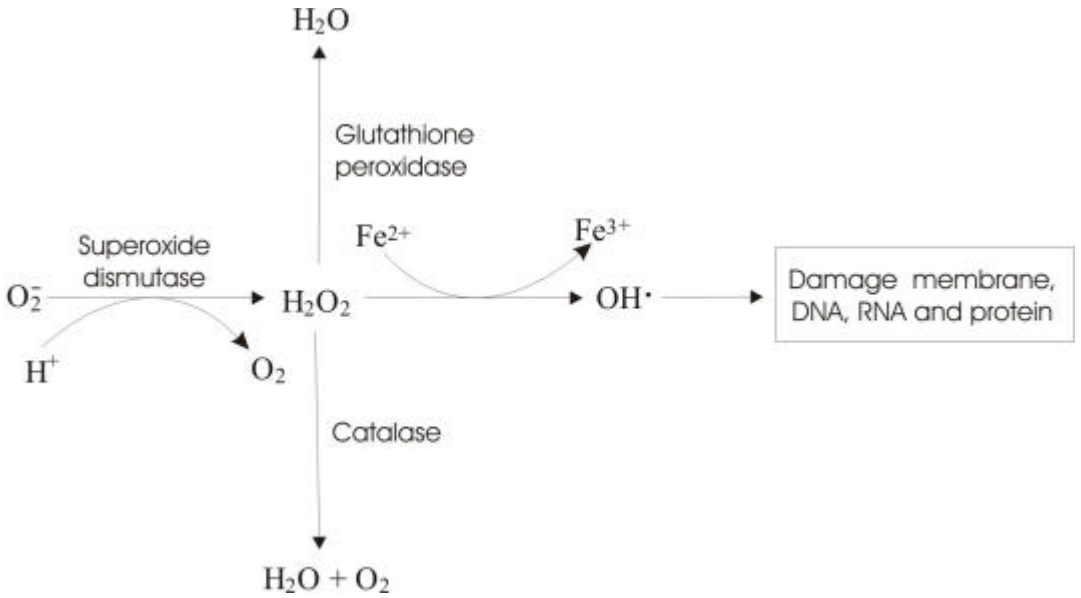


Figure I-3. Reactive oxygen species and the main detoxifying enzymes in living organisms.

Membrane lipids are a major target of free radical attack leading to lipid peroxidation. Some products of lipid oxidation are also highly reactive species and may modify proteins and DNA (Singh *et al.*, 2002; Mittler, 2002) (Fig. I-3). Lipid peroxidation is a self-propagating process including the following reactions (Frankel, 1998; He and Haeder, 2002; Montuschi *et al.*, 2004).

Initiation:	$R^\cdot$	+ LH	?	RH + $L^\cdot$
Propagation:	$L^\cdot$	+ O_2	?	$LOO^\cdot$
	$LOO^\cdot$	+ LH	?	LOOH + $L^\cdot$
Termination:	$L^\cdot$	+ AH	?	LH + $A^\cdot$
	$A^\cdot$	+ $LOO^\cdot$	?	LOO-A
	$2LOO^\cdot$		?	LOOL + O_2
	$2L^\cdot$		?	L – L

where R = free radical species; LH = lipid; $L^\cdot$ = lipid radical; $LOO^\cdot$ = lipid peroxyl radical; LOOH = lipid peroxide; A = antioxidant (usually ascorbate or tocopherol).

Under high light conditions photosystem I can produce superoxide anions ($O_2^{\cdot-}$), which can be converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (Asada, 1999). Hydrogen peroxide in turn can be detoxified by catalases in all organisms. In addition, H_2O_2 can also be metabolized by peroxidases, glutathione peroxidases, haloperoxidases, and ascorbate peroxidase (Regelsberger *et al.*, 2002; Miyake *et al.*, 1991; Srivastava *et al.*, 2005). Peroxiredoxins and thioredoxins are considered as important anti-

oxidative compounds found in cyanobacteria (Matsuda *et al.*, 2005; Perelman *et al.*, 2003; Stork *et al.*, 2005). In *Anabaena*, antioxidative enzymes (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) and antioxidants (glutathione, ascorbate, tocopherol and carotenoids) are induced by salt or copper stress (Srivastava *et al.*, 2005).

Most research on cyanobacterial stress so far has focused on *Synechocystis* in relation to photosynthesis. Changing from low light to high light conditions, *Synechocystis* and *Prochlorococcus* exhibit very sensitive transcriptional responses of stress-associated genes (Mary *et al.*, 2004). Transcription of more than 160 genes was modulated when *Synechocystis* was acclimated to high light (Hihara *et al.*, 2001). The *isiA* (iron stress induced protein A) genes have been proven to be highly stress-responsive under a variety of environmental conditions, including iron deficiency, high salt, and oxidative stress (Singh *et al.*, 2005). Under high light condition, *isiA* is induced and protects cyanobacteria from photo-oxidative stress (Havaux *et al.*, 2005) or photo-inhibition (Cadoret *et al.*, 2004). *isiA* plays an important role in adaptation of the photosynthetic electron transport chain in cyanobacteria to iron deficiency (Pistorius and Michel, 2004). When *Synechocystis* PCC 6803 was stressed with 75 µM H_2O_2 for 15 min, gene expression was modulated, particular the expression of *isiA* (Li *et al.*, 2004). *isiA* was also identified in *Anabaena* PCC 7120 and it was shown that *isiA* was regulated by *pkn22* (putative Ser/Thr kinase). The *pkn22* mutants cannot grow in an iron-limited environment (Xu *et al.*, 2003).

Under high UV-light conditions, cyanobacteria can produce secondary metabolites. For instance, enhanced ultraviolet radiation induced the formation of mycosporine-like amino acids (MAAs) and scytonemin in canobacteria to avoid adverse effects of short waves (Sinha *et al.*, 1998, 1999, 2001; Klisch *et al.*, 2002). He *et al.* (2002) showed that there is a relationship between UV stress, increase of oxidative stress and DNA damage in *Anabaena*. Cyanobacteria can also produce heat shock proteins under high temperature or intensive light stress (Nitta *et al.*, 2005). Effects of UV-B light on *Anabaena doliolum* were enhanced in combination with high temperature or chemical stress like heavy metals (Srivastava *et al.*, 2006).

Heavy metals, especially some trace elements are necessary nutrients for all living organisms at low concentration. Metals are important because they are involved in electron chain reactions in photosynthesis and respiration. On the other hand, toxicity of high heavy metal concentrations is a general problem for humans, animals, plants and bacteria. When exposed to a higher than appropriate dose, living organisms have to develop appropriate metabolic mechanisms to survive. Free metal ions can react with H_2O_2. By this reaction, the

well-kown Fenton reaction, highly reactive hydroxyl radical can be formed by oxidation of redox active metal ions, for instance, Cu^{1+} ? Cu^{2+} or Fe^{2+} ? Fe^{3+} (Fig. I-3).

Toxicity of heavy metal ions on cyanobacteria was shown in several studies i.e. for copper (Laube *et al.*, 1980; Surosz and Palinska, 2004; Hullebusch *et al.*, 2002; Villada *et al.*, 2004; Srivastava *et al.*, 2005), aluminum (Rai *et al.*, 1996), cadmium (Surosz and Palinska, 2004), lead (Zaccaro *et al.*, 2001), nickel and silver (Rai and Raizada, 1987). At low dose, heavy metals can cause growth inhibition or ultrastructure changes in cyanobacteria (Surosz and Palinska, 2004). In contrast, high levels of heavy metals cause cell lysis. For example, treatment of *Anabaena* PCC 7120 with copper (Cu^{2+}) inhibits growth (Laube *et al.*, 1980; Surosz and Palinska, 2004) and causes cell lysis at early time points of treatment (Laube *et al.*, 1980). Copper also alters the ultrastructural characteristics of vegetative cells, resulting in a degradation of chlorophyll a and reduced respiration in *Anabaena flos-aquae* (Surosz and Palinska, 2004). Interestingly, copper always exhibits a higher toxicity compared to cadmium when applied in equal concentrations (Surosz and Palinska, 2004). Because of its high toxicity to cyanobacteria, copper sulfate is used as algicide to control cyanobacterial bloom (Hullebusch *et al.*, 2002; Villada *et al.*, 2004). However, not only excess but also lack of metal nutrient can cause oxidative stress in cyanobacteria. For instance, iron deficiency leads to oxidative stress in *Anabaena* PCC 7120 via a significant increase of ROS (Latifi *et al.*, 2005). Metal toxicity in cyanobacteria can be lowered by chemical antioxidants. For example, nickel and silver toxicities on *Nostoc muscorum* can be reduced by antioxidants such as ascorbic acid and glutathione or sulfur amino acids such as L-cysteine and L-methionine (Rai and Raizada, 1987).

It is clear that heavy metal stress is one of the causes, which contribute to generate ROS leading to lipid oxidation in the membrane. Mechanisms of lipid peroxidation in plants and animals as well as the functions of their oxylipins will be discussed in the next parts.

I.3 Phytoprostanes

Products from oxidative fatty acids metabolism are generally called oxylipins. Cyclic fatty acids such as prostaglandins and jasmonates are well-known signal compounds in animals and plants. In addition, oxylipins can also be formed non-enzymatically via free radical catalyzed pathways. Chemical oxidation of fatty acids may yield oxylipins that structurally resemble prostaglandins and jasmonates such as isoprostanes (in animals) and phytoprostanes (in plants) (Blee, 2002, Thoma *et al.*, 2004). In mammals and higher plants,

isoprostanes and phytoprostanes have been described as archetypal compounds that display in part similar biological activities as prostaglandins and jasmonates (Mueller, 2004). Similar to 12-oxo-phytodienoic acid (precursor of jasmonic acid), phytoprostanes induce genes involved in plant defense and biosynthesis of secondary metabolites (Loeffler *et al.*, 2005). Formation of phyto-oxylipins is often among the first reaction of plants to different stress conditions (Blee, 2002; Feussner and Wasternack, 2002).

I.3.1 Prostaglandins and isoprostanes in animals

In animals, oxylipins are produced from arachidonic acid (C20:4), the major polyunsaturated fatty acid in mammal membranes. When arachidonic acid is metabolized through enzymatic and non-enzymatic pathways, prostaglandins and isoprostanes, respectively, are produced (Fig. I-4). Prostaglandins and isoprostanes are present in human and mammal blood or tissues at very low concentrations ranging from from pM to nM (Russell *et al.*, 1975; Morrow *et al.*, 1990). In general, prostaglandins are involved in many physiological processes of mammals. Prostaglandins play an important role in the regulation of inflammation and organ development (Vane *et al.*, 1998; Bazan *et al.*, 2002; Miller, 2006). In animals, prostaglandins are essential compounds for development and survival. For example, mice mutants lacking the prostaglandin H synthases cannot develop a mature renal system or die shortly postnatal (Morham *et al.*, 1995; Langenbach *et al.*, 1995). In addition, prostaglandins also activate many pro-inflammatory genes, which are triggered during infections such as cyclooxygenase 2 (COX-2), tumor-necrose-factor α (TNFα), and inducible NO-synthase (iNOS) (Straus and Glass, 2001).

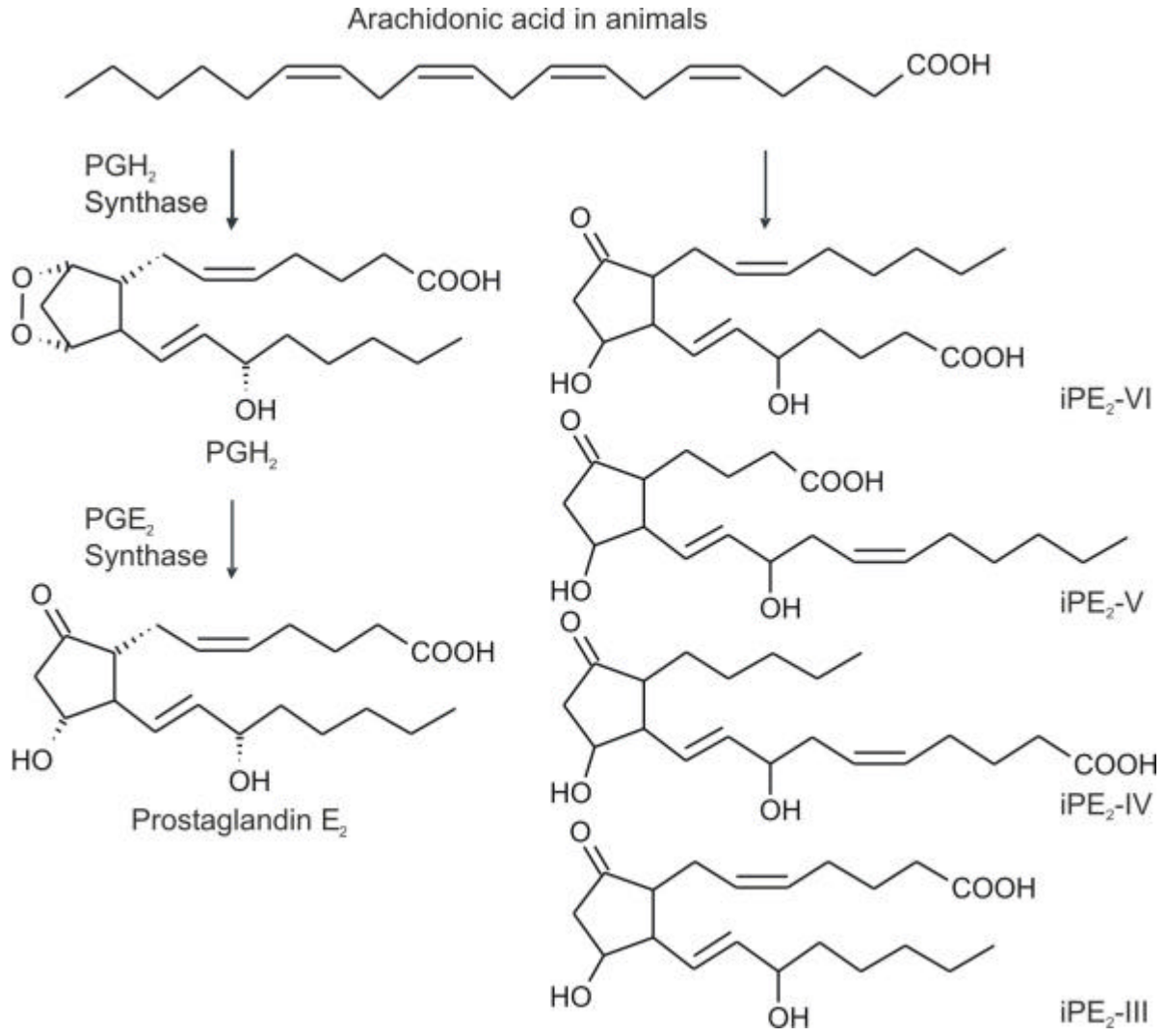


Figure I-4. Biosynthesis of prostaglandins and isoprostanes from arachidonic acid in animals. Prostaglandin E_2 (PGE_2) and isoprostanes E_2 (iPE_2) are structural examples of these classes of compounds. According to Mueller (1998).

Morrow *et al.* (1990) discovered that free radical catalyzed peroxidation of arachidonic acid forms a series of prostaglandin structure-like compounds termed isoprostanes (Fig. I-4). Among them, F_2-isoprostanes have been the most extensively studied in human physiology (Montuschi *et al.*, 2004). Isoprostanes accumulate as a consequence of oxidative stress (Roberts and Morrow, 2002) or diseases (Janssen, 2001) and are considered as accurate, reliable as well as sensitive markers and mediators of oxidative stress (Morrow and Roberts, 1996; Mueller, 1998; Montuschi *et al.*, 2004). *In vivo,* isoprostanes are biological active in nano- to micro-molar concentrations. They can induce mitogenesis as well as endothelium and fibric cell proliferation (Cracowski, 2004). It has also been postulated that isoprostanes may play an important role in the function and development of the circulatory and nervous system (Cracowski, 2004; Montuschi *et al.*, 2004).

I.3.2 Jasmonates and phytoprotanes in plants

Higher plants lack the enzymatic capacity to synthesize arachidonic acid. Instead, a-linolenic acid is the most prominent polyunsaturated fatty acid in their membranes, accounting for up to 40% of the total polyunsaturated fatty acids in plants (Conconi *et al.*, 1996). In addition, a-linolenic acid is the main precursor of oxylipins in plants.

I.3.2.1 Jasmonates

Jasmonates in plants are produced from a-linolenic acid by an enzymatic pathway (Mueller, 1997) (Fig. I-5). Jasmonic acid and 12-oxo-phytodienoic acid (OPDA) are commonly referred to as jasmonates. In plants, OPDA biosynthesis is carried out in chloroplasts and then transferred to peroxisomes, where OPDA can be converted to jasmonic acid by the action of an OPDA reductase and ß-oxidation (Mueller, 1997). Like prostaglandins in animals, jasmonates play a critical role in plant growth, defense, development, and reproduction although the involved signal transduction mechanisms and physiological reactions are completely different (Creelmann and Mullet, 1997). Jasmonates have an important function in plant defense against fungi, bacteria, and herbivores (Mueller, 1998). Jasmonic acid is known to modulate gene expression involved in plant defense, secondary metabolism and plant development. Among the genes induced by jasmonic acid are those that encode important key enzymes in all major pathways for secondary metabolites (Gundlach *et al.*, 1992; Mueller *et al.*, 1993; Gundlach and Zenk, 1998). For instance, methyl jasmonate induces the biosynthesis and accumulation of scopoletin in the extracellular medium of tobacco cell culture (Sharan *et al.*, 1998). Jasmonic acid plays a critical role for plant resistance to insects (Wasternack, 2007). Mutants of *Arabidopsis thaliana* defective in the formation of jasmonic acid produce only low level of jasmonic acid and are susceptible to insect attack. Exogenous application of jasmonic acid reconstitutes resistance nearly to the levels of the wild type plant (McConn *et al.*, 1997). Some important physiological functions of jasmonates are summarized in table I-1.

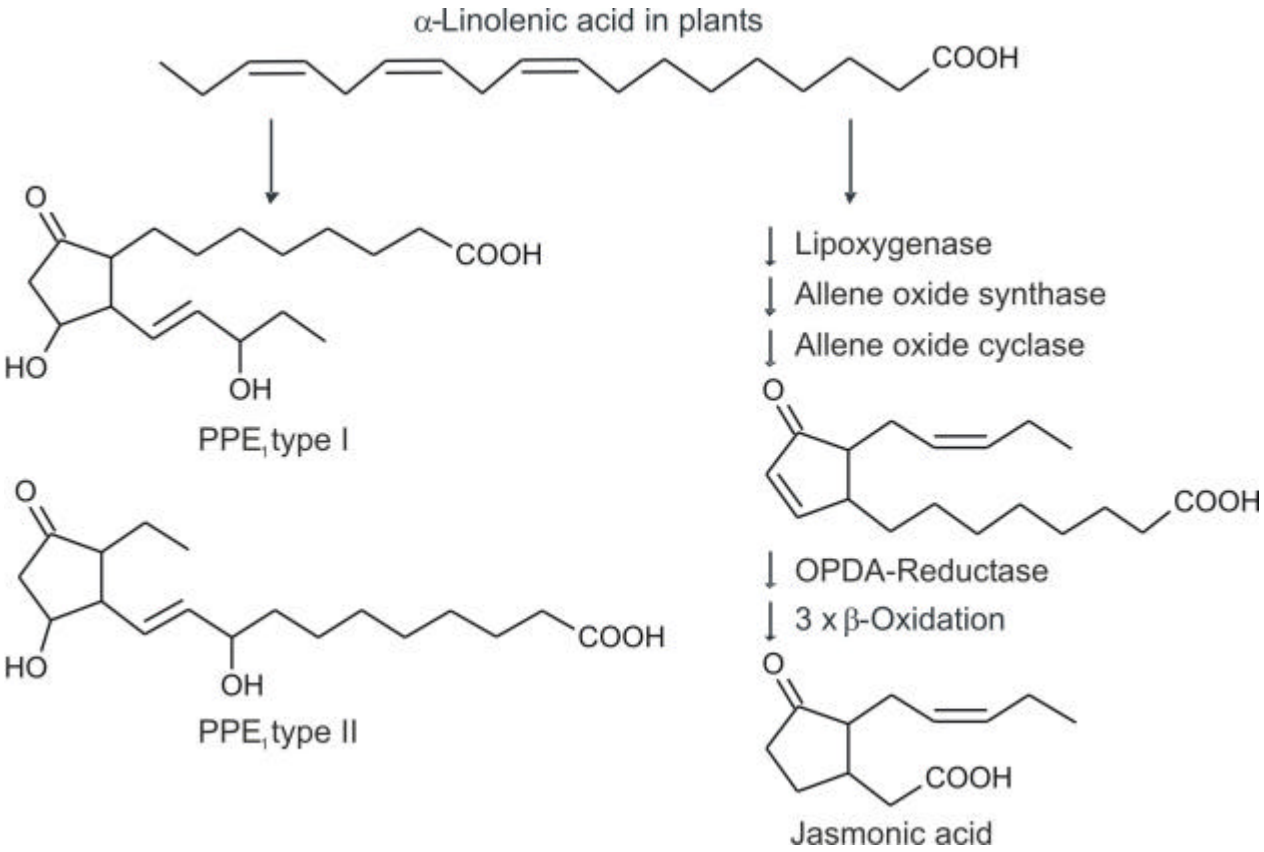


Figure I-5. Metabolic pathways of α-linolenic acid in plants. Enzymatic pathway: formation of OPDA and jasmonic acid (right). Non-enzymatic pathway: formation of phytoprostanes (left). Here, PPE_1 type I and type II are shown as representative examples.

Table I-1. Physiological functions of jasmonates in plants (simplified from Creelmann and Mullet, 1997)

Types of function	Physiological effect
Seed germination and growth	
Seed, pollen germination	-
Root growth	-
Tendril coiling	+
Photosynthesis/vegetable sinks	
Photosynthesis apparatus	-
Vegetative protein storage	+
Tuberization	+
Flower and fruit development	
Pollen viability	+
Fruit ripening, pigments	+
Seed development	+
Insect and disease resistance	
Insect resistance	+
Disease resistance	+

The well-known functions of jasmonates in plants are due to the availability of *Arabidopsis* mutants. Mutants lacking OPDA-reductase 3 (OPR3 mutants) cannot convert OPDA to jasmonic acid and are sterile. Fertility can be restored by exogenous application of

jasmonic acid (Stintzi and Browse, 2000). Hence, there is an absolute requirement for jasmonic acid for pollen development and fertility (Xie *et al.*, 1998). When wild type, *opr3* mutants, *fad3fad7fad8*-triple-mutants (lacking α-linolenic acid and jasmonates) and *coi1* mutants (jasmonic acid insensitive 1) were infected with necrotrophic fungus *Alternaria brassicicola*, only *opr3* mutants and wild type can survive long term, indicating that OPDA is sufficient to protect the plants (Stintzi *et al.*, 2001). Thus, OPDA is sufficient to confer resistance to *A. brassicicola*. Interestingly, although there are many jasmonate mutants available, jasmonate receptors have not yet been identified (Wasternack, 2007).

I.3.2.2 Discovery and biosynthesis of phytoprostanes in plants

Phytoprostanes are a novel family of cyclic compounds, which are formed non-enzymatically by a free radical catalyzed mechanism from a-linolenic acid (C18:3) (Parchmann and Mueller, 1998; Loeffler *et al.*, 2005) (Fig. I-5). Although dinor-isoprostanes in plants were already discovered in 1998 (Parchmann and Mueller, 1998), the name phytoprostane (PP) was suggested for this kind of substances in 2000 (Imbusch and Mueller, 2000a).

Linolenate can be released from membrane lipids by the action of a lipase and metabolized via lipoxygenase/allene oxide synthase pathway to prostaglandin-like plant hormones of the jasmonate family (Mueller, 1997) (Fig. I-5). In the non-enzymatic pathway, however, esterified a-linolenate in complex membrane lipids is oxidized in situ to a series of phytoprostanes that can be released by the action of so far unknown lipases, The autoxidation reaction is similar to the formation of isprostanes from arachidonic acid. The first intermediates G_1 -phytoprostanes (PPG_1, Fig. I-6). PPG_1 are unstable and may decompose in aqueous environment to PPD_1, PPE_1 and PPF_1. PPD_1 and PPE_1 may further dehydrate and isomerize to PPJ_1, deoxy-PPJ_1, PPA_1 and PPB_1 (Fig I-7) (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000b; Krischke *et al.*, 2003; Thoma *et al.*, 2003).

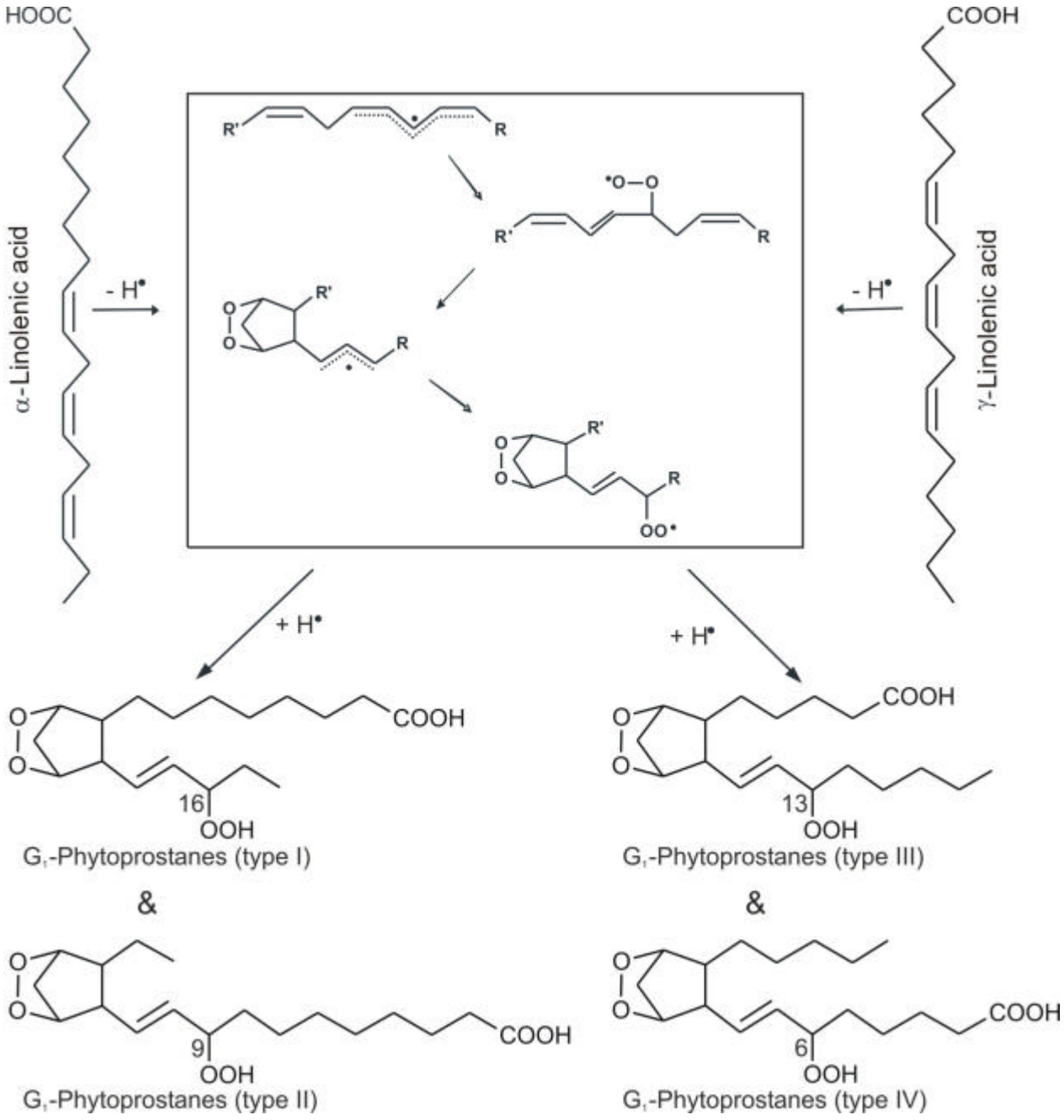


Figure I6. Formation of PPG_1 by autoxidation and cyclization of a-linolenic acid or ?-linolenic acid. Modified from Thoma *et al.* (2004).

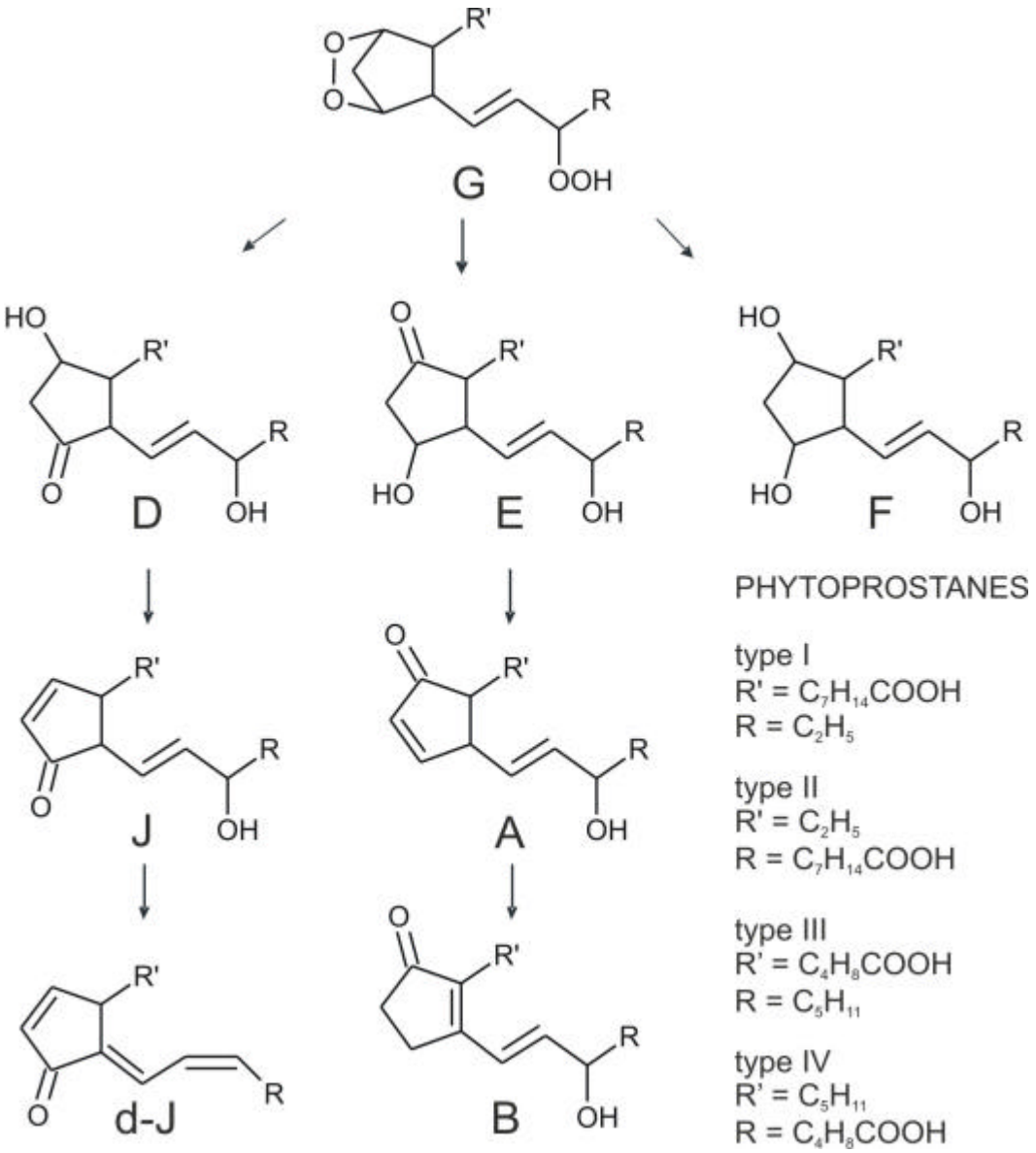


Figure I-7. Phytoprostane pathway in plants. PPG_1 accumulates not in plants and is rapidly decomposed to yield PPD_1, PPE_1 and PPF_1. PPF_1 is stable; PPD_1 and PPE_1 may dehydrate and isomerize to PPJ_1 and deoxy-PPJ_1 (d-J in this figure) or PPA_1 and PPB_1, respectively. According to Thoma *et al.* (2004).

I.3.2.3 Occurrence, analysis and classification of phytoprostanes in plants

PPE_1/PPB_1 were characterized in 1998 by Parchmann and Mueller (1998), both after autoxidation of a-linolenic acid *in vitro* and in plant cell cultures *in vivo.* Autoxidation of a-linolenic acid yielded 0.31% of PPE_1 (type I and type II). Levels of PPE_1 found in plant cell cultures were in the range of 4.5 to 60.9 ng/g dry weight (Parchmann and Mueller, 1998). In addition, PPF_1 were also identified in plants by Imbusch and Mueller (2000a; 2000b). The PPA_1, PPD_1, $dPPJ_1$ were subsequently identified and quantified by Thoma *et al.* (2003) and Krischke *et al.* (2003). However, quantification/determination of phytoprostanes is difficult

due to the presence of several structural classes and isomers (more than 136 molecular species in total can be formed via the phytoprostane pathway (Imbusch and Mueller, 2000a) and the low abundance of these compounds in comparison to the high levels of primary and secondary metabolites in plant materials (Thoma *et al.*, 2004). Therefore, highly sensitive and specific analysis methods have been developed i.e. using GC-MS or HPLC coupled to a fluorescence detector to quantify phytoprostanes. The internal standards, which have been so far used, are oxygen-18 labelled phytoprostanes that have been prepared by autoxidation of a-linolenic acid in an $^{18}O_2$ atmosphere (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000b). Some phytoprostanes are unstable and have to be converted into more stable compounds prior GC-MS and HPLC analysis. For example, PPE_1 could not be measured by GC-MS and were quantified as PPB_1 derivatives after isomerization of PPE_1 to PPB_1 (Parchmann and Mueller, 1998). In addition, derivatisation of hydroxy and carboxy groups is necessary to obtain volatile derivatives suitable for GC-MS analysis (Imbusch and Mueller, 2000a).

PPE_1 and PPF_1 have been found to occur ubiquitously in higher plants in the ng/g fresh weight range (Imbusch and Mueller, 2000b; Parchmann and Mueller, 1998). This range is within the range of their enzymatically synthesized congeners, OPDA and jasmonic acid. Phytoprostanes can be dramatically induced (up to 30-fold for PPF_1) in plants by oxidative stress caused by treatment with peroxides, heavy metals or wounding (Imbusch and Mueller, 2000a; Thoma *et al.*, 2003). After abiotic stress, phytoprostane levels may exceed basal levels of jasmonates in plant cells by more than an order of magnitude (Imbusch and Mueller, 2000a; Parchmann *et al.*, 1997). Thoma *et al.* (2003) also showed that jasmonic acid levels of tobacco cell cultures treated with 1 mM *t*-BuOOH was constant and below the concentration of phytoprostanes. These authors also showed that the infection of tomato leaves with a necrotrophic fungus (*Botrytis cinerea*) induced phytoprostanes levels up to four times higher than those found in control leaves. Infection of *Arabidopsis thaliana* by inoculation of an avirulent strain of *Pseudomonas syringae* resulted in 10-fold increase of PPF_1 5 hours after infection (Grun *et al.*, 2007).

Thoma *et al.* (2004) have classified phytoprostanes into 2 groups: cyclopentanone phytoprostanes and cyclopentenone phytoprostanes. Table I-2 shows the levels of phytoprostanes and jasmonates in tomato leaves.

Table I-2. Levels of free phytoprostanes and jasmonates in tomato leaves (means ± S.D) (according to Thoma *et al.*, 2004)

Cyclopentane phytoprostanes/DW (ng/g)

	Type I	Type II
PPD_1	459 ± 130	698 ± 161
PPE_1	35 ± 8	35 ± 12
PPF_1	75 ± 25	75 ± 25

Cyclopentene phytoprostanes/DW (ng/g)

	Type I	Type II
PPA_1	3.3 ± 2.0	1.2 ± 2.0
PPB_1	1.2 ± 1.1	0.75 ± 1.5
$dPPJ_1$	293 ± 20	1668 ± 202

Jasmonates/DW (ng/g)

12-Oxo-phytodienoic acid	Jasmonic acid
212 ± 51	20 ± 5

In plants, phytoprostanes occur in two forms: free and esterified in membrane lipids. Overall, concentrations of esterified phytoprostanes are generally higher compared to free phytoprostanes. For instance, the levels of free and esterified (total) PPF_1 in plants are in the range of 4 – 144 and 47 – 1124 ng/g of dry weight, respectively (Imbusch and Mueller, 2000a, b). Free A_1, B_1, D_1, E_1, F_1, and deoxy-J_1 phytoprostanes have been detected in all plants so far analyzed (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000a, b; Krischke *et al.*, 2003; Thoma *et al.*, 2003). Extremely high phytoprostane levels can be found in certain plant organs. For example, fresh birch pollens were shown to contain more than 32 µg/g of free PPF_1 (Imbusch and Mueller, 2000b) or 17.72 µg/g PPE_1 (Hoffmann *et al.*, 2005).

Notably, phytoprostane concentrations dramatically increase *post mortem* by autoxidation when plants are dried and stored after harvest (Thoma *et al.*, 2004). Levels of free and esterified PPF_1 in various dried plant are in the range of 3000 – 20000 ng/g and 10000 – 55000 ng/g of dry weight, respectively, which is up to 250-fold higher than in fresh plant parts (Imbusch and Mueller, 2000b). After cell death, a-linolenic acid in cell membranes is readily autoxidized after a lag period (during which endogenous antioxidants are preferentially oxidized), resulting in a dramatic increase of phytoprostane concentrations. When cultured tobacco cells were killed by shock freezing in liquid nitrogen and thawing,

PPE_1 and PPB_1 levels in dead cells remained constant for at least 8 h during storage at RT, but thereafter, a more than 40-fold increase of PPE_1 was observed within 20 h (Loeffler *et al.*, 2005).

Therefore, phytoprostanes occur not only ubiquitously at low levels in living plants but also at high levels in (partially or completely) autoxidized plant products. For example in commercial plant oils (fresh), levels of total F_1-phytoprostanes were found to range from 0.32 to 13.83 mg/l (Karg *et al.*, 2007). Thus, phytoprostanes could potentially be used as markers of oxidative degradation of plant derived foodstuff (Thoma *et al.*, 2004).

I.3.2.4 Biological functions of phytoprostanes

Recent studies show that some physiological and biological functions of phytoprostanes are similarly to those of jasmonates in plants, e.g. induction of phytoalexins like scopoletin (Thoma *et al.*, 2003). Application of a mixture of PPE_1 or PPA_1 (each comprising all isomers, obtained from *in vitro* autoxidation of a-linolenic acid) at a total concentration of 10 µM to tobacco cell cultures led to a 6- and 10-fold induction of scopoletin (an antimicrobial phytoalexin in the solanaceous family), respectively, in the culture medium after 4 hours (Thoma *et al.*, 2003). In another experiment, Loeffler *et al.* (2005) showed that PPB_1 type II (10 µM) induce scopoletin synthesis in tobacco cell culture better than PPB_1 type I and equivalent to jasmonic acid. Phytoprostanes induce not only scopoletin in solanaceous plants but also other phytoalexins in different plant families. For example, PBB_1 displayed an effective induction of benzophenanthridine alkaloids (macarpine, sanguinarine, and chelirubine) in cell cultures of *Eschscholzia californica* (Papaveraceae), flavanoids (isobavachalcone) in *Crotalaria cobalticola* (Fabaceae) cell cultures, and alkaloids (camalexin) in *Arabidopsis thaliana* plants (Loeffler *et al.*, 2005). In all examples studied, phytoprostanes were at least as active as jasmonates in eliciting secondary metabolites.

Pretreatment of tobacco cell culture with phytoprostane can activate an adaptive response of the cells. Tobacco cells were preincubated with 75 µM of PPB_1 for 17 hours and subsequently stressed with 10 mM $CuSO_4$. Determination of dead cells after additional 24 h revealed that more than 50% of PPB_1-treated cells survived. In control cells that were not preincubated with PPB_1 and only treated with copper ions a dramatic cell death (75 – 95%) was observed (Loeffler *et al.*, 2005). Therefore, phytoprostanes (PPB_1) may prime plants cells to resist oxidative stress. Similar to jasmonates, several phytoprostanes inhibit root growth (Mueller and Berger, unpublished).

Besides the induction of phytoalexin biosynthesis, cyclopentenone compounds such as PPA_1 and PPB_1 also induce MAPK (mitogen-activated protein kinase) activity in tomato cell suspension cultures at a concentration of 75 µM within 5 min (Thoma *et al.*, 2003). In addition, infiltration of 4 nmol of PPA_1 or PPB_1 into leaves of a GST1:GUS reporterline of *Arabidopsis thaliana* increased GUS-activity dramatically, 11 and 14-fold, respectively (Thoma *et al.*, 2003). Microarray analysis of 626 genes in *Arabidopsis thaliana* after treatment with 75 µM PPB_1 type I or type II revealed an alteration in gene expression of 60 genes. Notably, treatment with PPB_1 led to an induction of genes involved in detoxification, e.g. 17 putative glutathione S-transferases, 8 glycosyltransferases, 2 glutathione S-conjugate ABC transporters, and 3 ABC transporters (Loeffler *et al.*, 2005). Induction of expression of detoxification genes indicates that phytoprostanes might be rapidly metabolized which is supported by a fast decrease of PPB_1 levels after exogenous application in tobacco cell cultures (Loeffler *et al.*, 2005).

Biological and physiological functions of phytoprostanes in plants gradually become clear. Interestingly, phytoprostanes also exhibit pharmacological effects on mammalian cells. For example, PPA_1 and deoxy-PPJ_1 similar to the structurally related prostaglandins PGA_1 and deoxy-PGJ_2 induced apoptosis in Jurkat T cells and inhibited NFκB signalling (Karg *et al.*, 2007). In addition similar to the action of PGE_2, PPE_1 were shown to potently modulate human dendritic cell maturation and to inhibit interleukin 12 production in response to lipopolysaccharide (Hoffmann *et al.*, 2005; Gutermuth *et al.*, 2007).

Although phytoprostanes cannot be formed in humans endogenously, substantial amounts of phytoprostanes are contained in the daily diet and can be absorbed. After consumption of vegetable oils, PPF_1 was detected in human blood and urine (Karg *et al.*, 2007). However, research on the biological activities of phytoprostanes in humans has just begun and the relevance of phytoprostanes for physiological and/or pathophysiological processes in humans remains to be clarified.

I.4 Aims of the work

Animals and plants can form isoprostanes and phytoprostanes from arachidonic and a-linolenic acid by non-enzymatic pathway, respectively. Formation of these oxylipins is inducible in plants and animals under oxidative stress. Bacteria generally lack both polyunsaturated fatty acids as well as enzymatic oxylipin pathways. Cyanobacteria potentially could represent an exception since they utilize α- or γ-linolenate as structural fatty acid in

their membranes. Moreover, cyanobacteria are believed to be progenitors of plant chloroplasts in which the first enzymatic steps of jasmonate biosynthesis take place. One of the fully sequenced cyanobacteria, *Anabaena*, also contains putative lipoxygenase and allene oxide synthase genes that catalyse the first two steps of jasmonate biosynthesis in plant chloroplasts. While it remains to be clarified in this work if jasmonates are synthesized in *Anabaena* species, it is likely that non-enzymatic lipid oxidation pathways exist. Another species, *Synechocysis*, does neither contain a-linolenic acid nor homologs of plant jasmonate biosynthesis genes and is therefore not expected to produce jasmonates. However, the presence of ?-linolenate in their membranes suggests that *Synechocystis* may produce certain phytoprostane isomers that have previously not been characterized.

Since cyanobacteria belong to an evolutionary old group of organisms, it is exciting to study the biosynthesis and function of oxylipins. A metabolic model of cyclic oxylipin biosynthesis from polyunsaturated fatty acids is shown in Fig. I-8 is expected.

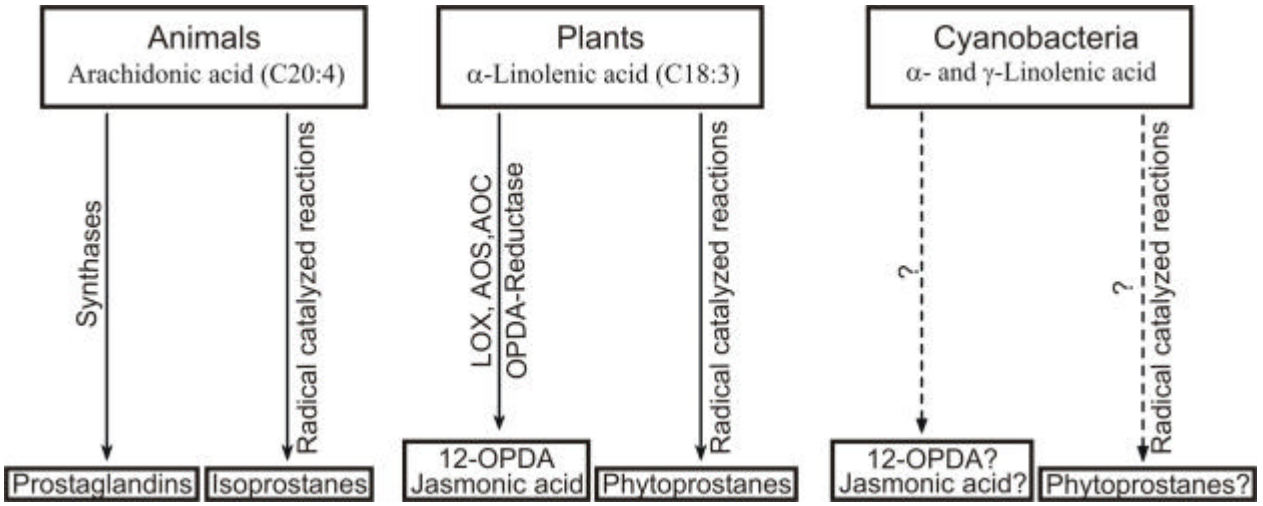


Figure I-8. Oxidative metabolism of fatty acids to prostaglandin-like oxylipins. Pathways displayed by solid line arrows are well-known. Dashed line arrows indicate so far unknown pathways. LOX: lipoxygenase; AOS: allene oxide synthase; AOC: allene oxide cyclase; 12-OPDA: 12-oxo-phytodienoic acid.

The cyanobacteria *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 have been fully sequenced and possess a- or ?-linolenic acid in their membranes; therefore, it is highly interesting to investigate the occurrence of phytoprostanes and jasmonates in cyanobacteria.

Until now biological activities and functions of jasmonates in plants have been well characterised on the gene expression and functional level. There have been several reports about biological activities of phytoprostanes in plants, however, their function and significance remains to be clarified. To this end, unraveling the putative biological activity of

oxylipins in cyanobacteria may provide us with insights about evolutionary ancient functions of oxidized lipids.

Hence, the aim of the work is to study biosynthesis and function of oxylipins in cyanobacteria using the filamentous growing *Anabaena* PCC 7120 and the unicellular *Synechocystis* PCC 6803 strain as model organisms.

II. MATERIALS AND METHODS

II.1 Chemicals

Chemicals used in experiments were obtained from Aldrich (Steinsheim), Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Invitrogen (Karlsruhe), AppliChem (Darmstadt), Sigma (Deisenhofen) or Fermentas (St. Leon-Rot, Germany).

Prostaglandines and 15-HETE (15-Hydroxyeicosatetraenoic acid) were from Cayman Chemicals (Ann Arbor, USA).

Borage oil was from Henry Lamotte Ltd, Bremen, Germany.

II.2 Materials

Anabaena PCC 7120 was provided by Dr. Dirk Schneider, Institute for Biochemistry and Molecular Biology, University of Freiburg. *Synechocystis* PCC 6803 was from CNCM (Collection Nationale de Cultures des Microorganismes), Pasteur Institute, Paris.

Aminopropyl (NH_2)- und Silica (SiOH)-material were from Macherey and Nagel, Düren, Germany. Silica-material 60 (particle size 60-100 µm) was obtained from Merck, Darmstadt, Germany.

UV block shield was a courtesy of Department of Botany II, Julius-von-Sachs-Institute, University of Würzburg.

II.3 Equipments

Gas Chromatography- Mass Spectrometry: GC-MS
Agilent 6890 Series Gaschromatography
(Agilent, Waldronn)
JMS-GC Mate II Massenspectrometry
(Jeol, Tokyo, Japan)

High Performance Liquid Chromatography- Mass Spectrometry: HPLC-MS
HPLC system: Agilent 1200 Series (Agilent, Waldbronn)
1200 Standard Autosampler
1200 Binary Pump
Pre-Column: Purospher Star RP 18ec 4 x 4 mm, 5 µm (Merck, Darmstadt)

Column: Purospher Star RP 18ec 125 x 2 mm, 5 µm (Merck, Darmstadt)

MS system: Quattro Premier Triple Quadrupole Mass Spectrometer (Waters/Micromass, Milford, USA)

HPLC: HPLC-system (Waters, Milford, USA)

W600 pump

W600 controller

W717 auto sampler

996 diode array detector

W474 fluorescence detector

Millenium 32 software

Microscope: Axioskop (Carl Zeiss, Jena)

Sonicator: cell disruptor B15 (G. Heinemann, Schwäbisch Gmünd)

UV-Spectrometer: Specord 200 (Analytic, Jena)

Mixer: Magnetic mixer IKA-Werke (Janke & Kunkel, Staufen)

Magnetic mixer KMO electronic 2 (Janke & Kunkel, Staufen)

Vortex: Vortex (Heidolph, Kehlheim)

Solid phase extraction (SPE): vacuumbox for SPE-columns (Diagen, Düsseldorf) with membrane vacuum pumpe MZ 2C (Vacuubrand, Wertheim)

Zentrifuges: Sorvall RC-5B and RC-5C with rotor SS34 and GSA (DuPont, Bad Homburg)

Eppendorf-table zentrifuges 5414R, 5417R, 5804R (Eppendorf, Hamburg)

Megafuge 1.0 R (Heraeus-Christ, Osterode)

Jouan KR25i (Thermo Scientific)

Rotary evaporator: Rotavapor R 110 (Büchi, Flawil, Schweiz)

Rotational-vacuum concentrator RVC 2-25 (Christ, Osterode, Germany)

Shaking: Rotation shaker G53 (New Brunswick Scientific, Nürtingen)

4020-shaker for multiple plate (Köttermann, Hänigsen)

Autoklave: Technomara-Autoklave (Fedegari, Albuzzano, Italy)

Temperation/Incubation: Thermo stable water bath Julabo EM und Thermomixer MM (Braun, Melsungen)

Dry chamber (Heraeus-Christ, Osterode)

- 20°C freezer (Bauknecht)

- 80°C freezer (New Brunswick Scientific, Nürtingen)

Weighing: Analytical balance 2006 MP (Sartorius, Göttingen)

Laboratory balance 1213 MP (Sartorius, Göttingen)

Balance BP 3100P (Sartorius, Göttingen)

Analytic balance BP 211D (Sartorius, Göttingen, Germany)

Microscope: Axioskop (Carl Zeiss, Jena)

PCR: Hybaid Limited PCR Sprint

Lamps: HPS 400 W for high light intensity was from Hortilux Schréder, Groeilicht – Holland.

UV-Lamp MinUVIS with λ = 254 nm and 356 nm (Desaga, Heihelberg)

RNA/DNA quantification: GeneQuant, Pharmacia Biotech, Cambridge – England.

Gel documentation: VisiLuxx Imager (Visitron Systems. Munich)

Gel electrophoresis system: Electrophoresis comb (Peqlab Biotechnologie Ltd., Erlangen, Germany)

Ettan DALTsix electrophoresis unit (GE Healthcare, Munich, Germany)

Xcell Sure Lock (Invitrogen, Karlsruhe, Germany)

Isoelectric focusing system: Ettan™ IPGphor 3, GE Healthcare, Munich, Germany

Immobiline™ dry strip reswelling tray, GE Healthcare, Munich, Germany

ZOOM IEF fractionator (Invitrogen, Karlsruhe, Germany)

Power supply system: Consort EV215 Electrophoresis Power Supply

Electrophoresis Power Supply – EPS 301 (Amersham Pharmacia Biotech, Munich, Germany)

Electrophoresis Power Supply – EPS 601 (GE Healthcare, Munich, Germany)

ZOOM Dual Power (Invitrogen, Karlsruhe, Germany)

Nano-HPLC-MS/MS

HPLC: Ultimate 3000 (LC Packings, Dionex, Idstein, Germany)

with Chromeleon software

Pre-Column C18, 100 µm I.D., 5 µm particles, 2 cm, nanospeparations, (Nieuwkoop, Netherlands)

Separation column, C18 pepmap, 75 µm I.D., 3 µm particles, 15 cm, (LC Packings, Dionex, Idstein, Germany)

Mass spectrometry: LCQ Deca XP Plus (Thermo Electron, San Jose, USA)

with Xcalibur software

Documentation: Microtek SM9800XL scanner (Microtech, Krefeld, Germany)

with SilverFast software

Other equipments: Laminar-Flow-Box (Prettl)

Ultra sonic water bath Sonorex TK 52 (Bandelin)

pH-Meter 766 Calimatic (Knick, Berlin)

All other equipments used in this work correspond to usual laboratory standards.

II.4 Cell culture of *Anabaena* PCC 7120 und *Synechocystis* PCC 6803

Anabaena and *Synechocystis* were grown in 1 L glass flasks containing 500 ml of BG11 medium (Rippka *et al.*, 1979) on stationary shelves under continuous light with an intensity of 10 $\mu E.m^{-2}.s^{-1}$ at 23°C. Flasks were shaken manual twice a week. Every 4 weeks, 500 ml medium were inoculated with 200 ml cell cultures. The composition of BG11 medium is shown in table II-1. Medium was autoclaved and stored at room temperature (RT).

Table II-1. Composition of BG11 medium

Macroelements	Concentration (g/l)
$NaNO_3$	1.5
$K_2HPO_43H_2O$	0.04
$MgSO_47H_2O$	0.075
$CaCl_22H_2O$	0.036
Citric acid	0.006
Iron Ammonium Citrate	0.006
EDTA	0.001
Na_2CO_3	0.02
Micro-elements	1 ml (see below)

Microelements	Concentration (g/l)
H_3BO_3	2.86
$MnCl_24H_2O$	1.81
$ZnSO_47H_2O$	0.222
$NaMoO_42H_2O$	0.390
$CuSO_45H_2O$	0.079
$Co(NO_3)_26H_2O$	0.049

II.5 Determination of dry weights of cell suspension by optical density

Concentrated cell cultures of *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 were diluted from 1 to 9 times (v/v) with BG11 medium and the optical density (OD) at 720, 730 and 750 nm was measured for each dilution. The cell cultures (50 ml) of each dilution were collected by centrifugation and dried at 60^{o}C until constant weight was reached. Dry weights were plotted against OD values. The correlation between OD and dry weight for each species is shown in Figure II-1. Although OD measurement of all wavelengths showed good correlations with cell dry weight, the best correlation was obtained at 720 nm (Fig. II-1).

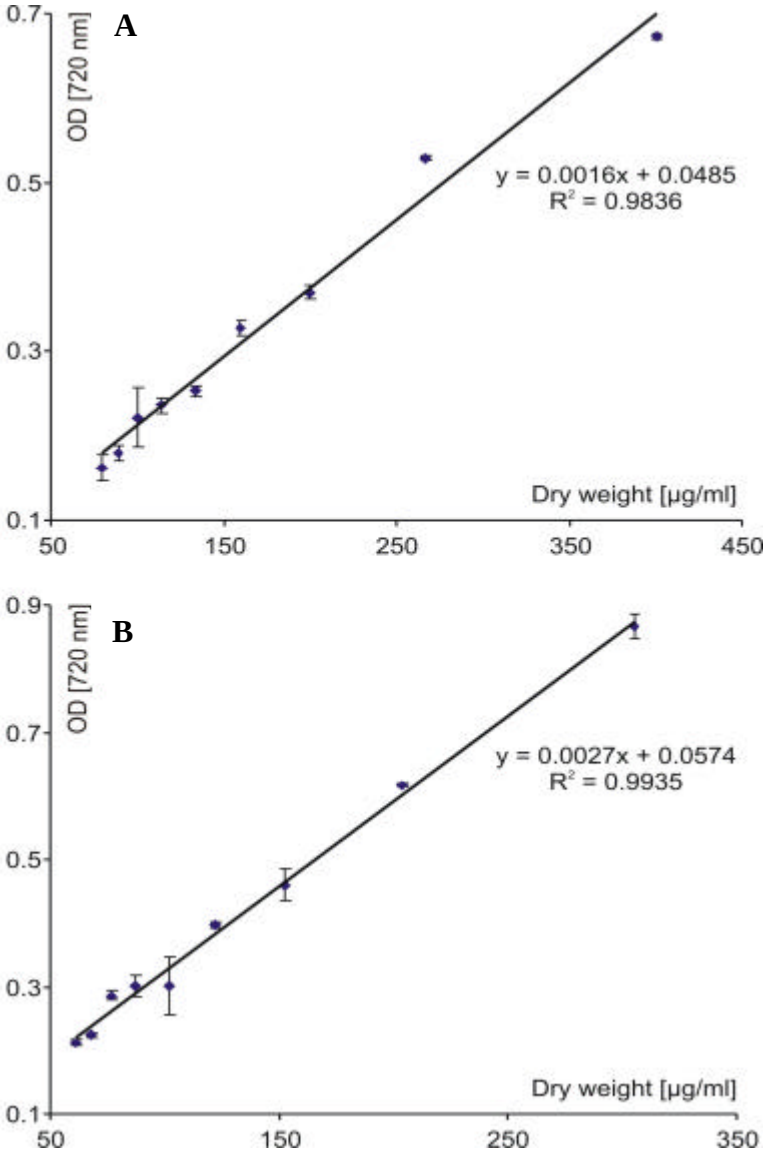


Figure II-1. Correlation between optical density at 720 nm (OD 720) and dry weight of two cyanobacterial strains used in experiments, *Anabaena* (A) and *Synechocystis* (B). Shown is the mean of three measurements for each data point ± sd.

In order to standardize cell culture conditions for further experiments of cyanobacterial cell cultures, optical density was adjusted to an OD (720 nm) of 0.3. Cell cultures for experiments were used one week after transfer into fresh medium.

II.6 Solid phase extraction (SPE)

Solid-phase extraction (SPE) is a separation method that uses a solid phase and a liquid phase to isolate analytes from a solution. In this work, SPE was used to isolate fatty acids and phytoprostanes from cyanobacterial samples. Amino-propyl (NH_2) and silica (SiOH) materials were used for seperation. Material (500 mg) were filled into glass columns. NH_2 columns were washed with 6 ml of 5% triethylamine in methanol, followed by 6 ml of methanol. For SiOH columns, 6 ml of methanol and 6 ml of diethyl ether was used for washing. Columns were washed directly before use to avoid column dryness. Column equilibration was performed depending on the solvent used for sample reconstitution before loading on the column.

II.7 Hydrogenation, methoximation, and derivatisation of oxylipins

For the oxylipin analysis, derivatisation steps are necessary. This procedure is done to convert oxylipins into volatile compounds for GC-MS analysis (Mueller *et al.*, 2006).

II.7.1 Hydrogenation

For GC-NCI-MS analysis of PPF_1, catalytic hydrogenation is the first derivatisation step (Imbusch and Mueller, 2000a). To perform the hydrogenation, PPF_1 obtained from NH_2-purification was taken to dryness and dissolved in 1 ml of methanol. Adams catalyst (PtO_2) (20 mg) were added and hydrogen gas was bubbled through the sample for 10 min (Fig. II-2). The catalyst was removed by filtering and the organic phase was dried under a stream of nitrogen.

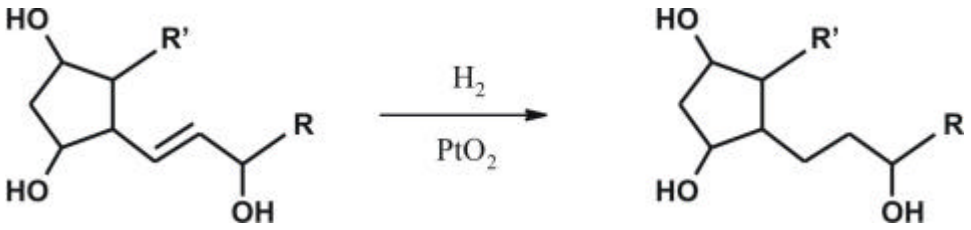


Figure II-2. Hydrogenation of PPF_1 (type I: R' = $C_8H_{15}O_2$, R = C_2H_5; type II: R' = C_2H_5, R = $C_8H_{15}O_2$; type III: R' = $C_5H_9O_2$, R = C_5H_{11}; type IV: R' = C_5H_{11}, R = $C_5H_9O_2$).

II.7.2 Methylation of carboxyl group by trimethylsilyl-diazomethane

For GC-EI-MS analysis of lipids, carboxyl group modification is needed. So, after purification using NH_2-SPE, the lipid extracts were methylated in 200 µl methanol with 20 µl of trimethylsilyl-diazomethane (Fig. II-3) for 10 min at RT. Reagents were removed under a stream of nitrogen.

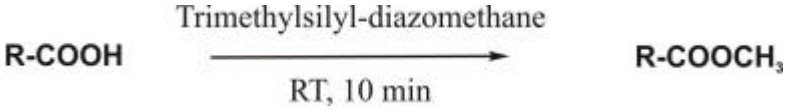


Figure II-3. Methylation of lipids.

II.7.3 Methoxymation of keto group by methoxyamine

Because of the high temperature (300°C) in the GC-MS injector, PPA_1 and PPE_1 isomerized to stable PPB_1 (Thoma *et al.*, 2004). Therefore, without derivatization of the keto function, PPA_1, PPB_1 and PPE_1 were as PPB_1 detected. To avoid this isomerization, PPA_1 and PPE_1 were methoximated before GC-MS analysis (Fig. II-4). PPA_1 and PPE_1 were incubated with 50 µl methoxyamine-HCl (5 mg in 100 µl dimethylformamide) for 1 h at 60°C. After the reaction, the solvent was removed under a stream of nitrogen. The sample was reconstituted in 2 ml water, acidified with 1 M citric acid to a pH of 3 and extracted twice with 5 ml diethyl ether. The organic phases were combined and the solvent was removed under a stream of nitrogen.

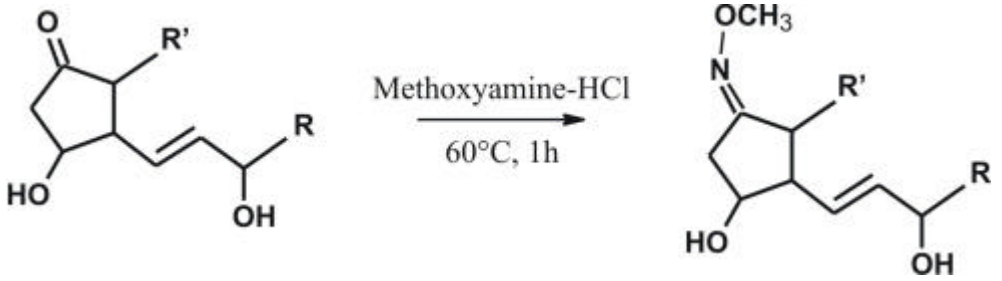


Figure II-4. PPE_1 as an example of methoximation (type I: R' = $C_8H_{15}O_2$, R = C_2H_5; type II: R' = C_2H_5, R = $C_8H_{15}O_2$; type III: R' = $C_5H_9O_2$, R = C_5H_{11}; type IV: R' = C_5H_{11}, R = $C_5H_9O_2$).

II.7.4 Esterification of carboxyl group by pentafluorbenzylbromide (PFB-Br)

Pentafluorbenzylbromide derivatives are used for analysis in NCI-MS (Mueller *et al.*, 2006). The purified lipid extracts were dissolved in 200 ml chloroform, 10 µl of N,N-diisopropylethylamine (DIEA), and 10 µl PFB-bromide (Fig. II-5). After 1 h at 40°C, the sample was dried, dissolved in 3 ml chloroform and applied to SPE using a silica column. The column was washed with 3 ml diethyl ether. PFB-esterified phytoprostanes were eluted with 6 ml diethyl ether/methanol 90/10 (v/v) and the sample was taken to dryness.

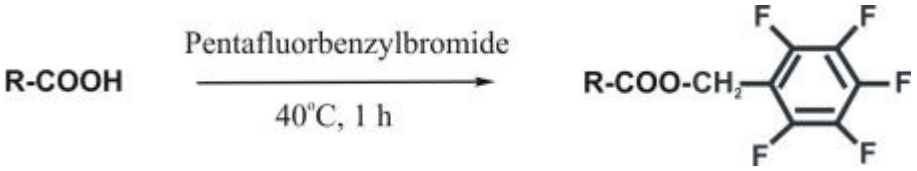


Figure II-5. PFB-esterification of oxylipins.

In the negative chemical ionisation mode (NCI) in the mass spectrometer, PFB-esterified oxylipins are dissociated and the lipid anion can be detected with high sensitivity and specificity (Fig. II-6).

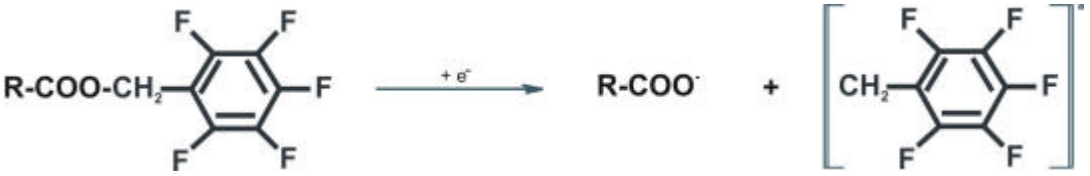


Figure II-6. Dissociation of PFB-esterified oxylipins in the NCI mode.

II.7.5 Preparation of trimethylsilyl ether derivatives with N,O-Bis-(trimethylsilyl)trifluoracetamide (BSTFA)

The sample was dissolved in 200 µl chloroform. BSTFA (50 µl) were added and the sample was incubated for 1 h at 40°C. Silylation of oxylipin occurred as shown in Fig. II-7. Finally, the chloroform phase was taken to dryness and the sample was ready for GC-MS analysis.

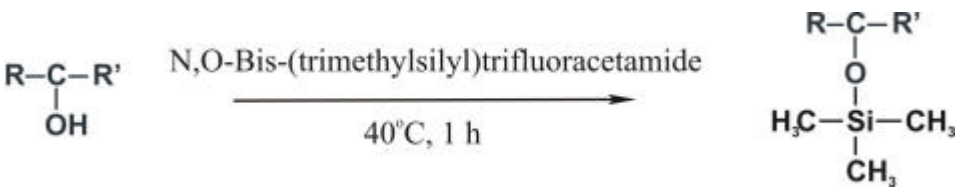


Figure II-7. Silylation of oxylipins.

II.8 Formation of phytoprostanes type III and IV by autoxidation of g-linolenic acid

II.8.1 Hydrolysis of Borage oil

γ-linolenate was obtained by hydrolysis of Borage oil. Borage oil (from seeds of *Borago officinalis*) contains about 25% γ-linolenic acid according to manufacturer's description. Borage oil (5 g) were hydrolyzed using a mixture of 25 ml methanol and 25 ml of 30% KOH (w/v) in a 250 ml round bottom glass flask. The mixture was stirred on a magnetic plate and hydrolyzed for 6 h at RT. After hydrolysis, the mixture was diluted with 100 ml water and the pH was adjusted to 2 – 3 by 6 N HCl. Fatty acids were extracted 3 times with 50 ml diethyl ether. The ether was removed in a rotary evaporator and the sample was reconstituted in 10 ml chloroform. Fatty acids were further purified with a silica column. The column was packed with 10 g of silica material with a particle size of 63 – 200 µm. The column was washed with 100 ml methanol and equilibrated with 100 ml chloroform. The sample was loaded on the column and the fatty acids were eluted with 200 ml hexane/diethyl ether 8/2 (v/v). The organic phase was taken to dryness.

II.8.2 Autoxidation of g-linolenic acid and quantification of PPF_1 type III and IV

The fatty acids mixture of hydrolyzed Borage oil were dissolved in 50 ml chloroform and stirred on a magnetic plate under atmospheric oxygen condition for 12 days at RT. The oxidation was stopped by the addition of 3 g triphenylphosphine (TPP) for 30 min. The autoxidized sample was stored at - 20°C until PPF_1 analysis.

An aliquot of the autoxidized mixture (equivalent to 50 µg Borage oil) with 250 ng of $[^{18}O]_3$-PPF_1 as IS was diluted in 3 ml chloroform and applied to a 500 mg silica column. The column was washed 6 ml diethyl ether/acetic acid (99:1, v/v) and 3 ml chloroform/acetone/acetic acid (80:20:1, v/v). The column was eluted with 6 ml diethyl ether/methanol/acetic acid (90:10:1, v/v). The organic phase was taken to dryness. PPF_1 was hydrogenated (II.7.1) and derivatized to its PFB-ester (II.7.4) and trimethysilyl ether (II.7.5) before analysis by GC-MS.

II.8.3 Autoxidation of g-linolenic acid and quantification of PPE_1 type III and IV by isomerisation to corresponding PPB_1 type III and IV

The fatty acids mixture of hydrolysed Borage oil was diluted in 50 ml water/methanol 1/1 (v/v). The mixture was stirred on a magnetic plate under atmospheric oxygen condition at RT. After 12 days, the sample was diluted with 50 ml chloroform and the oxidized mixture was reduced by the addition of 3 g TPP. Saturated NaCl solution (100 ml) was added and the pH of the mixture was adjusted to 3 with 1 M citric acid and 100 ml of was added. The sample was mixed for 1 min and the chloroform phase was collected. PPE_1 type III and IV in the autoxidized sample were extracted further 2 times with 50 ml chloroform. The organic phase was removed by a rotary evaporator and the PPE_1 mixture was stored at - 20°C prior analysis.

PPE_1 cannot be analyzed directly by HPLC, but can easily be detected after isomerisation to the corresponding PPB_1 derivatives (Parchmann and Mueller, 1998). Before the isomerisation to PPB_1, PPE_1 were purified by silica SPE. A silica column (500 mg) was preconditioned with 6 ml chloroform. The PPE_1 mixture (equivalent to 50 µg Borage oil) was mixed with 5 µg PGE_1 as internal standard and loaded on the column. The column was washed with 6 ml diethyl ether/acetic acid (98:2, v/v) and 6 ml chloroform/acetone/acetic acid (8:2:0.1, v/v). PPE_1 were collected by eluting the column with 6 ml diethyl ether/acetone/acetic acid (6:4:0.1, v/v). The eluate was taken to dryness, reconstituted in 100 µl of 0.25% BHT in methanol (w/v), and treated with 400 µl of 1M KOH for 10 min at 40°C. Under these conditions, PPE_1 and PGE_1 undergo base-catalyzed dehydration and isomerisation to form the corresponding PPB_1 and PGB_1 (Fig. II-8). Afterwards, the sample was diluted with 2.5 ml water, acidified with 1 M citric acid to pH 3, and extracted twice with 3 ml diethyl ether. The ether phase was taken to dryness and the sample was diluted in 200 µl methanol and analyzed by HPLC. Detection of PPB_1 (type III and IV) and PGB_1 were recorded at 279 nm by a diode array detector (see II.22.3). Quantification of PPE_1/PPB_1 was performed by GC-MS.

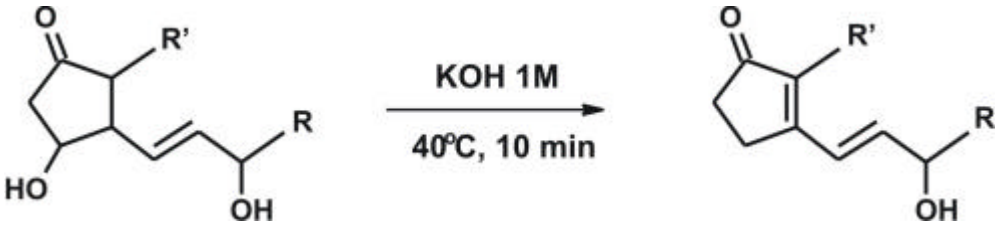


Figure II-8. Dehydration and isomerisation PPE_1 to the corresponding PPB_1. (type I: R' = $C_8H_{15}O_2$, R = C_2H_5; type II: R' = C_2H_5, R = $C_8H_{15}O_2$; type III: R' = $C_5H_9O_2$, R = C_5H_{11}; type IV: R' = C_5H_{11}, R = $C_5H_9O_2$).

II.8.4 Formation and isolation of PPA_1 type III and IV by acidic dehydration of PPE_1 type III and IV

PPA_1 are formed by acid catalyzed dehydration of PPE_1 (Thoma *et al.*, 2004) (Fig. II-9). A mixture of PPE_1 (equivalent to 200 µg Borage oil) was incubated with 2 ml of water/acetic acid/phosphoric acid (10:3:2, v/v) over night. PPA_1 in the sample were extracted twice with 3 ml diethyl ether. The ether phase was taken to dryness and the sample was diluted in 200 µl methanol and analyzed by HPLC using a PDA detector at a wave length oh 217 nm. The putative PPA_1 peak was collected and further identified as PPA_1 type III and IV by GC-MS in the EI mode after methoximation (II.7.3) and derivatisation with TMS-diazomethane and BSTFA (II.2.2 and II.7.5).

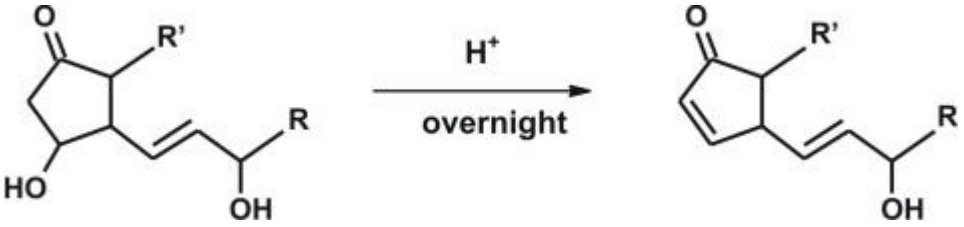


Figure II-9. Dehydration of PPE_1 to the corresponding PPA_1.
(type I: R' = $C_8H_{15}O_2$, R = C_2H_5; type II: R' = C_2H_5, R = $C_8H_{15}O_2$;
type III: R' = $C_5H_9O_2$, R = C_5H_{11}; type IV: R' = C_5H_{11}, R = $C_5H_9O_2$).

Quantification of PPA_1 type III and IV was carried out indirectly via their corresponding PPB_1 type III and IV. An aliquot of sample was dissolved in 100 µl methanol and treated with 400 µl of 1 M KOH at 40°C for 10 min. Under these conditions, PPA_1 isomerised to PPB_1 (Fig. II-10). After that, PGB_1 (5 µg) as IS was added. The sample was extracted with 5 ml diethyl ether. The solvent was removed under a stream of nitrogen and the sample was dissolved in 3 ml chloroform. The silica column (500 mg) was equilibrated with 6 ml chloroform before the sample was loaded. The column was washed with 6 ml chloroform and eluted with 6 ml diethyl ether/acetic acid (98:2, v/v). The eluate was taken to dryness and reconstituted in 200 µl methanol and quantified by HPLC as described in II.22.3.

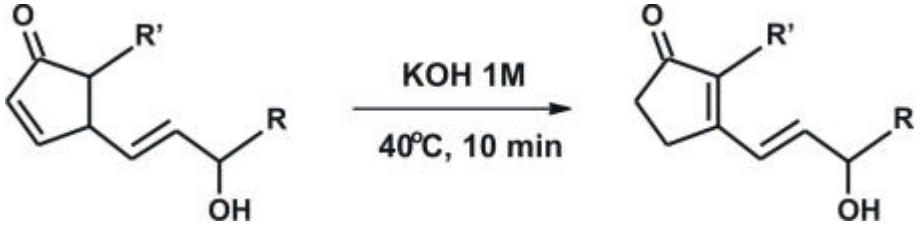


Figure II-10. Isomerisation from PPA_1 to the corresponding PPB_1.
(type I: R' = $C_8H_{15}O_2$, R = C_2H_5; type II: R' = C_2H_5, R = $C_8H_{15}O_2$;
type III: R' = $C_5H_9O_2$, R = C_5H_{11}; type IV: R' = C_5H_{11}, R = $C_5H_9O_2$).

II.9 Fatty acid quantification in *Anabaena* und *Synechocystis*

Cell suspensions (500 ml) were centrifuged at 4000 rpm for *Anabaena* or 6000 rpm for *Synechocystis* for 10 min to collect the cells. Cells were shock frozen in liquid nitrogen. About 750 mg of fresh weight was used for fatty acid quantification. Lipids were extracted according to Bligh and Dyer (1959) with a mixture of 1 ml water, 2.5 ml chloroform, 5 ml methanol, 250 µl 1 M citric acid, 300 µl 5% (w/v) butylated hydroxytoluene in methanol. Triphenylphoshine (TPP) (20 mg) was added to reduce peroxides to the corresponding alcohols. Margarinic acid (1 µg) was added as internal standard. Blank samples were processed and analyzed to exclude fatty acid contaminations in analytical solutions.

Samples were sonicated for 1 min and shaken by hand for additional 1 min. After centrifugation at 3000 rpm at 4°C for 10 min, the liquid phase was transferred to sealed glass tubes and the residue phase was reextracted with 2.5 ml chloroform. After centrifugation, the liquid phases were combined and 2.5 ml 0.88% (w/v) KCl were added. After centrifugation, the lower lipid containing phase was transferred to glass tubes and dried under a stream of nitrogen.

After sample extraction, lipid hydrolysis was necessary to determine both, free and esterified, fatty acids. For that purpose, the dry sample was reconstituted in 2 ml methanol and hydrolyzed after addition of 2 ml 15% (w/v) KOH for 1 h at 60°C. After hydrolysis, the sample was diluted with 10 ml water and acidified to a pH about 2 – 3 with 11 ml 1 M citric acid. Total fatty acids were extracted 2 times with 10 ml diethyl ether. Afterwards, the combined organic phase was taken to dryness and the residue was dissolved in 3 ml ethyl acetate.

Fatty acid separation was performed by SPE using NH_2 material. The column was equilibrated with 6 ml ethyl acetate. After the sample was applied onto the column, the column was washed with 6 ml chloroform/isopropanol 2/1 (v/v) and fatty acids were eluted with 6 ml of diethyl ether/acetic acid 98/2 (v/v). The ether phase was taken to dryness and the residue was reconstituted in 200 µl diethyl ether. Fatty acids were methylated with 20 µl trimethylsilyl-diazomethane for 10 min at RT (II.7.2). Finally, the solvent was removed and the residue was dissolved in 1 ml hexane. 2µl of solution were subjected to GC-MS analysis using the electron impact mode (EI) (II.23).

II.10 Analysis of total hydroxy fatty acid in *Anabaena* by GC-MS

Extraction procedure was performed as described in II.9 and 200 ng of 15-HETE were added as internal standard. After the SPE, the organic phase was taken to dryness and sample was hydrogenated (II.7.1), methylated (II.7.2) and silylated (II.7.5) prior to GC-MS analysis (II.23).

II.11 Determination of phytoprostanes from *Anabaena* and *Synechocystis* by GC-MS

II.11.1 Extraction of free PPF_1 from cells and medium by GC-MS

The OD 720 of cell suspensions was determined before cell cultures were collected for further analysis. The levels of PPF_1 in the cells and in the medium were quantified in 1 and 6 weeks old cultures. Cell suspension (500 ml) was divided into two 250 ml centrifuge tubes and spinned down at 4000 rpm and 4°C for 10 min. The pellets were resuspended and combined into a 50 ml plastic tube and centrifuged again. The cells were frozen immediately in liquid nitrogen and stored at - 80°C until use.

The supernatant was acidified with 1 M citric acid to pH 2 – 3. PPF_1 in 500 ml medium was extracted with 200 ml diethyl ether and 500 ng of $[^{18}O]_3$-PPF1 as internal standard (IS). The ether phase was taken to dryness and PPF_1 was purified by aminopropyl SPE. The residue was reconstituted in 3 ml chloroform and loaded onto the column. The column was washed with 6 ml chloroform/isopropanol 2/1 (v/v) and eluted with 6 ml diethyl ether/methanol/acetic acid (80/20/2, v/v/v). The eluate was taken to dryness and the residue was derivatized. Quantification of PPF_1 in the sample was carried out using GC-MS in NCI mode. Derivatisation reactions necessary before analysis were: hydrogenation, esterification and silylation (described in II.7).

For the extraction of free PPF_1 from cells, 10 ml saturated NaCl, 200 µl 1 M citric acid, 400 µl BHT (5% (w/v) in methanol), 20 mg TPP, 250 ng of $[^{18}O]_3$-PPF_1 as IS, and 15 ml diethyl ether were added. The mixture was sonicated for 1 min, additionally shaken by hand for 1 min, and centrifuged at 3000 rpm and 4°C for 10 min. The upper phase was transferred to a glass tube and taken to dryness. The residue was resolved in 3 ml chloroform and applied onto a NH_2 column. The column was washed with 6 ml chloroform/isopropanol 2/1 (v/v) and eluted with 6 ml diethyl ether/methanol/acetic acid (80/20/2, v/v/v). The eluate was taken to dryness and derivatized as mentioned above for PPF_1 in medium.

II.11.2 Analysis of free PPE_1 from cells and medium by GC-MS

Cells and medium collection was done exactly the same as described in II.11.1. Before extraction of PPE_1 from the medium, 50 ng of prostaglandin E_1 (PGE_1) was added as internal standard. Diethyl ether (200 ml) was used for extraction. Ether phase was taken to dryness and the sample was dissolved in 100 µl methanol. PPE_1 were isomerized to the corresponding PPB_1 by addition of 400 µl 1 M KOH to the sample for 10 min at 40°C. After isomerization, 2.5 ml water was added. The solution was adjusted to a pH of 3 with 1 M citric acid and PPB_1/PGB_1 in the sample were then extracted twice with each 5 ml diethyl ether. The ether phase was taken to dryness; the residue was reconstituted in 3 ml diethyl ether and loaded onto a NH_2 column. The column was washed with 6 ml of chloroform/isopropanol 2/1 (v/v) and PPB_1/PGB_1 were eluted with 6 ml of diethyl ether/acetic acid 98/2 (v/v). Samples were taken to dryness for further derivatisation (esterification and silylation) prior to GC-MS analysis in the NCI mode.

For the extraction of free cellular PPE_1 extraction, 10 ml saturated NaCl, 200 µl 1 M citric acid, 400 µl BHT (5% (w/v) in methanol), 20 mg TPP, 25 ng of PGE_1 as IS, and 15 ml diethyl ether were added. The mixture was sonicated for 1 min, additionally shaken by hand for 1 min, and centrifuged at 3000 rpm and 4°C for 10 min. The upper phase was transferred to a glass tube and taken to dryness. The residue was resolved in 3 ml chloroform and applied onto a NH_2 column. The column was washed with 6 ml chloroform/isopropanol 2/1 (v/v) and eluted with 6 ml diethyl ether/methanol/acetic acid (80/20/2, v/v). After elution, the eluate was taken to dryness and the sample was dissolved in 100 µl of methanol. Isomerization of PPE_1/PGE_1 to PPB_1/PGB_1 and additional SPE purification were carried out according to the analysis of free PPE_1 in the medium.

II.11.3 Analysis of total PPF_1 and PPE_1 from the cells by GC-MS

The extraction of total (free and esterified) PPF_1 from the cells was carried out as described for free cellular PPF_1 in II.11.1 using 250 ng $[^{18}O]_3$-PPF_1 as IS. After extraction, esterified PPF_1 were hydrolyzed by adding 2 ml of methanol and 2 ml of aqueous KOH solution (15%, w/v) and incubating the mixture at 60°C. After 1 h hydrolysis, the sample was diluted with 10 ml water and 11 ml 1 M citric acid and PPF_1 was extracted twice with 10 ml diethyl ether. The organic phases were combined, the solvent was removed under a stream of

nitrogen and the residue was reconstituted in 3 ml chloroform. The solution was further purified by SPE and subsequently derivatized for GC-MS analysis (see II.7).

The extraction procedure for total PPE_1 was carried out the same way as described for total PPF_1 except for using of 25 ng PGE_1 as IS. Total PPE_1 and PGE_1 in the sample were isomerized to corresponding PPB_1 and PGB_1 during hydrolysis. The hydrolysis and subsequent extraction was carried out as described for the determination of total PPF_1. The sample was reconstituted in 3 ml chloroform. The solution was loaded on a NH_2 column. The column was washed with 6 ml chloroform/isopropanol 2/1 (v/v) and eluted with 6 ml diethyl ether/acetic acid (98/2, v/v). The eluate was taken to dryness and afterwards esterified and silylated for GC-MS analysis (II.7.4 and II.7.5). Quantification of PPE_1 in the sample was done by GC-MS in negative chemical ionisation mode (NCI) (II.23).

II.12 Determination of free and total PPF_1 and PPE_1 in *Synechocystis* by HPLC-MS/MS

The procedure for the extraction of free PPF_1 and PPE_1 from the medium was carried out as described in II.11.1 and II.11.2 using 500 ng $[^{18}O]_3$-PPF_1 and 500 ng $[^{18}O]_3$-PPE_1 as IS. After removal of the organic phase, the residue was reconstituted in 50 µl of buffer containing of 1 mM ammonium acetate/acetonitrile 80/20 (v/v) adjusted to a pH of 6.8. The solution was transferred to a reaction tube and centrifuged at 14000 rpm for 5 min. The supernatant was transferred to a HPLC vial and analyzed via HPLC-MS/MS (II.24).

For the extraction of free PPF_1 from cyanobacterial cells, 2 ml methanol, 100 µl acetic acid and 100 ng of $[^{18}O]_3$-PPF_1 as IS were added. The mixture was sonicated for 1 min and then centrifuged at 4000 rpm and 4°C for 10 min. The supernatant was taken to dryness and reconstituted in 50 µl of a mixture of 1 mM ammonium acetate/acetonitrile 80/20 (v/v). Sample was centrifuged again at 14000 rpm for 5 min and the supernatant was used for HPLC-MS/MS analysis.

For the analysis of free cellular PPE_1, 100 ng of $[^{18}O]_3$-PPE_1 were used as IS. After extraction, isomerization of PPE_1 to PPB_1 was carried out by addition of 100 µl methanol and 400 µl of 1 M KOH. After 10 min at 40°C, the sample was diluted with 2.5 ml water and acidified with 1 M citric acid to a pH of 3. Free PPB_1 were extracted twice with 5 ml diethyl ether. The ether phases were combined and the solvent was removed under a stream of nitrogen. The residue was reconstituted in 50 µl of a mixture of 1 mM ammonium acetate/acetonitrile 80/20 (v/v). The solution was transferred to a reaction tube and

centrifuged at 14000 rpm for 5 min. The supernatant was transferred into a HPLC vial and analyzed by HPLC-MS/MS.

For the extraction of free and esterified cellular PPE_1 and PPF_1 extraction, 2 ml methanol, 100 µl acetic acid, 100 ng of $[^{18}O]_3$-PPE_1 and 100 ng of $[^{18}O]_3$-PPF_1 were added as IS. The sample was sonicated for 1 min and centrifuged at 4000 rpm and 4°C for 10 min. The upper phase was transferred into a new reaction tube and 2 ml of 15% (w/v) KOH were added. The hydrolysis of esterified PPE_1 and PPF_1 was carried out by incubating the sample at 60°C for 1 h. After hydrolysis, the sample was transferred to a plastic tube, which contained 10 ml water and 11 ml 1 M citric acid. The total PPE_1/PPF_1 were extracted twice with 10 ml diethyl ether. The organic phases were combined and taken to dryness. The sample was reconstituted in 50 µl of a mixture of 1 mM ammonium acetate/acetonitrile 80/20 (v/v). The solution was transferred to a reaction tube and centrifuged at RT at 14000 rpm for 5 min. The upper phase was transferred to a HPLC vial and analyzed by HPLC-MS/MS.

II.13 Analysis of PPF_1 and PPE_1 in *Anabaena* grown under high light intensity and oxidative stress

Experiments were accomplished with 3 replicates (500 ml cell suspension for each replicate). Cell cultures were treated with either 10 µM $CuSO_4$ or 200 µM H_2O_2 in combination with low or high light intensity (low light (LL): 10 $\mu E.m^{-2}.s^{-1}$ and high light (HL): 330 $\mu E.m^{-2}.s^{-1}$). HL was generated by Phillips 400 W lamps. Stress treatments were carried out for 8 h at a constant temperature of 23°C to avoid warming from the light source. Medium and cell collection, phytoprostanes extractions and analysis were performed as described in II.11.

II.14 Investigation of protective effects of oxylipins on *Anabaena* under oxidative stress condition

Development of a vital staining method for determination of cell death in *Anabaena*: 20 ml of cell culture were boiled for about 2 min to ensure that all cells are dead. Dead cell suspension (50 µl) were stained with 100 µl aqueous Eosin solution (3.3%, w/v) for 5 min. In parallel, 50 µl of untreated cell suspension were stained in the same way. Stained cells were analyzed by a light microscope. Dead cells were red colored whereas living cells were still green.

After the staining method was developed, volume of 1.5 ml of cell culture was transferred into each well of a 12-well plate. The cell suspension was incubated with 100 µM of different oxylipins for 16 h under normal growth conditions. Oxylipins were applied in a methanol solution with final concentration of 1% methanol, therfore 1% methanol was used in control experiments. The oxylipins that used in experiments were PGA_1, PGB_1, PGB_2, PGE_2, PGF_1, PGJ_2, JA, OPDA, PPA_1 (mixture of type I and II), PPB_1 type I, PPB_1 type II, PPE_1 type I, PPF_1 type I, PPF_1 type II, PPJ_1 type I, PPJ_1 type II, and cyclopentenone (CP). Each oxylipin was tested in three replicates. Subsequently, the cells were treated with 50 µM of $CuSO_4$ or 1 mM of H_2O_2. After 5 h, cells were transferred into 2.5 ml reaction tubes and centrifuged at 3000 rpm for 10 min. The medium was removed and the cell pellet was resuspended in 1.5 ml of fresh BG11 medium. In the experiments with $CuSO_4$, additional washing steps were neccessary because of Eosin precipitations which interfere with the light microscopy. Additionally, removal of $CuSO_4$ was required to stop the reaction of copper ions with *Anabaena* during the time-consuming light microscopy analysis. H_2O_2 also had to be removed to avoid further hydrogen peroxide toxicity on the cells during the counting experiments. The protective effects of oxylipins in *Anabaena* were evaluated by determining the cell death rate using 3.3% Eosin solution. Cell suspension (15 µl) were used for staining with 30 µl of Eosin solution. Cells were incubated for 5 min and 10 µl of the sample were loaded on a double counting plate (Fuchs-Rosenthal-Marienfield, Hartenstein). For each sample, three parallel preparations were stained and counted. Dead cells were counted in 10 squares on the counting plate in diagonal direction. The percentage of dead cells for each oxylipin was calculated by dividing the dead cell number in the treatment by the total cell number in the control. In the stress treatments, lytic cells were considered as dead cells.

II.15 Analysis of H_2O_2 and PPF_1 effects on *isiA* (iron stress induced protein A) expression in *Anabaena*

A standard cell suspension was concentrated 20 times and 5 ml (about 100 mg FW) were transferred to a well in a 6-well plate. Cells were treated with 1.5 mM of H_2O_2, 100 µM PPF_1 or methanol (< 1%) as control. Cells were incubated in light (40 $\mu E.m^{-1}.s^{-1}$) at 27°C for 90 min. Cells were collected by centrifugation at 4000 rpm at 4°C for 10 min. Cells were shock frozen in liquid nitrogen and stored at – 80°C until use. Three replicates for each treatment were performed.

II.15.1 RNA isolation from *Anabaena*

RNA isolation from *Anabaena* was performed using TRItidy G solution (AppliChem) according to manufacturer's instruction. 1 ml TRItidy was added to 100 mg cell material and cells were ruptured. The homogenate was incubated at RT for 5 min and then 0.3 ml chloroform was added. The sample was shaken by hand (30 times) and left at RT for 10 min. RNA was obtained in the upper phase after centrifuging the sample at 4°C by 12000 rpm for 15 min. The upper phase was transferred to a new tube and the same volume of isopropanol was added. The sample was kept in ice for 30 min to allow RNA precipitation. RNA was collected by centrifugation at 12000 rpm for 15 min at 4°C. The RNA pellet was washed twice with 750 µl of 3 M aqueous $LiCl_3$ and once in 750 µl 70% ethanol. After centrifugation at 7500 rpm for 5 min at 4°C, RNA was dried for a few minutes at 37°C. The RNA was dissolved in 20 µl of DEPC-treated water by shaking the sample at 1400 rpm at 60°C for 10 min. The RNA concentration was quantified by photometer at 260 nm.

II.15.2 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Analysis of expression of the *isiA* gene was done by RT-PCR. The *rnpA* gene (encoding a subunit of the ribonuclease P) was used as internal control (Havaux *et al.*, 2005). The RT-PCR was essentially performed according to the protocol provided with the reverse transcriptase (MBI, Fermantas). 1 µg RNA from each treatment was used for RT-PCR. To remove DNA in the RNA samples, RNA samples were treated with 1 µl DNAse (18068-015 from Invitrogen, 1U/µl) for 15 min at RT. After 1µl of EDTA was added, the samples were incubated at 65°C for 10 min. Then, 1 µl oligodT primer was added and the samples were incubated for 5 min at 70°C. After short centrifugation, samples were mixed with 4 µl reaction buffer, 1 µl RNAse inhibitor, and 2 µl dNTPs and incubated at 37°C for 5 min before 1 µl RevertAid H Minus M-MULV (200 U/µl) was added. .Reverse transcription occurred at 42°C for 1h. Enzyme inactivation was done by incubating the samples at 70°C for 10 min.

The reverse transcription product (cDNA) was used as a template in PCR. PCR samples consisted of 2.5 µl cDNA from each treatment for *rnpA* or 5 µl cDNA from each treatment for *isiA* together with 1 µl *rnpA* primer, 1 µl *isiA* primer, 1 µl dNTPs, 3 µl PCR buffer, and 1 µl Taq-Polymerase in a total volume of 20 µl. A sample containing water instead of template DNA was used as a negative control. A sample containing plasmid-DNA served as a positive control for the PCR. The plasmid contained the PCR products of genomic

DNA of *Anabaena* amplified by the same primers used for RT-PCR in the vector pCR®2.1. The PCR running programme:

Process	Temperature (^oC)	Time	Number of cycle
Denaturation	95	3 min	1
Amplification	94	30 s	30
	45.2 for *isiA*	45 s	
	36 for *rnpA*	45 s	
	72	45 s	
Elongation	72	10 min	1

Primer sequences: *rnpA* (forward: AGAGAGTAGGCGTTGG; reverse: ACACGAGGGCGATTAT); *isiA* (forward: GCCCGCTTCGCCAATCTCTC; reverse: CCTGAGTTGTTGCGTCGTAT).

PCR products were separated by 1% agarose gel electrophoresis (0.8 g agar in 80 ml TEA buffer). 20 µl DNA from each treatment was mixed with 1 µl ethidium bromide (5 mg/ml), shortly centrifuged and loaded on the gel for electrophoresis at 100V for 30 min. 5 µl of 1 kb ladder DNA marker were employed as standard. A gel photo was taken by gel documentation system.

II.16 Analysis of PPF_1 effect on the *Anabaena* proteome

Standard cell suspension (2l) was centrifuged at RT at 4000 rpm for 10 min. Supernatant was discarded and the cell pellet was resuspended in 100 ml fresh BG11 medium. Cell suspension was centrifuged and the medium was removed again. Finally, the cell pellet was suspended in another 100 ml fresh BG11 medium. Possible contaminations in the medium of cell culture were removed by the washing procedure. Cell suspension (100 ml) was used for control (methanol treated, 0.5%) or 100 µM PPF_1 (mixture of type I and II) treatment. Treated cell suspensions were kept under normal cell growth condition for 6 h. Treated cells were collected by spinning down at 4000 rpm and 4^oC for 20 min. Cell pellet was frozen in liquid nitrogen and stored at – 80^oC for protein analysis.

II.16.1 Protein extraction in *Anabaena* cells

Cell pellets were ground in liquid nitrogen using pestle and mortar. 100 mg powdered cells were used for protein extraction according to Wang (Wang *et al.*, 2006). Cells were washed by mixing with 1 ml of ice cold 10% TCA in acetone. This solution inactivates proteases and minimizes protein degradation. Liquid phase was removed by centrifugation at 4°C and 14000 rpm for 3 min. The sample was washed with 1 ml ice cold solution of 80% methanol in 0.1 M ammonium acetate. Additionally, precipitated proteins were washed with 1 ml cold 80% acetone. The pellet was dried at RT for about 15 min. Proteins in the sample were extracted by mixing with 1 ml mixture of phenol/SDS buffer 1/1 (v/v). SDS buffer consists of 30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0. Mercaptoethanol (5%) was added to the SDS buffer directly before use. Proteins were diluted in the extraction buffer (*Wang et al.*, 2003). The sample was centrifuged at 14000 g and 4°C for 5 min. The phenolic phase was transferred to a new tube which contained 1 ml 80% methanol in 0.1 M ammonium acetate. Sample was incubated at – 20°C overnight for protein precipitation. Proteins were collected by centrifugation at 4°C and 14000 rpm for 5 min. Protein pellet was washed with 1 ml 100% cold methanol, afterwards using 1 ml of 80% acetone. After discarding the supernatant, the sample was dried at RT and stored at – 20°C for further use.

II.16.2 Determination of *Anabaena* proteins by 1D-gelelectrophoresis

Extracted protein was denatured by incubating at 80°C for 10 min after mixing with 25 µl water and 25 µl double SDS buffer. The SDS buffer is composed of 100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue and 20% glycerine. Proteins were separated according to Laemmli (Laemmli, 1970). Separation gel (12.5%) and stacking gel (4%) were used as following:

Separation gel (12.5%)	**Stacking gel (4%)**
4.2 ml acrylamide/bisacrylamide (30%), a mixture of 37.5:1	0.88 ml acrylamide/bisacrylamide (30%), a mixture of 37.5:1
2.5 ml 1.5 M Tris, pH 8.8	1.66 ml 0,5 M Tris, pH 6.8
0.1 ml 10 % SDS	66 µl 10 % SDS
3.2 ml H_2O	4.06 ml H_2O
50 µl 10 % Ammoniumpersulfate	50 µl 10 % Ammoniumpersulfate
40 µl TEMED	50 µl TEMED

Running buffer consisted of 250 mM Tris, 1.92 M glycine, and 1% SDS. Protein ladder (4 µl) including of ß-Galactosidase (116 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp98I (25 kDa), ß-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) (Fermentas) was used as molecular weight marker. Sample was loaded with 5 and 10 µl for each treatment. After electrophoresis, the gel was stained using colloidal Coomassie according to Neuhoff *et al.* (1990). Protein amounts were verified visually.

II.16.3 Liquid isoelectric focusing

Protein content in 100 mg *Anabaena* was reconstituted in rehydration buffer until a concentration of about 0.5 mg/ml was reached. Rehydration solution included 7 M urea, 2 M thiourea, 2 % CHAPS in 50 ml H_2O and 50 µl 1% bromophenol blue (*tetrabromophenolsulfonephthalein*). 250 µl 2 M DTT and 200 µl ampholytic solution pH 3-10 were added to the sample.

A multiple compartment isoelectric focusing system with isoelectric membranes (pH 3.0, 4.6, 5.4, 6.2, 7.0 and 10.0) was used to separate proteins according to their isoelectric points (Righetti *et al.*, 1997). Protein sample (650 µl) was loaded in the chamber. The multiple compartments system was filled with 17.5 ml of anode and cathode buffers (see below). The electrophoresis was carried out at 100 V for 30 min, subsequently 200 V for 90 min and 600 V for 135 min. After isolectric focussing is finished, samples in each chamber were collected and concentrated for SDS-PAGE separation.

Anode buffer (pH 3.0)	**Cathode buffer (pH 10.4)**
8.4 g urea	8.4 g urea
3.0 g thio-urea	3.0 g thio-urea
3.3 ml Novex IEF anode buffer (50X)	2.0 ml Novex IEF cathode buffer (10X)
20 ml water	20 ml water

About 200 µl protein solution was mixed with 1 ml ice cold TCA 10% and incubated overnight for precipitation at – 20°C. After centrifuging at 14000 g and 4°C for 3 min, protein pellet was washed with 1 ml ice cold solution of methanol 80%/0.1 M ammonium acetate. Afterwards, sample was washed with 80% acetone and dried at RT for about 15 min.

II.16.4 Protein separation by SDS-PAGE

The protein pellet was diluted in 20 µl LDS buffer (10% glycerine, 141 mM Tris, 106 mM Tris-HCl, 2% LDS, and 0.51 mM EDTA). The sample was heated at 80^{o}C for 10 min before 18 µl were loaded on a Bis-Tris-Gel (NuPAGE, 4-12%). Electrophoresis was accomplished at 50 V for 30 min and subsequently by 150 V for 60 min. A mixture (4 µl) of ß-Galactosidase (116 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp98I (25 kDa), ß-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa) was used as protein marker (Fermentas).

The gel was silver stained according to Blum *et al.* (1987). Protein bands of the different treatments were compared visually and differential bands were cut out and stored in Teflon coated plastic tubes for analysis.

II.16.5 Two-dimensional gel electrophoresis according to Görg *et al.* (2004)

Anabaena (50 mg FW) was used for protein isolation according to Wang *et al.* (2006). First, IPG strips were first rehydrated overnight with buffer (7 M urea, 2 M thio-urea, 2 % CHAPS, in 50 ml H_2O, and 50 µL 1% bromophenol blue). Rehydration buffer (1 ml) was mixed with 25 µl 2 M DTT and 20 µl IPG buffer pH 3-11. This mixture (450 µl) was used for rehydration for each strip. After rehydration, IPG strips were covered with 108 ml paraffin oil to avoid drying during isoelectric focusing. Protein sample was loaded on the acidic side of strip. The IPGphor was programmed as followed:

Phase	Mode	Volt (V)	Time (h)
1	Constant	100	2
2	Constant	200	2
3	Constant	300	3
4	Gradient	500	3
5	Gradient	1000	3
6	Gradient	4000	3
7	Constant	4000	10

Afterwards, gel strips were washed with water and were equilibrated twice for 15 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerin, 2% SDS and

0.002% bromophenol blue). First, strips were shaken lightly in buffer with 130 mM DTT. Second, gels were incubated in buffer with 280 mM IAA.

Subsequently, proteins were separated by 12.5% SDS gel. Second dimension was carried out in an IsoDalt-Electrophoresis chamber with 5W/gel for 30 min and then 17W/gel for 4 h. Fixing and staining procedures were carried out as described in II.14.4. Differences between treatments in protein patterns were done by Delta 2D (Decodon) after gels scanned using the SilverFast software. Spots with a pixel volume more than 20 and a 3-fold intensity change were classified as different. Differential spots were cut out and stored at – 20°C for protein analysis by nano-LC-MS/MS.

II.16.6 In-gel digestion of protein spots

Set the pH at 7.8 and remove enzyme inhibiting compounds such as detergents (Reinders *et al.*, 2004), spots have to be washed several times. Gel pieces were washed 3 times alternatively with 100 µl 50 mM ammonium hydrogen carbonate (pH 7.8) and 25 µl 25 mM ammonium hydrogen carbonate (pH 7.8) in 50% acetonitrile. For NuPAGE gel pieces, they were washed with 100 µl DTT 10 mM in ammonium hydrogen carbonate 50 mM (pH 7.8) by 56°C for 30 min and with 100 µl 5 mM IAA in ammonium hydrogen carbonate 50 mM (pH 7.8) at RT for another 30 min. After washing, gel spots were dried in a vacuum centrifuge at 60°C for about 30 min. Proteins were digested with 4 µl trypsin solution at 37°C overnight. Trypsin solution was prepared by mixing 2.5 µg trypsin in 25 µl 1 mM HCl. This mixture was added to 175 µl ammonium hydrogen carbonate 50 mM (pH 7.8). Protein fragments were extracted with 50 µl of 5% formic acid in acetonitrile. The sample was concentrated to 5 µl in a vacuum centrifuge and another 10 µl 5% formic acid was added. The eluted peptides were analyzed using nano-HPLC-MS/MS (as described in II.25).

II.16.7 Protein analysis by nano-LC-MS/MS

The HPLC-Ultimate 3000-HPLC and a LCQ Deca XP plus mass spectrometer were used for protein analysis. First, proteins were concentrated and separated on a RP-HPLC column before MS/MS analysis. After sample injected, the sample was washed on a trapping column (C18) with 0.1% trifluoroacetic acid for 8 min to remove salt contamination in the sample. Afterwards, peptides were eluted using a mixture of formic acid and acetonitrile. The

eluted proteins were transferred directly to MS/MS system for peptides analysis. Spectra were processed using the server algorithm Mascot™.

II.17 Shinorine analysis in *Anabaena* after ultra violet light (UV light), copper sulphate, jasmonate and phytoprostane treatment

Anabaena cell suspension (5 ml, adjusted to 5 mg DW/5 ml) were pipetted into each well of a 6 well-plate. The cell culture was treated with UV light in combination with 1 mM of $CuSO_4$, 50 µM of OPDA, 50 µM JA, 50 µM dihydro-JA, 50 µM PPB_1 type I or water as control at 23°C. In parallel, an experiment was carried out with UV light. To avoid UV effects in untreated controls, a UV shield was used. The shield was transparent, allowing white light to pass. Cells were collected 24, 48, and 72 h after treatment. Shinorine reference was a courtesy of Professor Donat-P Häder from the University of Erlangen.

The cell suspension was collected by centrifugation at 4000 rpm for 10 min. The cell pellet was shock frozen in liquid nitrogen and then extracted with 2 ml 20% ethanol (v/v) for 2.5 h at 45°C in a water bath (Sinha *et al.*, 1999). After extraction, the sample was centrifuged and 1.5 ml of the upper phase was transferred into a 2 ml reaction tube. The solvent was removed in a rotary evaporator at 45°C. The residue was reconstituted in 250 µl water and shinorine was analyzed by HPLC (II.22.1).

II.18 Analysis of tocopherols in *Anabaena* after ultra violet light (UV light), copper sulphate, jasmonate and phytoprostanes treatment

The sample collection was carried out as described in II.17 the cells were extracted with 1.5 ml hexane/isopropanol 2/1 (v/v) in an ultrasonic water bath for 10 min. Subsequently, 1.125 ml of saturated NaCl solution was added, the sample was mixed for one minute and centrifuged at 4000 rpm for 10 min. The organic phases were removed in a rotary evaporator at 45°C. The residue containing tocopherols were reconstituted in 250 µl methanol and analyzed by HPLC with fluorescence detection (II.22.2). A mixture sample of α-, β-, δ-, and γ-tocopherol was used as external standard and retention time reference.

II.19 Salicylic acid, jasmonate, and phytohormone analysis in *Anabaena*

Anabaena cell culture (500 ml) was centrifuged at 4000 rpm at 4°C for 10 min and the medium was poured off. The cell pellet was shock frozen in liquid nitrogen. The sample was extracted with 2 ml methanol plus 100 µl acetic acid. 50 ng of norvaline, $[^{18}O]_3$-salicylic acid, $[D]_6$-absicic acid. 100 ng of $[D]_5$-OPDA and 25 ng of $[D]_2$-indole-3-acetic acid (IAA) were used as internal standards. Samples were sonicated for 1 min, shaken for 1 min and centrifuged at 3600 rpm at 4°C for 10 min. The methanol phase was transferred into a plastic tube and dried in a speed vacuum concentrator at 50°C. Thereafter samples were reconstituted in 50 µl of acetonnitrile/1 M ammonium acetate (20/80, v/v), pH 6.8 and transferred into new tubes. After centrifugation at 14000 rpm for 5 min, the aqueous phase was transferred into HPLC-micro-vials and analyzed by HPLC-MS (II.24.2).

II.20 Investigation of protective effects of PPF_1 on *Pseudomonas syringae* and *Escherichia coli* under oxidative stress condition

Pseudomonas syringae bacteria were grown in King's medium consisting of 20 g glycerol (87%) and 40 g proteose peptone 3 in 1l of water. After autoclaving, 10 ml of 10% K_2HPO_4 (w/v) and 10 ml of 10% $MgSO_4$ (w/v) were sterile filtrated and added.

Escherichia coli bacteria were grown in LB (lysogeny broth) medium that included 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1l of distilled water. The LB medium was autoclaved and stored at RT.

E. coli (DH5a) was cultured in 12 ml plastic tubes containing 5 ml LB medium on a shaking plate (180 rpm) at 37°C until cell density reached an OD_{600} of 0.8. A volume of 50 µl from this cell suspension was transferred to 5 ml LB medium containing 1% methanol as control or 100 µM PPF_1. Cultures were incubated over night and cells were harvested by centrifugation at 3500 rpm for 15 min. The cell pellet was resuspended with fresh LB medium to an OD_{600} of 0.8. The cell suspension (50µl) were added to a final volume of 5 ml LB medium and subjected to the second treatment which was either distilled water (as control), 1000, or 2000 µM H_2O_2. The experiment was done with 3 replicates. *E. coli* growth rate was determined by measuring the OD_{600} after incubation for 3 and 6 hours.

In another experiment, avirulent strain *P. syringae* pv tomato DC3000 *avr*RPM1 was used. Bacteria were grown in LB medium containing 50 mg/l rifampicin (Rif) at 28°C on a shaking plate (220 rpm). The experiment was carried out as described for *E. coli* except that the H_2O_2 concentration was changed from 0, 100, 500 to 1000 µM and growth was determined after 8 und 24 hours of culture.

II.21 Analysis of free PPF_1 and PPE_1 in hay by GC-MS

Dried hay (stored for at least several months) was used in this experiment. Hay was ground by a small electric café grinder. About 100 mg of hay powder were incubated with 20 ml of distilled water and shaken with a rotary shaker for 14 h at RT. The sample was filtered through a Whatman paper filter. 500 ng of $^{18}[O]_3$-PPF_1 and 250 ng PGE_1 were used as IS. The aqueous extract was acidified to pH 3 with 1 M of citric acid. Free PPF_1 or PPE_1 were extracted 2 times with 10 ml diethyl ether. The ether phases were combined and taken to dryness.

For PPF_1 analysis, the sample was diluted in 3 ml chloroform and loaded on an NH_2-column. After washing with 6 ml chloroform/isopropanol 2/1 (v/v), the column was eluted with 6 ml diethyl ether/methanol/acetic acid 80/20/2 (v/v). The eluate was taken to dryness. Derivatisation included hydrogenation (II.7.1), PFB-Br esterification (II.7.4), and silylation (II.7.5) before the samples were analyzed by GC-MS.

For PPE_1 analysis, sample was reconstituted in 100 µl methanol, isomerized to PPB_1 by adding 400 µl of 1 M KOH and incubated for 10 min at 40°C. After hydrolysis, the sample was transferred to a 50 ml Falcon tube containing 10 ml water and 11 ml 1 M citric acid and extracted twice with 10 ml diethyl ether. The sample was taken to dryness, re-diluted in 3 ml diethyl ether, purified using an NH_2-column, esterified with PFB-Br (II.7.4), silylated with BSTFA (II.7.5) before being analyzed by GC-MS. For details of the reactions and GC-MS conditions, see II.23.

II.22 HPLC methods

II.22.1 HPLC parameters for MAA analysis

HPLC:	Waters
Column:	LiChrospher 100 RP 18 (250 x 4 mm with precolumn, 5 µm)
UV detection:	λ = 334 nm
Mobile phase:	0.2 % acetic acid
Gradient:	isocratic
Flow rate:	1 ml/min
Retention time:	3.2 min

II.22.2 HPLC parameters for tocopherols analysis

HPLC:	Waters	
Column:	Purospher Star RP 18ec (250 x 4 mm, 5 µm)	
Column operating temperature:	40°C	
Mobile phase:	water/methanol 5/95 (v/v)	
Gradient:	isocratic	
Flow rate:	1.5 ml/min	
Fluorescence detection:	$\lambda_{excitation}$:	295 nm
	$\lambda_{emission}$:	325 nm
Retention time:	δ-tocopherol	10.6 min
	β-tocopherol	12.8 min
	γ-tocopherol	12.9 min
	α-tocopherol	15.4 min

II.22.3 HPLC method for PPB_1 and PPA_1 analysis

HPLC:	Waters
Column:	LiChrospher 100 RP 18ec (250 x 4 mm, 5 µm)
Mobile phase:	water/methanol/acetonitrile/acetic acid (48:28:24:0.1, v/v)
Flow rate:	1 ml/min
UV detection:	279 nm
Retention time:	PPB_1 type IV: 13.2 min
	PPB_1 type III: 19.8 min
	PGB_1 (standard): 34.6 min
	PPA_1 type III/IV: 15.6 min

Gradient timetable:

Time (min)	0.1% acetic acid (%)	Methanol (%)	Acetonitrile (%)
0	48	28	24
25	48	28	24
25.01	30	35	35
35	30	35	35
35.01	0	50	50
50	0	50	50

II.23 GC-MS methods

Gas chromatograph:	Agilent 6890
Column:	ZB 5 (25 m x 0.25 mm, 0.25 µm, Phenomenex)
Pre-column:	methyl deactivated column (5 m x 0.25 mm, 0.25 µm Phenomenex)
Carrier gas:	Helium
Flow rate:	1 ml/min
Injector:	Insert-Liner filled with glass wool, 300°C
Split:	splitless
Detector:	JMS-GCMate II (Jeol, Tokyo, Japan)

Separation of methyl ester and TMS ether of fatty acids and phytoprostanes

Time (min)	ΔT (°C/min)	Temperature (°C)
0	-	140
1	-	140
5.8	20	235
21	1	250
23	29	300
28	-	300

Separation of PFB ester and TMS ether of phytoprostanes and corresponding methoximated phytoprostanes

Time (min)	ΔT (^{o}C/min)	Temperature (^{o}C)
0	-	175
0.5	-	175
4.2	30	185
10.2	2.5	300
15.2	-	300

Setting parameters of GC-MS

Parameters	Positive EI mode	Negative/Positive CI mode
Ion source temperature	200^{o}C	200^{o}C
Electron energy	70 eV	200 eV
Reactant gas	-	Methane
Gas pressure	-	2 x 10^{-4} bar

II.24 HPLC-MS/MS methods

II.24.1 HPLC-MS/MS setting for PPF_1

Source of electro spray	ESI-
Capillary voltage (kV)	3
Cone voltage (V)	41
Source temperature (^{o}C)	120
Desolvation temperature (^{o}C)	400
Cone gas flow (l/h) – N_2	47
Desolvation gas flow (l/h) – N_2	797
Collision gas (ml/min) – Ar	0.27
Column**:**	Purospher Star RP-18e (125 x 2 mm, 5 µm, MERCK)
Flow rate:	0.2 ml/min
Mobile phase:	ammonium acetate/acetonitrile

Gradient timetable:

Time (min)	Ammonium acetate 1 mM (%)	Acetonitrile (%)
0.00	90	10
15.00	60	40
15.01	10	90
20.00	10	90
20.01	90	10
35.00	90	10

Multiple Reaction Monitoring (MRM) for PPF_1 analysis

Substances	Ionisation mode	Parent (*m/z*)	Daughter (*m/z*)	Cone voltage (V)	Collision energy (eV)
PPF_1	-	327	283	41	26
$^{18}O_3$-PPF_1	-	333	287	41	26

II.24.2 HPLC-MS/MS setting for phytohormones

Source of electro spray:	ESI-
Capillary voltage (kV)	3
Cone voltage (V)	variable
Source temperature (^oC)	120
Desolvation temperature (^oC)	400
Cone gas flow (l/h) – N_2	48
Desolvation gas flow (l/h) – N_2	796
Collision gas (ml/min) – Ar	0.27
Column:	Purospher Star RP-18e (30 x 2 mm, 3 µm, MERCK)
Flow rate:	0.2 ml/min
Mobile phase:	ammonium acetate/acetonitrile

Gradient timetable:

Time (min)	Amonium acetate 1 mM (%)	Acetonitrile (%)
0.00	95	5
10.00	5	95
15.00	5	95
15.01	95	5
30.00	95	5

Multiple Reaction Monitoring (MRM) for phytohormones analysis

Substances	Ionisation mode	Parent (*m/z*)	Daughter (*m/z*)	Cone voltage (V)	Collision energy (eV)
ACC	+	102	56	20	9
Norvaline	+	118	72	17	10
SA	-	137	93	17	17
$^{18}O_2$-SA	-	141	93	17	17
IAA	+	176	130	20	15
D_2-IAA	+	178	132	20	15
ABA	-	263	153	26	14
D_6-ABA	-	269	159	26	14
OPDA	-	291	165	22	26
D_5-OPDA	-	296	170	22	26
JA	-	209	59	19	17
DHJA	-	211	59	19	17

II.24.3 HPLC-MS/MS setting for PPB_1

Source of electro spray	ESI-
Capillary voltage (kV)	3
Cone voltage (V)	31
Source temperature (^oC)	120
Desolvation temperature (^oC)	400
Cone Gas Flow (l/h) – N_2	47

Desolvation Gas Flow (l/h) – N_2	797
Collision gas (ml/min) – Ar	0.27
Column:	Purospher Star RP-18ec (30 x 2 mm, 3 µm, MERCK)
Flow rate:	0.2 ml/min
Mobile phase:	ammonium acetate/acetonitrile

Gradient timetable:

Time (min)	Amonium acetate 1 mM (%)	Acetonitrile (%)
0.00	85	15
30.00	60	40
30.01	0	100
35.00	0	100
35.01	85	15
60	85	15

Multiple Reaction Monitoring (MRM) for PPB_1 analysis

Substances	Ion mode	Parent (*m/z*)	Daughter (*m/z*)	Cone voltage (V)	Collision energy (eV)
[^{18}O]-PPB_1 type I	-	309	235	31	23
[^{18}O]-PPB_1 type II	-	309	187	31	23
PPB_1 type I	-	307	235	31	23
PPB_1 type II	-	307	185	31	23
PPB_1 type III	-	307	113	31	23
PPB_1 type IV	-	307	144	31	23

II.25 Nano-HPLC-MS/MS method for proteome analysis

Parameters for HPLC system:

Column:	C18, 15 cm x 75 µm
Solution A:	0.1% formic acid
Solution B:	0.1% formic acid in 84% acetonitrile
Flow rate:	250 nl/min
UV detector:	214 nm and 295 nm

Gradient timetable:

Time (min)	Solution A (%)	Solution B (%)
0	95	5
8	95	5
10	85	15
25	40	60
26	5	95
30	5	95
31	95	5
40	95	5

Parameters for MS/MS system:

Voltage: 2 kV

Temperature: 200^{o}C

Collision gas: helium

Ionisation: electro spray ionisation (ESI) in positive mode

Proteins were identified by their peptide fragmentation spectra via the Mascot Algorithm with following parameters:

Data bank: CyanoBase

Enzyme: Trypsin

Fixed modification: Carbamidomethylation

Variable modification: Oxidation (M), Pyroglutamate N-terminal

Mass value: Average

Peptide mass tolerance: ± 1.5 Da

Fragment mass tolerance: ± 1.5 Da

Max missed cleavage: 1

Instrument type: ESI-ion TRAP

III. RESULTS

III.1 Fatty acids composition in *Anabaena* and *Synechocystis*

Fatty acids, particularly polyunsaturated fatty acids, are the main component of cell and organelle membranes and are important to maintain cell membrane fluidity and also contribute to the resistance of cells or organisms to stress. In plants, more than 200 fatty acids have been identified. Among them, a-linolenic acid is the main polyunsaturated fatty acid in chloroplast that is essential for the proper function of the photosynthetic apparatus. On the other hand, trienoic fatty acids are prone to autoxidation by singlet oxygen and reactive oxygen species (ROS). Since the chloroplast photosynthetic apparatus is a main source of singlet oxygen and free radicals in plants and cyanobacteria, non-enzymatic formation of lipid oxidation products can not be avoided especially under high light conditions. Radical catalyzed oxidation of a-linolenic acid lead to the formation of phytoprostanes while enzymatic oxidation may yield jasmonates. Similar to plants, almost all cyanobacteria maintain a high level of polyunsaturated fatty acids in their membranes. The relative fatty acid composition is dependent on the cyanobacterial species, the growth medium and environmental factors especially light and temperature.

Since unsaturated fatty acids are precursors for enzymatic and non-enzymatic oxylipin pathways, the fatty acid compositions of the two cyanobacterial strains used in this work (*Anabaena* PCC 7120 and *Synechocystis* PCC 6803) were analysed under our standard incubation conditions. Total fatty acid profiles of the two cyanobacterial strains were determined after lipid hydrolysis and methylation by GC-MS (Fig. III-1 and III-2).

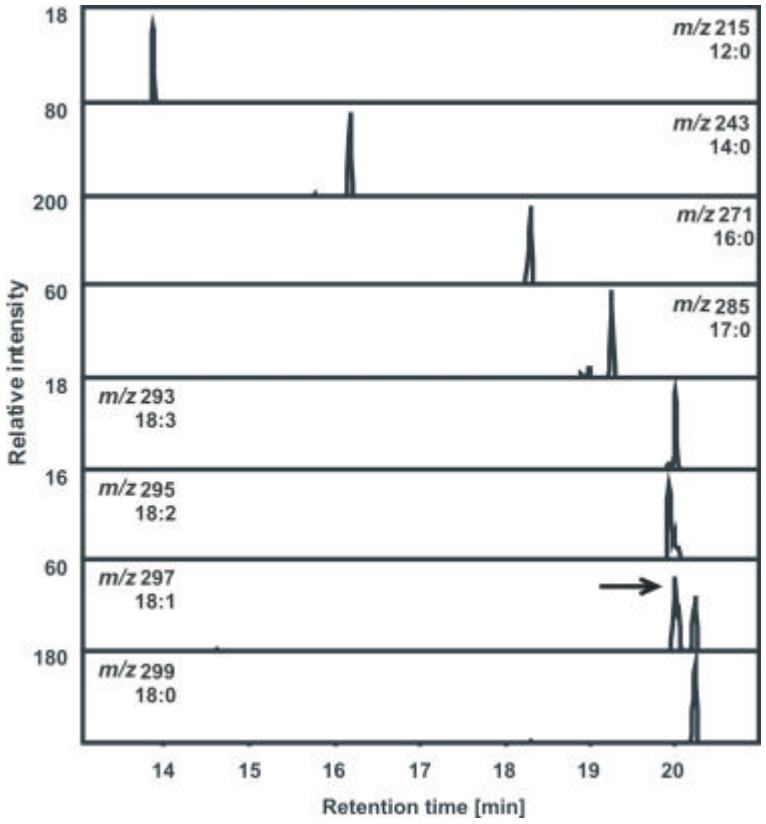


Figure III-1. CG-EI-MS chromatograms of fatty acids of *Anabaena* PCC 7120. Lipid extracts were hydrolysed and fatty acids were methylated prior analysis. Arrow shows the peak of integration.

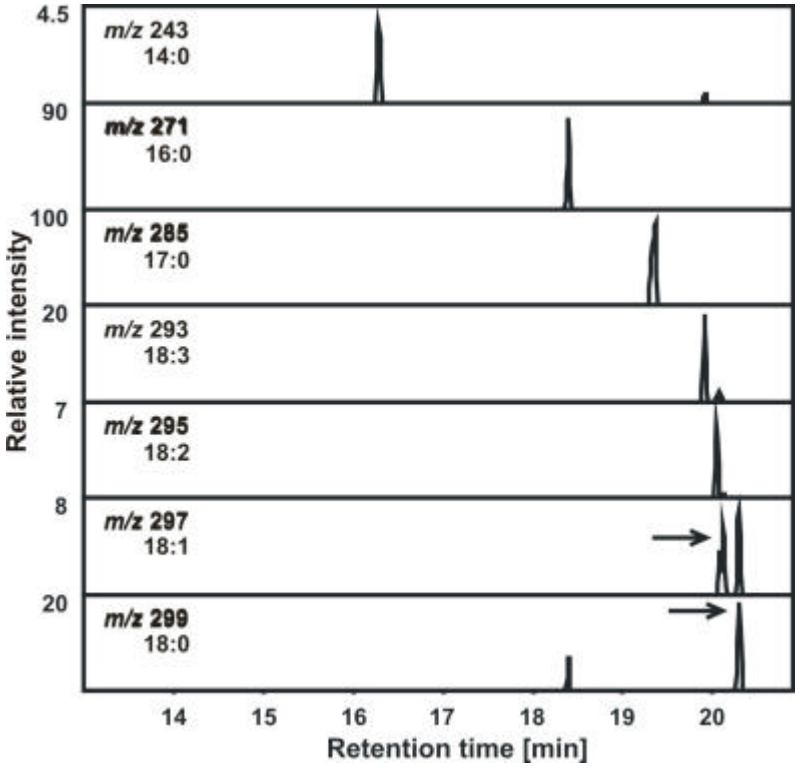


Figure III-2. CG-EI-MS chromatograms of fatty acids of *Synechocystis* PCC 7120. Lipid extracts were hydrolysed and fatty acids were methylated prior analysis. Arrow shows the peak of integration.

Table III-1. Total relative fatty acid composition (%).

Fatty acid	*Anabaena* PCC 7120	*Synechocystis* 6803
12:0	0.1 ± 0.00*	n.d.**
14:0	1.3 ± 0.19	1.5 ± 0.17
16:0	26.5 ± 1.46	49.8 ± 2.47
18:0	6.8 ± 1.08	10.2 ± 3.26
18:1	26.7 ± 0.46	8.0 ± 0.95
18:2	11.7 ± 0.37	6.3 ± 0.73
18:3(a**)**	**27.0** ± **2.37**	n.d
18:3(g**)**	n.d	**24.3** ± **2.59**

*(mean ± SD, n = 3); **n.d.: not detectable

The level of unsaturated fatty acids is higher in *Anabaena* with 65% than in *Synechocystis* with 38%. As shown in Tab. III-1, *Anabaena*, only utilizes a-linolenic acid (27 ± 2.37% of total fatty acids) as trienoic acid and is devoid of ?-linolenic acid. In contrast, *Synechocystis* only contains ?-linolenic acid (24.3 ± 2.59%) and no a-linolenic acid.

These results indicate the two cyanobacterial strains have a different potential capacity to form oxylipins. For instance, non-enzymatic oxidation of linolenate in both species will lead to the formation of two different series of oxidized lipids or two series of regioisomers. From the fatty acid analysis it can be predicted that *Anabaena* may form phytoprostanes (regioisomers of the type I and II) from α-linolenate similar to plants while *Synechocystis* may be capable to form animal-like dinor isoprostanes (phytoprostane regioisomers of the type III and IV) from γ-linolenate.

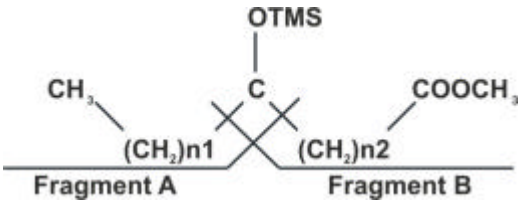
In plants and animals, linolenate and linoleate are substrates for lipoxygenases that lead to the exclusive formation of 9- and/or 13-hydroxy fatty acids. In contrast, non-enzymatic oxidation results in accumulation of hydroxy fatty acids oxygenated in all theoretical possible positions. Therefore, the presence of enzymatic and non-enzymatic products was investigated.

III.2 Hydroxy fatty acids analysis in *Anabaena*

Cyanobacteria are known as the unique bacteria which can convert light radiation into biochemical energy. This photosynthetic process produces free oxygen and reactive oxygen

species (ROS) as by-products. These products play a very important role in inducing the formation of hydroxy fatty acids (OH-FAs) and phytoprostanes in plants. Here, the natural occurrence of hydroxy fatty acids in *Anabaena* was determined. As shown in Table III-2, all OH-FAs that are present in plants, can also be found in *Anabaena* (Table III-2 and Fig. III-3). The levels of total OH-FAs are relatively high (µg/g of dry weight range), however, similar levels were reported for *Arabidopsis thaliana* (Grun *et al.*, 2007). Levels of 9- and 13-hydroxy fatty acids that potentially can be formed by enzymatic as well as non-enzymatic oxidation are similar to levels of hydroxy fatty acids that are exclusively non-enzymatically synthesised. Hence, results indicate that the extend of non-enzymatic lipid oxidation in *Anabaena* is comparable to *Arabidopsis*. Therefore, synthesis of phytoprostanes should also occur in *Anabaena*. In addition, the data provides no evidence for a functional lipoxygenase pathway in *Anabaena*. However, in higher plants (all expressing lipoxygenases) levels of non-enzymatic and enzymatically synthesized may be similar, at least under non-stressed, basal conditions. All higher plants thus far analysed produce via a lipoxygenase pathway jasmonates. In order to check wheather or not *Anabaena* is also capable to synthesise phytoprostanes and jasmonates, the oxylipin pattern was analysed.

Table III-2. Hydroxy fatty acids fragmentation and levels in *Anabaena*.

	m/z		
OH-FA derivative			**Total OH-FA in *Anabaena* (µg/g DW)**
8-OH-(C18:0)	243	245	5.59 ± 0.33
9-OH-(C18:0)	229	259	51.55 ± 5.72
10-OH-(C18:0)	215	273	33.04 ± 2.56
11-OH-(C18:0)	201	287	17.15 ± 1.65
12-OH-(C18:0)	187	301	25.27 ± 1.55
13-OH-(C18:0)	173	315	32.48 ± 2.09
15-OH-(C18:0)	145	343	4.11 ± 0.40
16-OH-(C18:0)	131	357	7.17 ± 0.78
15-OH-(C20:0)	173	343	Internal Standard

Hydroxy fatty acids were hydogenated, methylated, and silylated before being analyzed on GC-MS in the positive electron ionization mode. Values represent mean ± SD (n = 3). Calculation was done with response factors from Mueller *et al.* (2006).

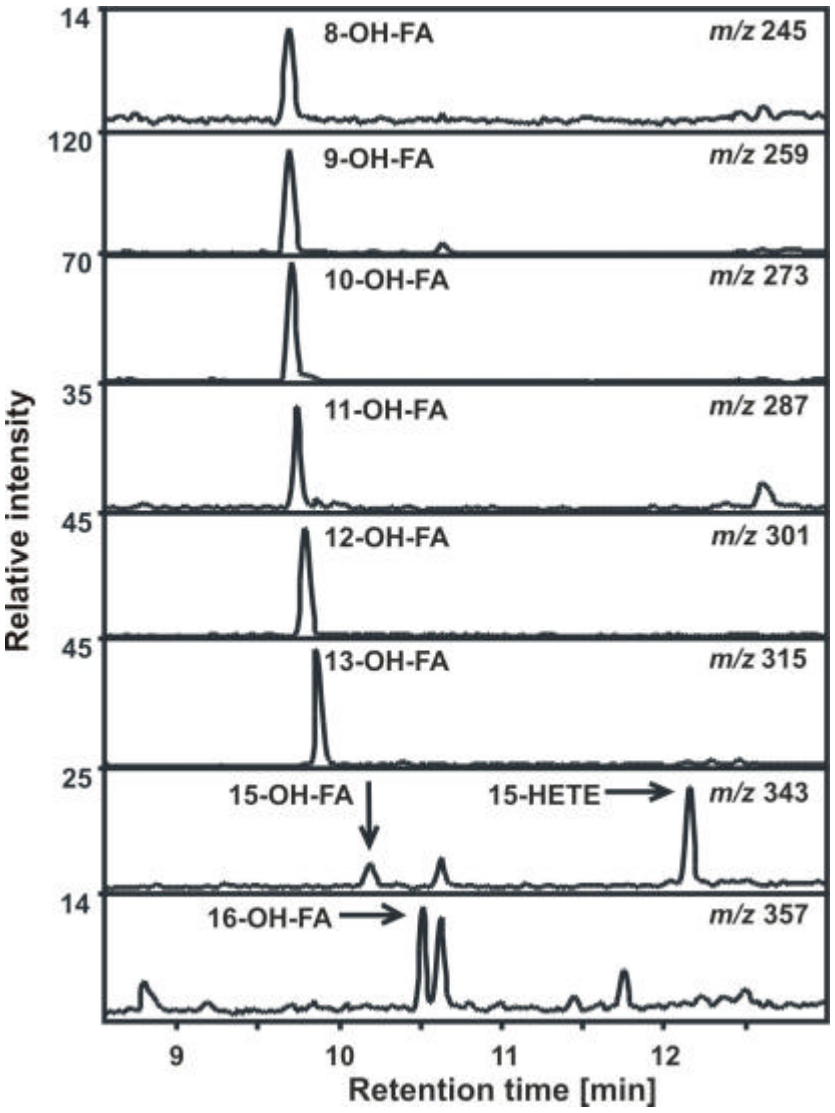


Figure III-3. GC-MS-EI chromatograms from *Anabaena* extraction. Peaks show the detected B-fragments after hydrogenating, methylating, and silylating of hydroxy fatty acids. Internal 15-HETE standard gives the same mass/charge ratio (*m/z* 343) with 15-OH-FA but differences in absorption intensity and retention time. See table III-2 for fragments from each hydroxy fatty acid. Arrows show the peaks of interest.

III.3 Evidence of phytoprostane F_1 and E_1 type I and II in *Anabaena*

The precursor of phytoprostanes, a-linolenic acid, is present in relative high proportion in cyanobacterial membranes. In addition, free radical-catalzed oxidation of linolenate leads to levels of hydroxy fatty acids comparable to levels found in higher plants. Therefore, phytoprostanes should also be present in *Anabaena*. As predicted, phytoprostanes were found in *Anabaena*, PPF_1 and PPE_1 (type I and II) could easily and for the first time be identified in *Anabaena* by GC-MS analysis (Fig. III-4 and III-5). This is also the first evidence for the presence of phytoprostanes in the bacterial kingdom.

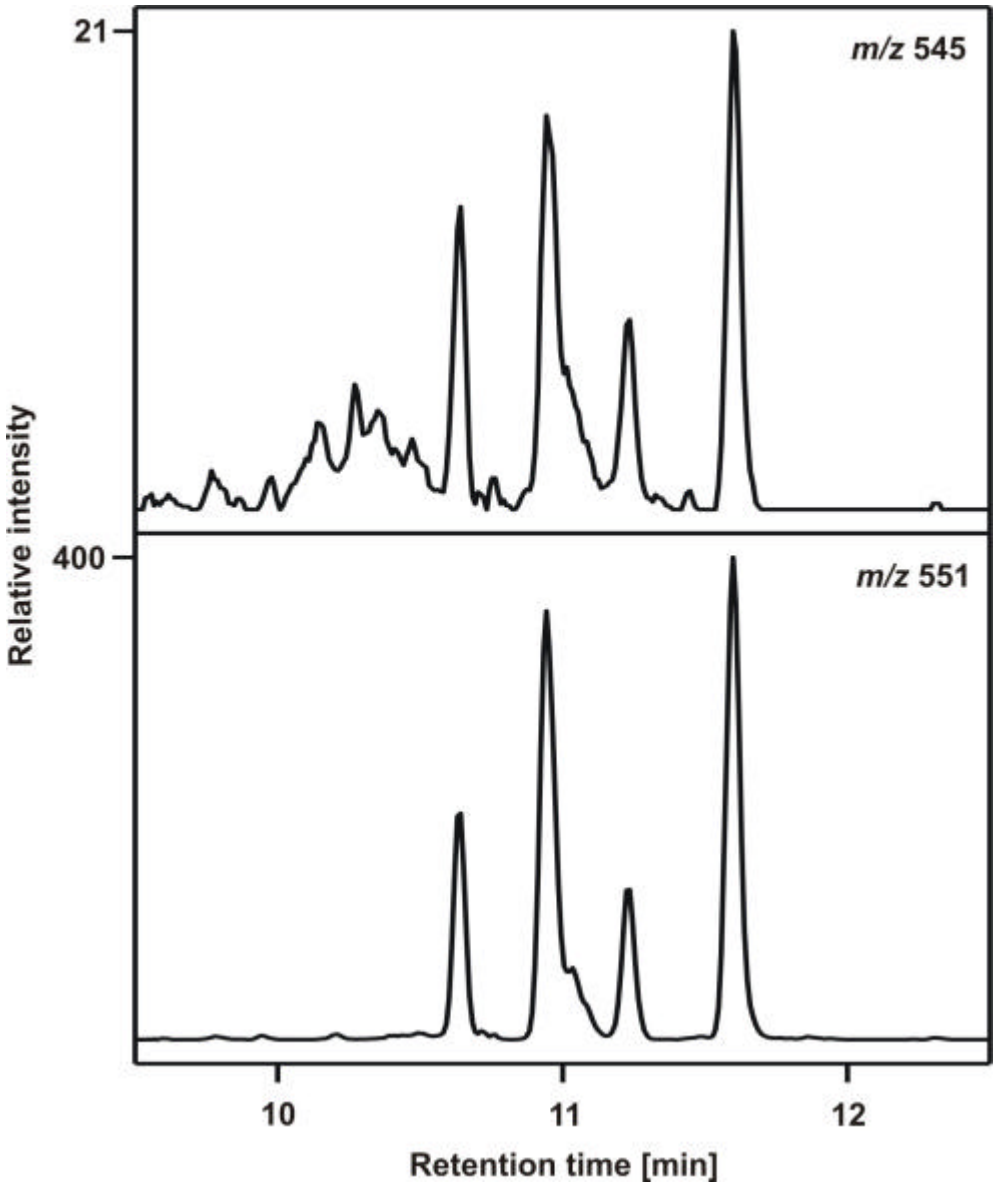


Figure III-4. GC-NCI-MS analysis of PPF_1 type I and II in *Anabaena*. PPF_1 were hydrogenated, esterified with PFB-Br, and silylated prior analysis. Endogenous PPF_1 (type I and II) were detected at *m/z* 545 and $[^{18}O]_3$-PPF_1 (type I and II, internal standard at *m/z* 551).

PPE_1 type I and II in *Anabaena* were converted to PPB_1 and quantified as a sum of PPB_1 and PPE_1. As explained in the method part, PPE_1 can not be measured directly by HPLC or GC-MS. However, careful analysis of *Anabaena* extracts revealed that PPB_1 type I and II are not present or below the limit of detection in *Anabaena*. Therefore levels of PPB_1 determined after conversion of PPE_1 to PPB_1 represent endogenous levels of PPE_1 (type I and II). This procedure was applied throughout this thesis.

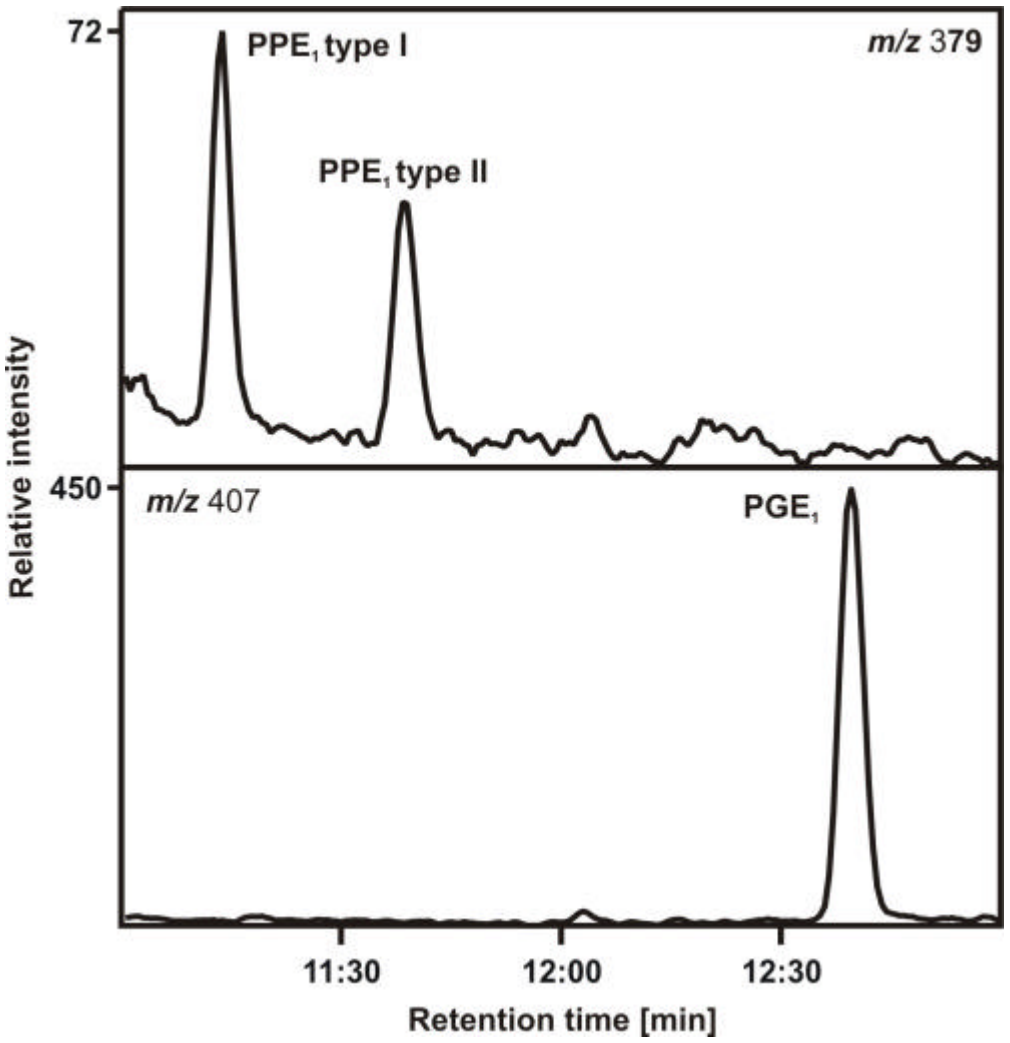


Figure III-5. GC-NCI-MS chromatograms of PPE_1 type I and II analysis in *Anabaena*. PPE_1 and PGE_1 were esterified with PFB-Br and silylated with BSTFA after isomerization to the corresponding PPB_1-and PGB_1-analogues. When measured in GC-NCI-MS, the mass/charge ratio of PPB_1 type I and II is 379 and that of PGB_1 is 407.

GC-MS quantification of the two main cyclic products of a-linolenic acid oxidation, PPF_1 and PPE_1, in *Anabaena* are shown in Table III-3. Their levels are comparable with those in plants. Both PPF_1 and PPE_1 were found in free and esterified form in cell as well as in the medium. In one week old of cell culture, PPF_1 could not be detected in the medium. After 6 weeks, PPF_1 accumulated up to 142.6 ± 41.8 ng/l in the medium. In contrast, PPE_1 were present in the medium at lower levels after one and six weeks of culturing (Table III-3). In addition, PPE_1 and PPF_1 occur both free and esterified form in the cells. Total phytoprostanes levels presented in Table III-3 are the sum of their free and esterified forms in the cells.

Table III-3. PPF_1 and PPE_1 in *Anabaena*. Values are means ± SD, n = 3.

Old of culture	PPF_1			PPE_1		
	Medium [ng/l]	Free [ng/g DW]	Total [ng/g DW]	Medium [ng/l]	Free [ng/g DW]	Total [ng/g DW]
1 week	n.d*	24.1 ± 10.9	211 ± 78.5	21.1 ± 10.4	80.5 ± 23.6	153.2 ± 39.8
6 weeks	142.6 ± 41.8	23.3 ± 3.2	159.7 ± 84.2	32.1 ± 7.7	114.1 ± 8.7	148.7 ± 63.7

*n.d: not detectable; SD: standard deviation

III.4 No evidence for the presence of jasmonates in *Anabaena*

After discovery of phytoprostanes in *Anabaena*, it is interesting to know if also the jasmonate pathway exists in this cyanobacterium. In plants, jasmonates are formed from a-linolenic acid by an enzymatic pathway. In this experiment, OPDA and JA in *Anabaena* were analyzed together with other plant hormones such as indole-3-acetic acid (IAA), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC, precursor of ethylene), and salicylic acid (SA) by HPLC-Tandem-MS. Multiple phytohormone analysis by GC-MS has been reported (Schmelz *et al.*, 2003). Here, an efficient and time saving HPLC-MS that does not require derivatisation of target compound has been developed. The detection limit for all phytohormones was below one ng per injection (Fig. III-6). Jasmonic acid and OPDA could not be detected in *Anabaena*. The trace peak observed in Fig. III-6-G corresponds to unlabelled OPDA that is present in the isotopically labelled OPDA standard (< 2%). In addition, *Anabaena* is also not able to form prostaglandins (not analyzed in this experiment) since it is devoid of the prostaglandin precursor arachidonic acid.

Thus far, enzymatically oxidized oxylipins typical for plants and animals as well as some fungal species, the jasmonates or prostaglandins, have never been detected in the bacterial kingdom. Since cyanobacteria are believed to be ancestors of chloroplasts, the enzymatic capacity to synthesize jasmonates evolved in chloroplasts of plants, or even higher plants since unicellular algae also appear to be devoid of jasmonates.

From the other plant hormones analyzed in *Anabaena*, only salicylic acid (63.1 ± 22.9 ng/g DW) and indole acetic acid (67.1 ± 6.6 ng/g DW) were found. IAA has previously been identified in cyanobacteria (Sergeeva *et al.*, 2002).

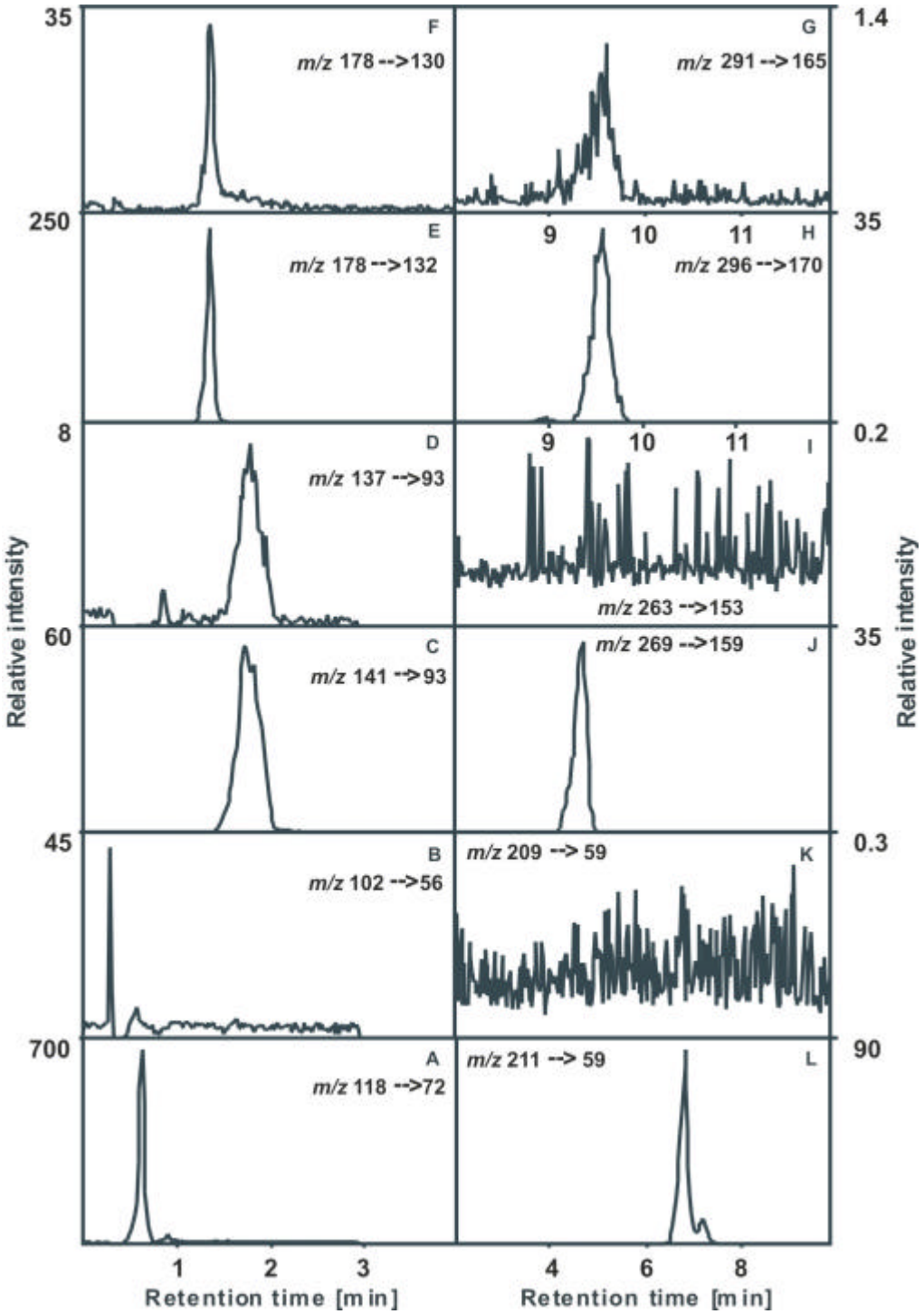


Figure III-6. Phytohormones analysis by HPLC-MS chromatogram in *Anabaena*. A: Norvaline (IS); B: endogenous 1-Aminocyclopropane-1-carboxylic acid (ACC); C: [$^{18}O_2$]-salicylic acid (SA, IS); D: SA; E: [D_2]-indole-3-acetic acid ([D_2]-IAA, IS); F: IAA; G: OPDA; H: [D_5]-OPDA (IS); I: abscisic acid (ABA); J: [D_6]-ABA (IS); K: jasmonic acid (JA); L: dihydro jasmonic acid (IS).

III.5 Induction of F_1- and E_1-phytoprostanes in *Anabaena* by oxidative stresses

Phytoprostanes are oxidative stress inducible compounds in plants. After determination of phytoprostanes in *Anabaena*, the question arose if phytoprostane levels

increase in *Anabaena* under oxidative stress and high light condition. When cells were grown under low light condition (LL, 10 µE.m^{-2}.s^{-1}), total cellular PPE_1 levels were 98.1 ± 27.3 ng/g DW. Same levels (92.6 ± 21.0 ng/g DW) were observed when cells were grown under high light (330 µE.m^{-2}.s^{-1}) for 8 h. Surprisingly, treatment of cells with 200 µM H_2O_2 under LL condition did not change the PPE_1 concentration (87.4 ± 29.5 ng/g DW). However, the same treatment under HL condition induced cellular PPE_1 levels more than 3 times (328.4 ± 41.0 ng/g DW, Fig. III-7A).

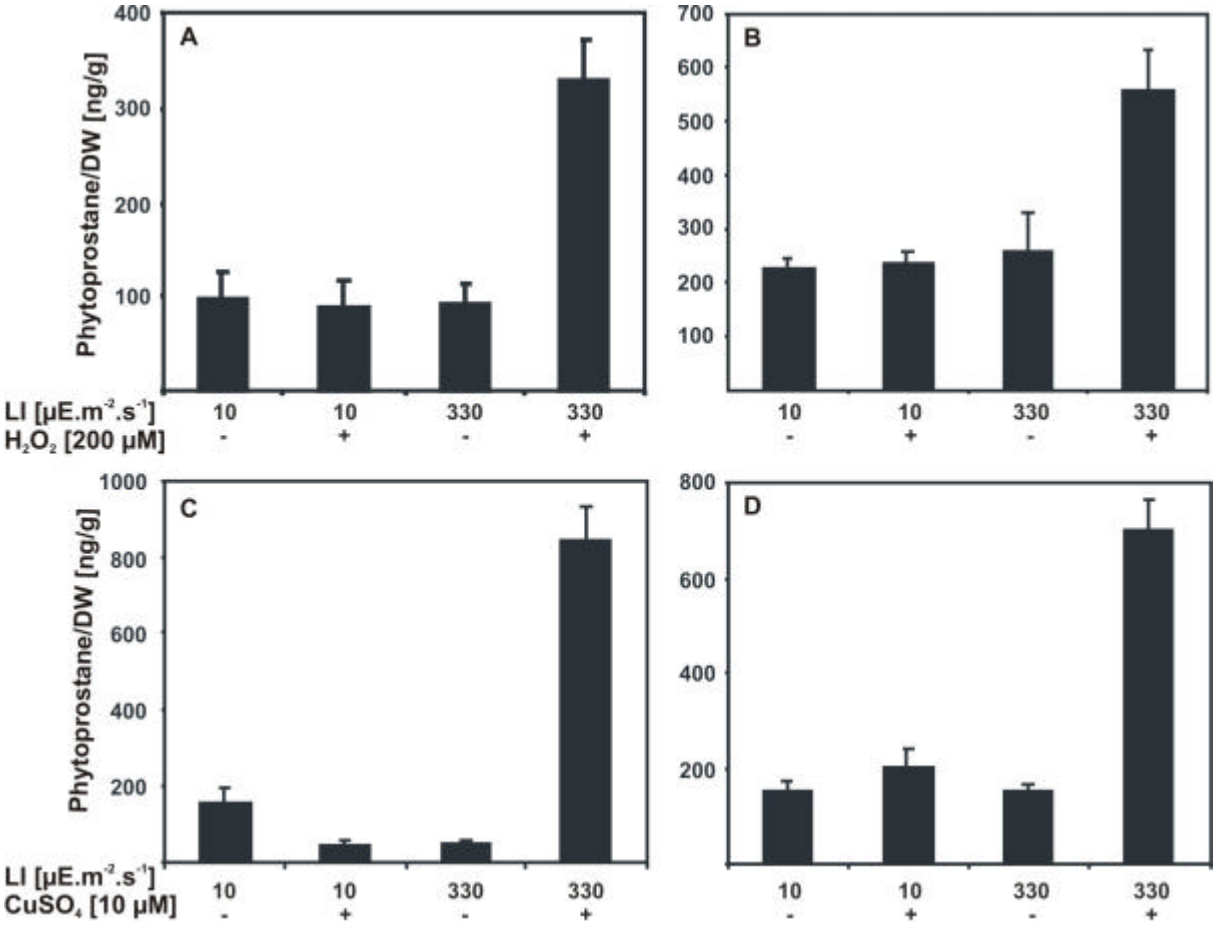


Figure III-7. Induction of PPE_1 (A, C) and PPF_1 (B, D) type I and II in *Anabaena* after 8h exposure to LL or HL and hydrogen peroxide or copper sulfate. LI: light intensity.

Another oxidative stress inducing condition (10 µM $CuSO_4$) did also not increase basal PPE_1 levels (157.0 ± 41.6 ng/g DW) under low light conditions; instead levels slightly decreased to 45.5 ± 14.7 ng/g DW. These levels were comparable with levels found after high light treatment (50.3 ± 9.8 ng/g DW) alone. Again, only the combination of oxidative stress (10 µM $CuSO_4$) and HL increased PPE_1 level more than 4-fold (842.5 ± 92.5 ng/g DW) (Fig. III-7C).

For PPF_1 levels in *Anabaena* cells, the effects of stress treatment are comparable to the in PPE_1 induction experiment. Under LL condition alone or in combination with 200 µM H_2O_2, the total PPF_1 concentration in the cell were similar (227.8 ± 19.5 ng/g DW and 234.8 ± 18.3 ng/g DW, respectively). HL condition alone did not alter total PPF_1 levels (260.8 ± 66.2 ng/g DW). Combination of HL with 200 µM H_2O_2 induced a 2-fold increase of total PPF_1 levels (Fig. III-7B).

Application of HL and 10 µM $CuSO_4$ at the same time gave a similar outcome but the induction factor is about 3 folds (Fig. III-7D). PPF_1 levels for LL and HL alone are 149.4 ± 19.7 ng/g DW and 151.9 ± 14.9 ng/g DW, respectively. A slight change in PPF_1 concentration (204.3 ± 32.8 ng/g DW) is observed in the treatment of LL and 10 µM $CuSO_4$. However, PPF_1 induction becomes more pronounced in combination of HL and 10 µM $CuSO_4$ (4,7-fold increase to 700.2 ± 64.2 ng/g DW).

Notably, basal levels of PPE_1 and PPF_1 in one week old *Anabaena* cultures (either LL or HL conditions) were slightly different in different experiments albeit the cell density was kept constant (OD720 ~ 0.3). Hence, phytoprostane levels appear to be somewhat sensitive to the culture conditions. However, HL or oxidative stress conditions alone did not trigger phytoprostane hyperaccumulation. Increases were only seen after combination of the two stress treatments. In cell cultures of higher plants, it has been shown that total phytoprostane levels could be strongly induced by peroxides or copper ions (6 to 15-fold increase) even under low light conditions (Imbusch and Mueller, 2000a). Hence, it appears that *Anabaena* cells are more resistant to oxidative stress – at least with respect to lipid oxidation. One possibility to explain the lower increase of lipid oxidation products in *Anabaena* would be to assume that phytoprostanes are excreted into the medium. However, PPF_1 could not be detected in the medium throughout the experiments and PPE_1 levels remained constant in the medium (about 20 ng/g DW).

III.6 Oxidized lipids protect *Anabaena* from oxidative stresses

Loeffler *at al.* (2005) first reported that copper ions induce the accumulation of phytoprostanes. Preincubation of plant cell cultures with phytoprostanes protects them from subsequent copper intoxication. It was hypothesized that recognition of oxidised lipids and priming of cells to resist oxidative stress could be an evolutionary old conserved mechanism. In order to test this hypothesis, a similar approach was used to explore if phytoprostanes display this activity in cyanobacteria. At the beginning, some observations indicated that the

sensitivity of *Anabaena* to the toxicity of copper sulfate was depended on the density of the cell suspension. At a high cell density, *Anabaena* can easily overcome the toxicity of 50 µM $CuSO_4$ or even higher concentrations. After several preliminary experiments, it was found that a cell suspension with the optical density at 720 nm (OD720) of about 0.3 was suitable for conducting the experiment because at this cell concentration, *Anabaena* is very sensitive to 50 µM $CuSO_4$, about 98% of the cells are lysed or dead 5 h after this treatment. In addition, the number of cells in the sample is relatively easy to count under the light microscope.

Results from the first phytoprostane preincubation experiments were unexpected as shown in Fig. III-8. The difference can be recognized by eyes. In the control, pre-treatment with 0.1 % methanol (solvent used to dissolve phytoprostanes) for 16 h followed by addition of water (equivalent to the volume of $CuSO_4$ stock solution) for 5 h, yielded only 0.5% dead cells and the cell suspension was green. In contrast, the 50 µM $CuSO_4$ treatment resulted in 98% of dead cells and the culture became yellowish. Interestingly, in a culture pre-incubated with 100 µM PPB_1 type I, only 16% of cells were dead after application of 50 µM $CuSO_4$ and the colour of cell suspension remained greenish. In comparison to the controls, we can see that PPB_1 type I in this case protected 84% of the cells from cell death. Pre-treatment with PPB_1 alone did not change the proportion of dead cells (0.6%, Fig. III-8) indicating that PPB_1 is not toxic for *Anabaena*.

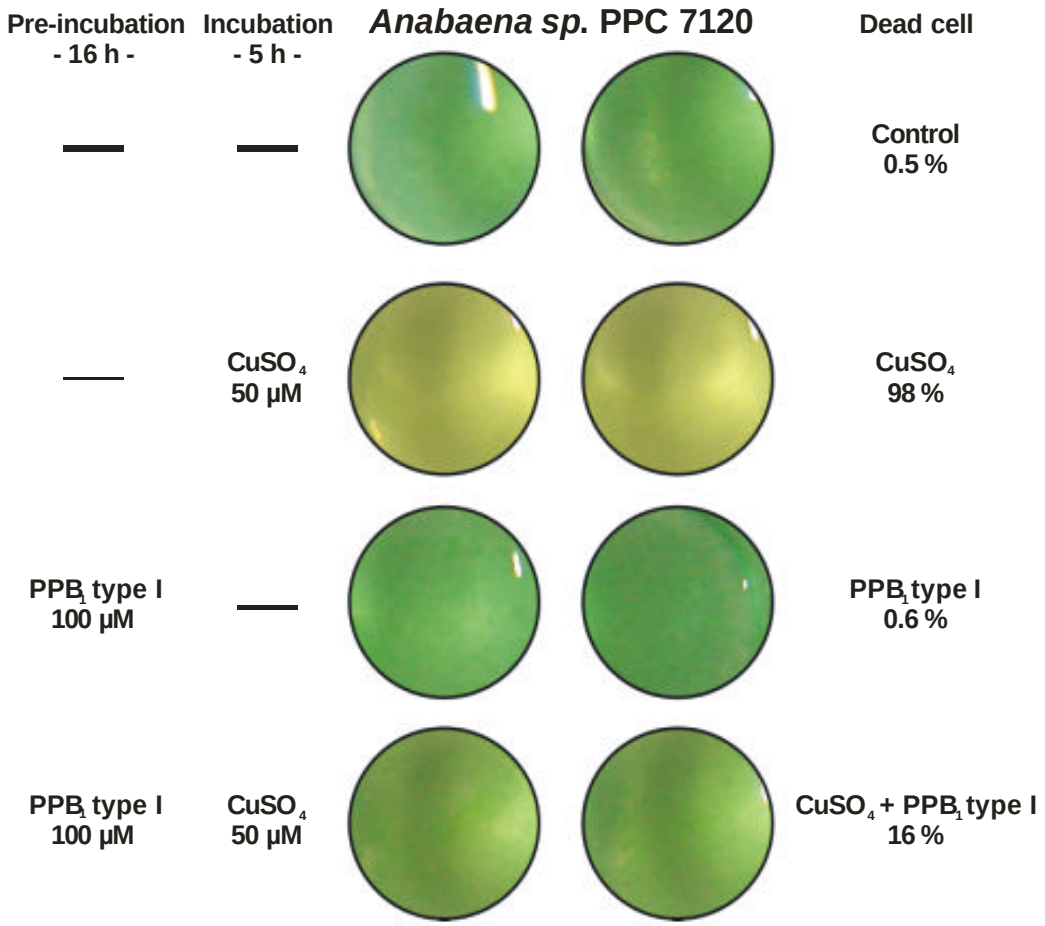


Figure III-8. Efficacy of pre-incubation of PPB_1 type I in protection of *Anabaena* from 50 µM copper sulfate stress.

Based on the dramatic protective effect of PPB_1 type I, it was tested if also other oxylipins exert similar effects. In this experiment, 100 µM of PPB_1 type I, OPDA, and PGB_1 were used for pre-incubation. Fig. III-9 shows that oxylipins differ in their efficiency in protecting *Anabaena* from toxicity of 50 µM copper sulfate. PGB_1, a mammalian oxylipin, is the least effective, saving only 13% of cells. OPDA, an oxilipin synthesized in plant chloroplasts, protects 37% of *Anabaena* cells. PPB_1, a plant oxylipin and likely an endogenous *Anabaena* oxylipin, saves 81% of the cells (similar to the first experiment (84%)). Without pre-incubation with oxylipins a massive cell death is caused by copper ions (98%) (Fig. III-9).

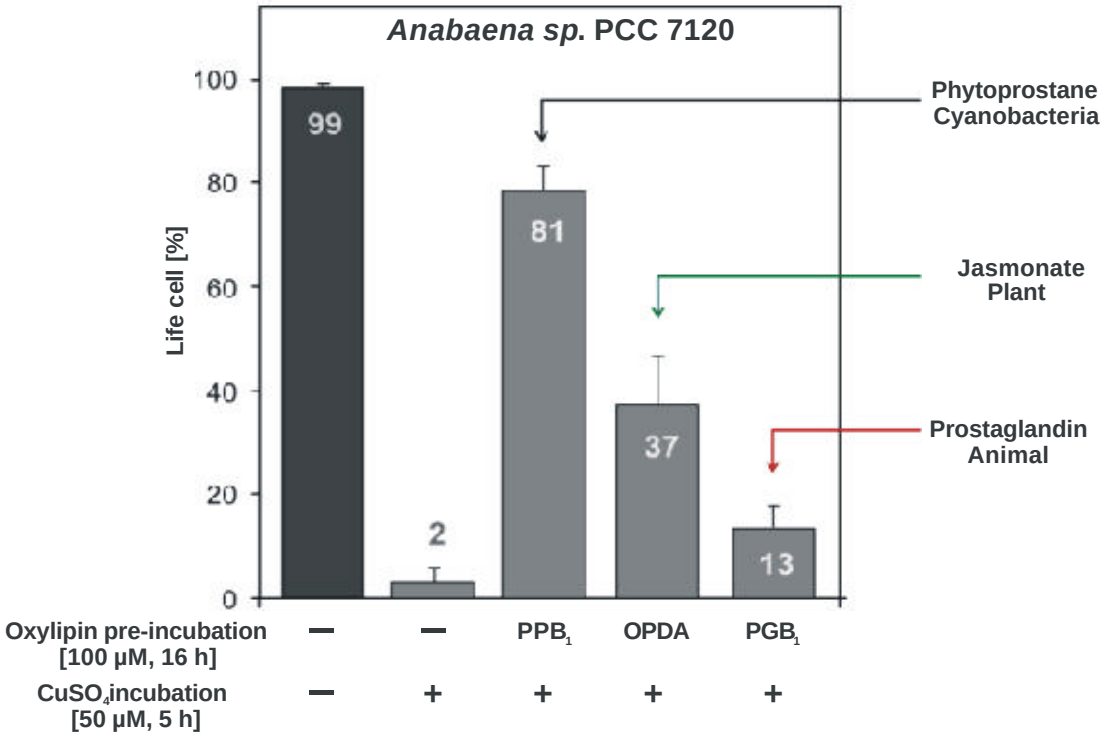


Figure III-9. Efficiency of different sources of oxylipins in protection of *Anabaena* from copper stress. + or – presents with or without treating of oxylipin or stress factor.

Notably, the experiment reveals that not only PPB_1 but also other oxidized lipids that do not occur in *Anabaena* protect *Anabaena* from cell death. Therefore, it was hypothesized that *Anabaena* cells potentially recognize a broad range of oxidized lipids. However, PPB_1, OPDA and PGB_1 are structurally related. In order to test the biological effects of oxylipins with different structures, a series of oxylipins endogenously found in cyanobacteria, plants and animals were tested. For these experiments two types of lethal oxidative stresses were employed: 1 mM H_2O_2 that kills about 97% of all cells within 5 h and 50 µM $CuSO_4$ that kills 98% of all cells within 5 h (both treatments without oxylipin preincubation). Oxylipin isomers were used in the experiment. Results are shown in Fig. III-10. Among all oxylipins tested phytoprostanes displayed the highest level of protection. The only exception being PPE_1 type I (Fig. III-10). All other phytoprostanes rescued between 36 and 84% of the *Anabaena* cells from undergoing cell death. Notably, there was a strong correlation between the protective effect against H_2O_2 and $CuSO_4$. Assuming that *Anabaena* cells can recognise and respond to a broad spectrum of oxidized lipids, one would expect that structurally highly related compounds would be equally effective. However, there is a big difference, for instance between PPA_1 and PGA_1. In the plant and animal literature it is well documented that cyclopentenone oxylipins have a common mechanism of action and therefore display similar biological activities.

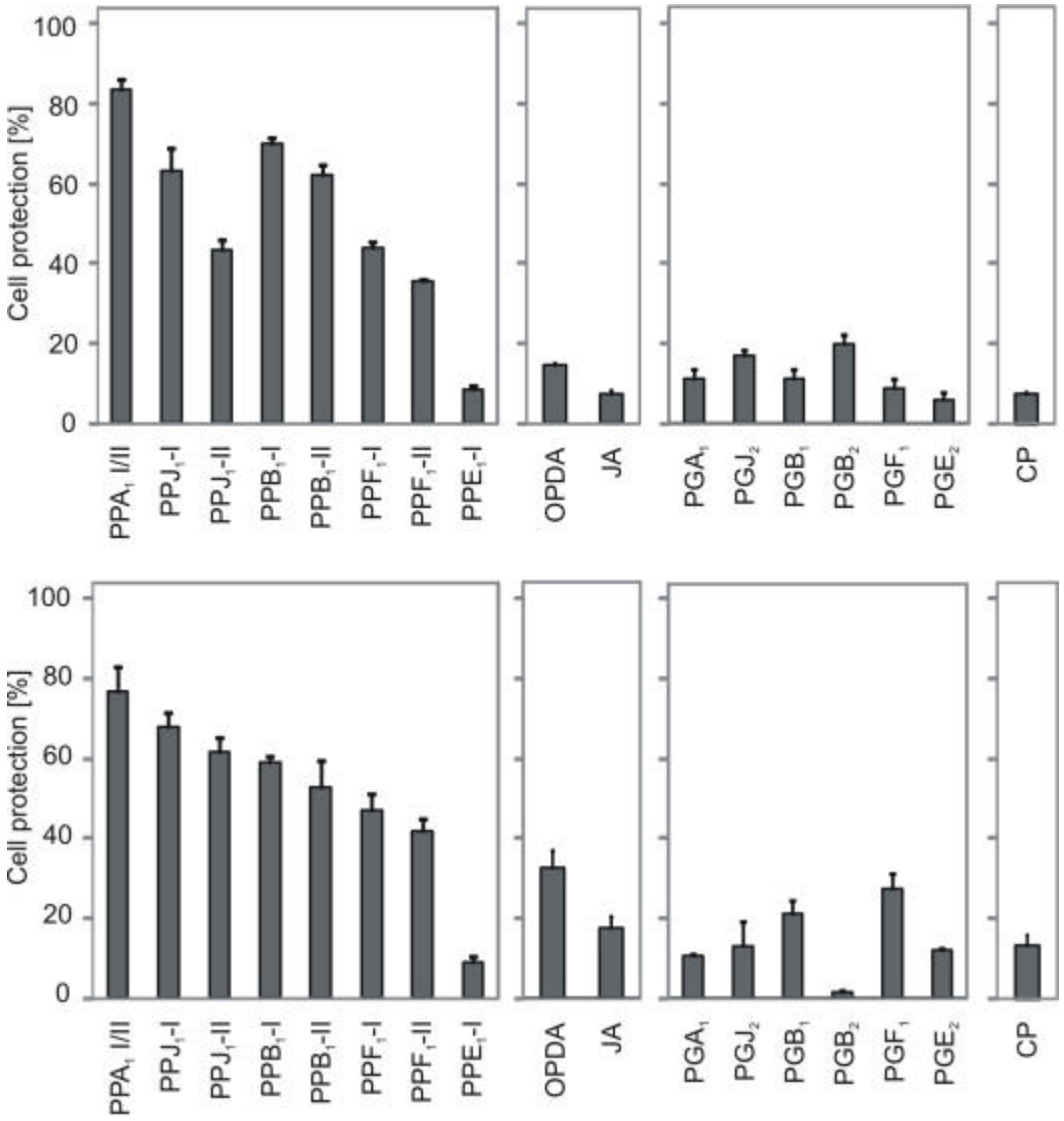


Figure III-10. An overviewed efficiency of different sources of oxylipins in protection of *Anabaena* from 50 µM copper sulfate (bottom) and 1 mM hydrogen peroxide (top).

A common feature of most cyclopentenone compounds is their high chemical reactivity. Cyclopentenones have been shown to bind *in vivo* (plants and animals) to cystein residues of proteins and peptides. Covalent binding to target enzymes and transcription factors has been shown to inactivate enzymes and to alter gene expression. However, the protective effect of highly reactive cyclopentenone phytoprostanes (PPA_1 and $dPPJ_1$) was in the same range as that of a completely unreactive but structurally related cyclopentenone (PPB_1). Moreover, unreactive cyclopentanone phytoprostanes such as PPF_1 were also highly active in protecting *Anabaena* cells from cell death.

Surprisingly, when the oxylipins were grouped according to their occurrence in different organisms, phytoprostanes (occuring in cyanobacteria and plants) were much more

effective than jasmonates (exclusively synthesised from plants), prostaglandins (synthesised from animals and some fungi) and cyclopentenone (chemical).

Table III-4. Efficiency of oxylipins in protection of *Anabaena* from stress factors

Oxylipins (100 µM)	**Protective effect (%) against stress***	
	1 mM H_2O_2	50 µM $CuSO_4$
PPA_1 I/II	84.2 ± 2.7	77.5 ± 6.6
$dPPJ_1$-I	64.0 ± 5.8	68.8 ± 3.2
$dPPJ_1$-II	44.0 ± 2.6	62.2 ± 3.9
PPB_1-I	70.9 ± 1.3	59.4 ± 2.1
PPB_1-II	63.1 ± 2.3	53.4 ± 6.7
PPF_1-I	44.5 ± 1.2	47.6 ± 4.3
PPF_1-II	35.9 ± 0.8	42.1 ± 3.2
PPE_1-I	8.6 ± 1.1	9.3 ± 1.7
OPDA	15.0 ± 0.6	33.0 ± 4.4
JA	7.7 ± 1.0	17.7 ± 2.8
PGA_1	11.2 ± 2.7	10.7 ± 0.5
$dPGJ_2$	17.0 ± 1.5	13.2 ± 6.4
PGB_1	11.2 ± 2.7	21.4 ± 3.4
PGB_2	19.8 ± 2.4	1.4 ± 0.5
PGF_1	8.9 ± 2.3	27.6 ± 3.9
PGE_2	6.1 ± 1.9	12.2 ± 0.5
CP	7.7 ± 0.7	13.6 ± 2.7

*data represents the percentage of cells rescued by oxylipin pre-incubation (mean ± SD, n = 3). CP: cyclopentenone.

Among them, a mixture of type I and II of PPA_1 displayed the best protective effect: 84.2% protection against 1 mM H_2O_2 and 77.5% protection against 50 µM $CuSO_4$. The detailed protective values of other oxylipins are presented in Table III-4 and the structures of the oxylipins are shown in Fig. III-11.

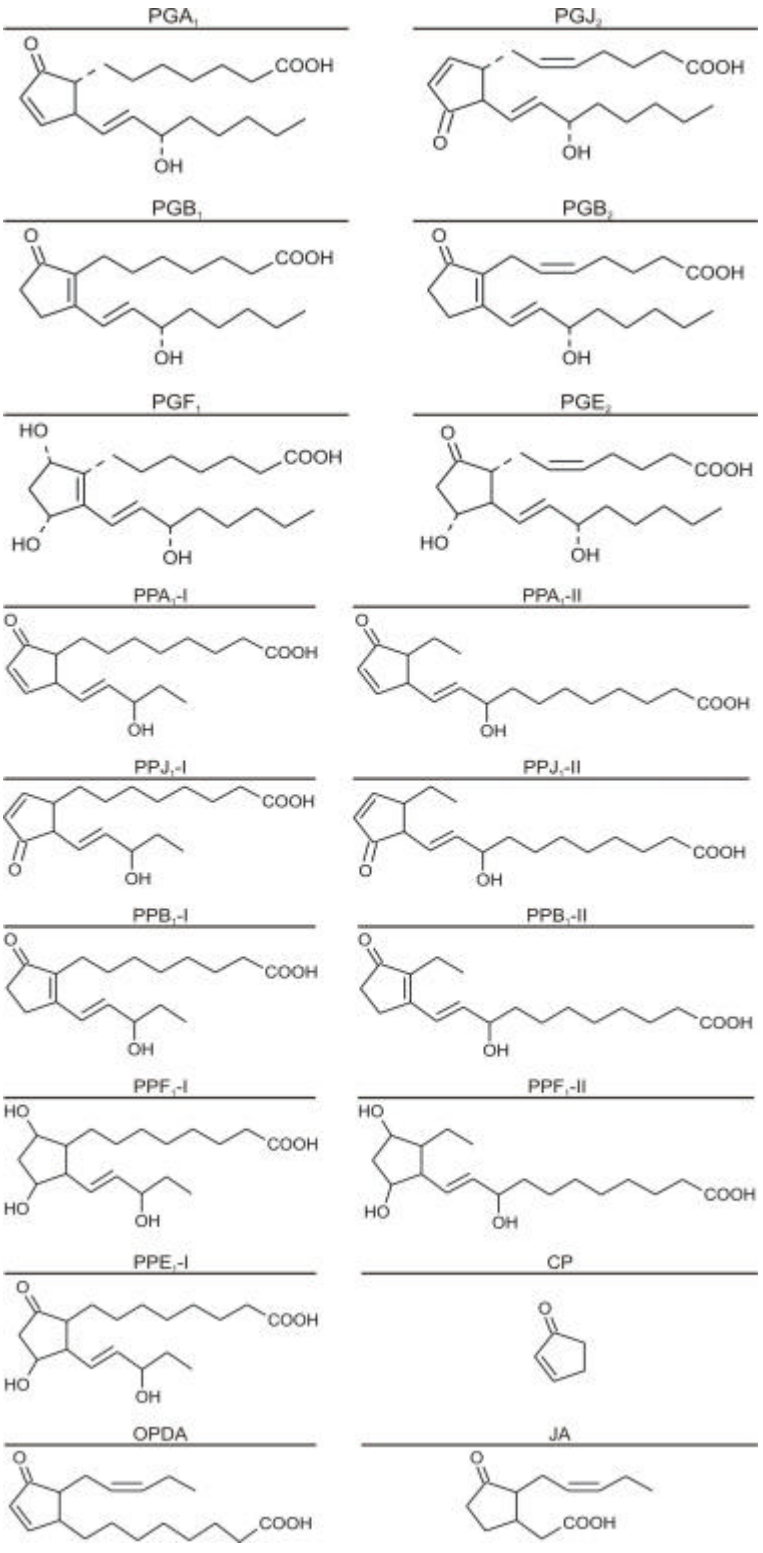


Figure III-11. Structures of the used oxylipins.

III.7 F_1-phytoprostanes have no protective effect on *Pseudomonas syringae* or *Escherichia coli* under peroxide stress

Since phytoprostanes display a powerful effect in protecting *Anabaena* from oxidative stress, it was interesting to investigate if this effect is unique to the cyanobacterium *Anabaena* or if this is a general mechanism operative in all or many bacteria. In this work, *Pseudomonas syringae* and *Escherichia coli* were employed. *E. coli* is a gram negative bacterium living in the lower intestines of mammals. *P. syringae* is a gram negative bacterium that can infect plants and cause plant disease. Experiments were carried out by pre-incubating these bacteria with 100 µM PPF_1 overnight. Pre-treated PPF_1 cells were resuspended in fresh medium and treated with a series of hydrogen peroxide concentrations. Bacterial growth was recorded by measuring the density of the cultures in a photometer. Observations after several experiments showed that PPF_1 did not have any biological activities in protection of these bacteria against hydrogen peroxide. For example, the cell density of *P. syringae* by 600 nm at 24 h after treated with 500 µM H_2O_2 were 0.512 ± 0.04 in the control (pre-treated with methanol/water) and 0.519 ± 0.05 in the PPF_1 pre-treatment. At higher hydrogen peroxide (1 mM) both treatments showed no increase in cell density. For *E. coli*, these values at 6 h after incubated with 1 mM H_2O_2 were 1.21 ± 0.02 and 1.24 ± 0.02, respectively. At a higher concentration (2 mM) of hydogen peroxide, there was no growth of *E. coli* in the cultures.

Results indicate that phytoprostanes have no priming effect in *E. coli* and *P. syringae* bacteria. Notably, both of these bacteria lack the enzymatic capacity to synthesise polyunsaturated fatty acids. Among all lipids, polyunsaturated fatty acids are most sensitive to chemical oxidation and, hence, the vast majority of all oxidised lipids in living cells is derived from these fatty acids. Since polyunsaturated fatty acids are generally absent in bacteria (with the exception of photoautotrophic cyanobacteria), lipid peroxidation is not a problem encountered by bacteria. Therefore, recognition mechanisms for oxidized lipids and appropriate defense mechanisms might be dispensable and possibly have never evolved in the bacterial kingdom (except cyanobacteria).

III.8 Exogenous sources of phytoprostanes: phytoprostanes release from dead plant materials

When comparing the endogenous levels of phytoprostanes found in *Anabaena* cultures (in cells and medium) with the concentration used for the priming experiments there is a huge

difference between the concentrations. For instance, endogenous PPF_1 levels in the cell culture medium and *Anabaena* cells (basal levels) were 0 nM and 0.64 nM, respectively. In contrast, oxylipin concentrations used to prime *Anabaena* cells were 100 µM. In plant oxylipin research, oxylipin concentrations used for exogenous application are typically one to three orders of magnitude higher than the basal endogenous levels. This is because some oxylipins are slowly taken up by plants cells and rapidly metabolized. In order to achieve biologically significant internal concentrations, exogenously applied oxylipins are often in the range of 50 – 100 µM (as used in this work).

Although cyanobacteria are producers of phytoprostanes, they are also exposed to phytoprostanes from external sources. For instance, *Anabaena* lives in rice paddy fields. Typical for this environment is the high proportion of dead plant material in the water. Previously, it has been shown that lipids in dead plants rapidly autoxidize. Oxidized lipids including phytoprostanes can be released into the medium. Thus, dead plants may represent an additional source of phytoprostanes.

In order to estimate the level of free phytoprostanes in hay (stored in dried form for several months), free levels of PPF_1 and PPE_1 were analysed. Results show that 1 g of dry hay contains 5.29 ± 0.78 µg PPF_1 and 5.48 ± 0.16 µg PPE_1 (Fig. III-12). These levels are several orders of magnitude higher than levels in living plant tissue (Imbusch and Mueller, 2000a). After complete release of the readily water soluble phytoprostanes, an aqueous suspension of hay in water (1 g/ml) phytoprostane concentrations are calculated to be 16 µM PPF_1 and 17 µM PPE_1. Results suggest that phytoprostanes released from dead plants can be significant source of external phytoprostanes. However, phytoprostanes levels in natural habitats of *Anabaena* and their significance remains to be investigated.

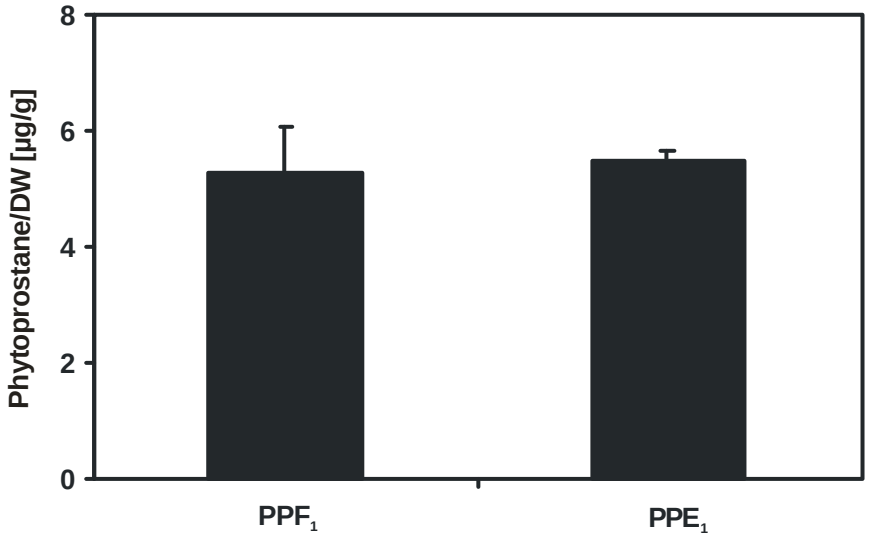


Figure III-12. Phytoprostane F_1 and E_1 level in dry hay.

III.9 Iron stress induced gene A (*isiA*) in *Anabaena* is neither induced by hydrogen peroxide nor phytoprostane F_1

There are several reports about expression of the *isiA* gene when cyanobacteria live under iron free conditions or suffer oxidative stress. Most of the *isiA* research has been so far focused on *Synechocystis*. Here, the expression of *isiA* in *Anabaena* after treating the cells with 1.5 mM hydroxyl peroxide or 100 µM PPF_1 for 90 min was tested by using RT-PCR. As a control for reverse transcription, the expression of gene *rnp* as a high constitutively expressed gene was determmined. No *isiA* RT-PCR product was detectable in controls and *Anabaena* cells treated either with 1.5 mM hydrogen peroxide or 100 µM PPF_1. The *rnp*-control showed RT-PCR product within all samples (Fig. III-13). This result indicated that even after H_2O_2 and PPF_1 treatment *isiA* mRNA levels are low. Hence, *isiA* transcription is not induced by F_1-phytoprostanes in *Anabaena*.

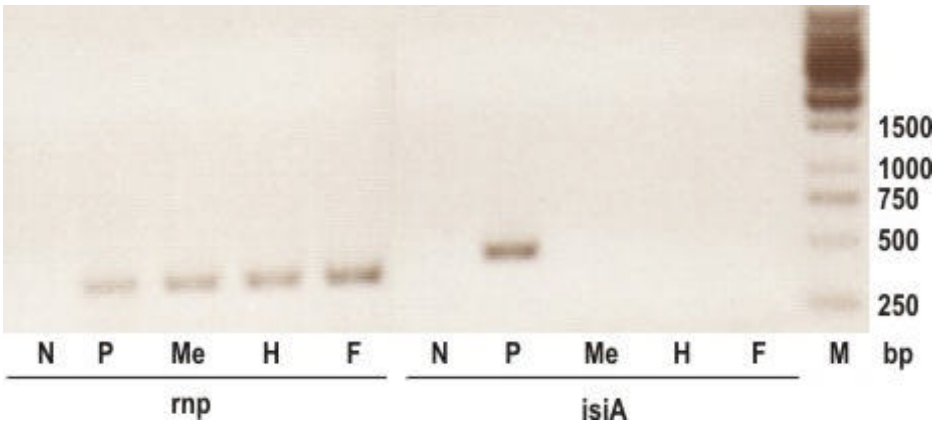


Figure III-13. Gene expression analysis of *isiA* and rnp in *Anabaena*. RT-PCR was performed and products were separated on agarose gel and stained with lithium bromide. M: 1kb DNA ladder; N: negative control (without DNA); P: positive control (with plasmid DNA); Me: methanol (0.5%); H: 1.5 mM H_2O_2; F: 100 µM PPF_1 (mixture of type I and II). Size of PCR products: *rnp*: 398 bp; *isiA*: 465 bp.

III.10 Induction of shinorine in *Anabaena* by UV light, copper sulphat, phytoprostanes and jasmonates

A typical biological activity displayed by many oxylipins in higher plants is the induction of secondary metabolites that are involved in defense against oxidative stress, microorganisms, herbivores or function as UV-light protection such as flavanoids. In contrast to higher plants, cyanobacteria synthesize only few secondary metabolites. Among them,

mycosporin-like amino acids such as shinorine (Fig. III-14, A) are UV-light-protective compounds produced by *Anabaena* under UV light stress (Sinha *et al.*, 1999; 2001). A recently published analysis method was used to determine shinorine levels in *Anabaena*. To test if the method is producible, *Anbaena* cell suspension was grown under UV light for 24 h and then cells were collected for shinorine analysis. As shown in Fig. III-14, no shinorine or low levels of shinorine were detectable in the control. Upon UV-light treatment, a strong accumulation of shinorine was observed. After method establishment, *Anabaena* cell culture (5 ml, about 5 mg DW) was treated with 50 µM jasmonates, phytoprostanes or 1 mM copper sulfate in combination with or without UV light. Cells were collected and analyzed for shinorine at 24, 48, and 72 h after treatment. Shinorine accumulated after all UV-light treatments. However, oxylipin or $CuSO_4$ did not have any effect on the induction of shinorine, both in the presence or absence of UV-light. Hence, it can be concluded that oxylipins and oxidative stress imposed by copper ions are not involved in the induction of shinorine.

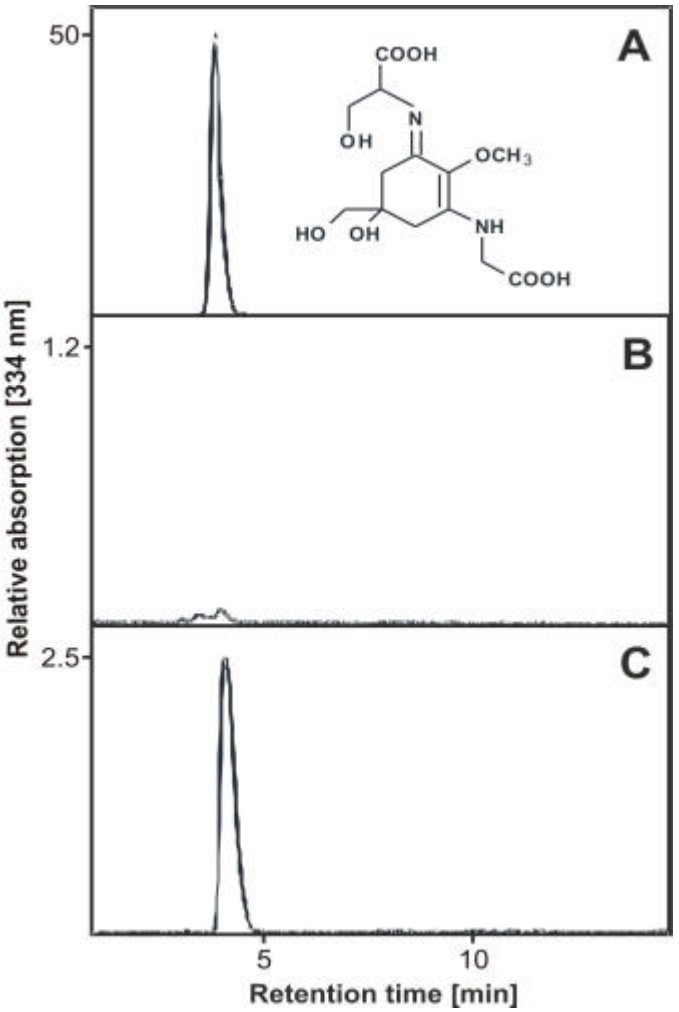


Figure III-14. HPLC chromatograms of *Anabaena* extracts.
A: shinorine reference; B: without UV light; C: with UV light.

III.11 Induction of tocopherols production in *Anabaena* by UV light, copper sulfate, phytoprostanes and jasmonates

Tocopherols are important anti-oxidant compounds in many living organisms including cyanobacteria, plants and animals. It has been shown that jasmonic acid can induce *de novo* tocopherol biosynthesis (Sandorf and Hollaender, 2002). To test if tocopherols synthesis is activated by oxylipins, *Anabaena* was treated with jasmonates, phytoprostanes, or copper ions. Although incubations were analysed for tocopherol levels up to 72 h, there was no difference in tocopherols levels between treatments (Fig. III-15). This result indicates that there is no induction of tocopherols by oxylipins and oxidative stress in *Anabaena*.

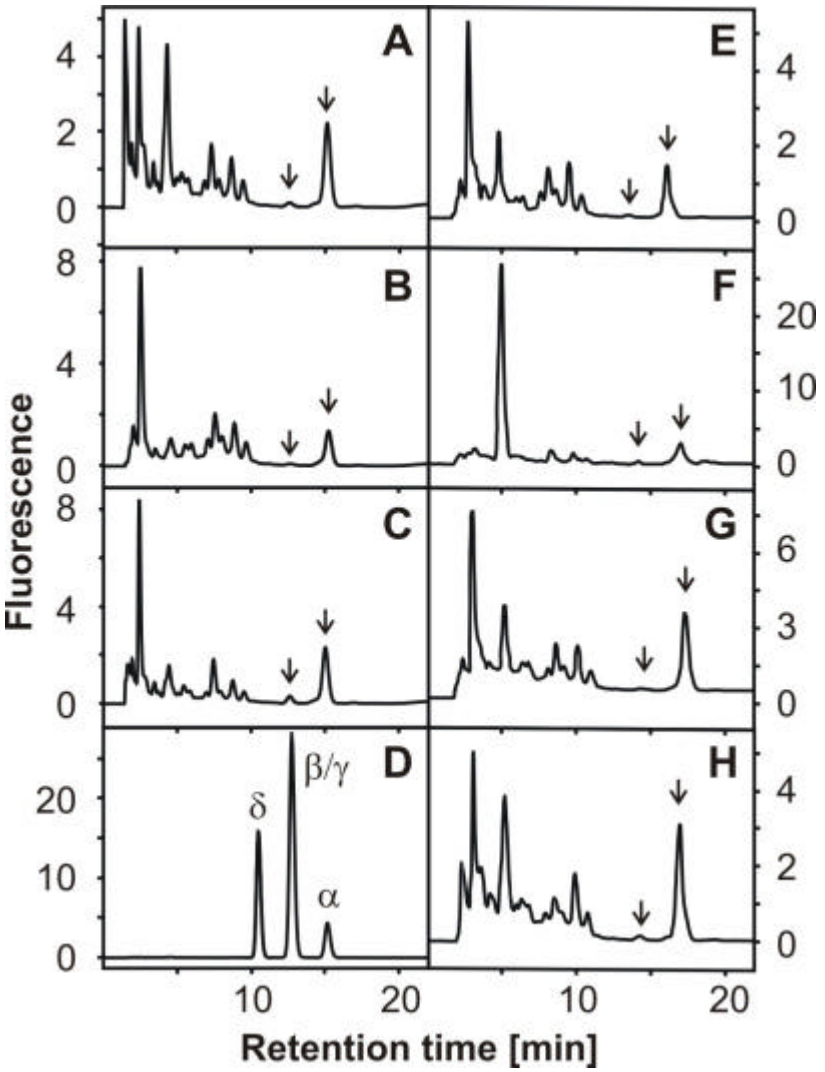


Figure III-15. HPLC chromatograms of tocopherols analysis in *Anabaena* after 24 h application of inducible factors. A: UV light, B: 1 mM copper sulfate, C: control, D: standard (a: a-tocopherol; b: ß-tocopherol; d: d-tocopherol; g: ?-tocopherol), E: JA, F: OPDA, G: PPB_1, and H: PPF_1. All oxylipins were used at 50 µM. Arrows show the peaks of analytic compounds. ß- and ?-tocopherol have an overlapping retention time.

III.12 F_1-phytoprostanes modulate protein expression/modification in *Anabaena*

The previous experiments have clearly demonstrated that phytoprostanes and other oxidised lipids prime *Anabaena* cells to cope better with oxidative stress. However, it is not known by which mechanism oxylipins prime the cells. It is highly likely that oxylipins modulate gene and protein expression and/or alter protein modification. To discover global gene expression changes induced by phytoprostanes microarray analyses would be best suited to explore this question. However, microarray chips are currently not available to study *Anabaena* gene expression. Since the genome of *Anabaena* has recently be analysed and published it is possible to analyse the impact of phytoprostanes on the proteome level. Therefore, different methods were established for differential proteome analysis (in the presence and absence of PPF_1). PPF_1 have been used as stimulus since PPF_1 have been quantified in *Anabaena* cells and increase in response to oxidative stress. Moreover, enough amount of compound (several milligrams) was available to conduct these studies.

III.12.1 Protein quantification by 1D gelelectrophoresis

Protein quantification in each treatment is necessary to determine a comparable protein amount from samples before loading on gel. In this experiment, proteins extracted from 100 mg of FW of *Anabaena* cells was diluted in 50 µl SDS buffer. 5 and 10 µl were used for loading on a gel. Fig. III-16 show a comparable bands between treatments and between amounts of loaded protein. It was estimated that protein yield was 4 – 5 mg per 100 mg FW.

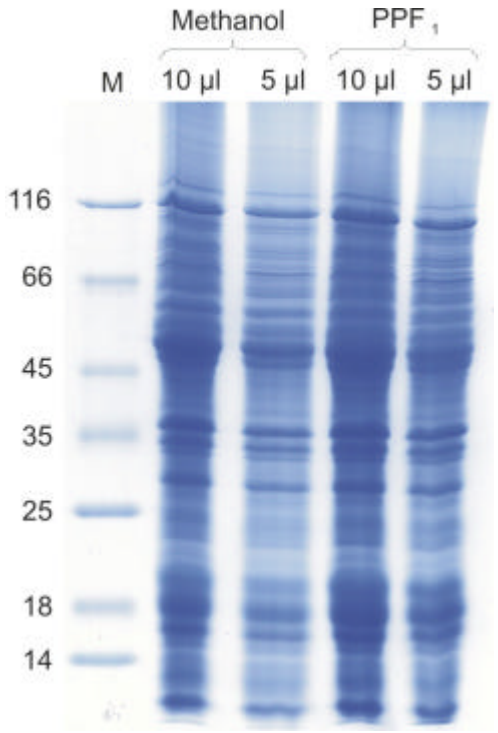


Figure III-16. Coomassie staining of 12.5 % SDS gel for protein quantification in *Anabaena*. 5 µl and 10 µl from each treatment were loaded on gel. M is protein marker.

III.12.2 Protein analysis by two-dimensional gel electrophoresis with IEF

In this experiment, *Anabaena* proteome was extracted by Wang method (Wang *et al.*, 2006) (see materials and methods for details). Protein concentration was estimated as being done in III.12.1. The same amount of protein was loaded on wells containing isoelectric membranes with the pH value from 3 to 10 of an isoelectric focusing apparatus. After programmed running for 135 min, proteins in each pH fraction was separated by a ready-for-use SDS-PAGE gel. Fig. III-17 shows protein bands in different pH range from each treatment. Although the same protein amount from each sample was loaded, cells treated with 100 µM PPF_1 show fewer protein bands in each pH range. Most of proteins have their isoelectric points in the mild acidic region (pH 4.6 – 5.4) resulting in more detected bands. At very acidic region (pH 3.0 – 4.6), protein levels were too low to exhibit significant bands. In order to get stronger bands, proteins in this pH range were concentrated and loaded on another gel and differential bands were detected as shown on left side of Fig. III-17. The higher pH values were, the fewer protein bands detected. This phenomeon can be easily recognized in the pH range 5.4 – 6.2, 6.2 – 7.0, and 7.0 – 10.0. Protein bands named with numbers on Fig. III-17 were chosen for analysis.

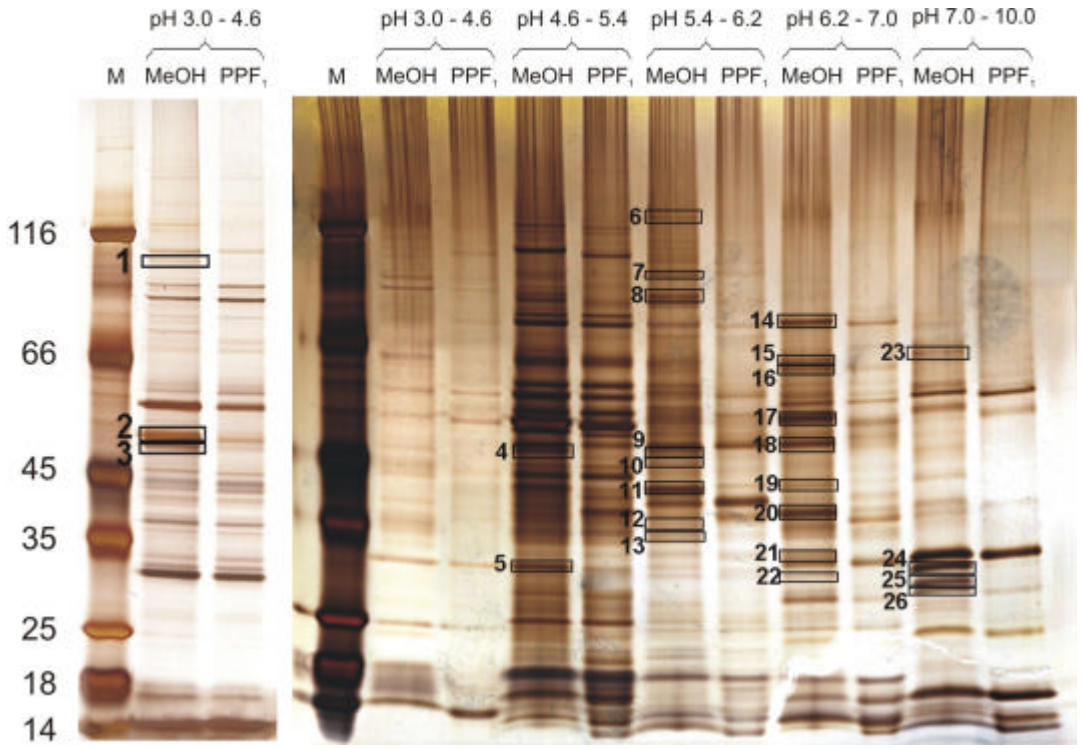


Figure III-17. Silver stained proteins in a SDS – PAGE gel after isoelectric focusing at different pH ranges and treatments (right). Proteins with a pH value from 3.0 to 4.6 were concentrated and loaded on another PAGE gel to exhibit stronger bands as shown on the left. 26 protein bands were chosen for analysis (marked in gels). Cell cultures were either treated with 0.5% methanol (MeOH) or 100 µM PPF_1 for 6h under 10 $\mu E.m^{-2}.s^{-1}$ light intensity at 23°C.

Complete names of proteins found in each selected band are presented in Table-5. Most identified proteins relate to photosynthesis system in cyanobacteria such as bicarbonate transport, bicarbonate-binding protein, phycobilisome core-membrane, fructose 1,6-biphosphate aldolase, carbon dioxide concentrating mechanism protein, ribulose 1,5-bisphosphate, etc. In total, 37 proteins were identified in 25 bands (except for the band 23). Detailed information about them can be found in Table-5.

Table-5. List of indentified proteins from selected bands in Figure III-15.

Band number	Protein name	Matched peptides	Sequence coverage (%)	Mascot Score	Molecular weight (kDa)	Code number
1	alr4550	2	3	127	60,694	gi|17133686
2	alr4550	13	35	952	60,694	gi|17133686
2	all4499	12	32	931	58,677	gi|17133635
2	porin; major outer membrane protein	5	14	356	54,553	gi|17130179
3	porin; major outer membrane protein	7	41	1232	54,553	gi|17130179
3	alr1819	8	18	469	58,090	gi|17130909
3	alr4550	6	12	356	60,694	gi|17133686
4	bicarbonate transport bicarbonate-binding protein	8	23	581	50,463	gi|17131971

4	alr4550	3	8	171	60,694	gi|17133686
5	manganese-stabilzing protein precursor	15	64	771	29,866	gi|17132988
5	all4688	2	10	148	32,063	gi|17133825
6	phycobilisome core-membrane linker protein	29	30	1739	127,058	gi|17134998
7	endopeptidase Clp ATP-binding chain	2	2	77	93,196	gi|17132093
7	all1463	2	3	74	70,217	gi|17135284
8	translation elongation factor EF-G	4	7	186	76,894	gi|17133519
9	bicarbonate transport bicarbonate-binding protein	2	6	139	50,463	gi|17131971
10	glutamate-1-semialdehyde 2,1-aminomutase	2	8	118	43,792	gi|17132360
11	fructose-1,6-bisphosphate aldolase	6	21	462	38,787	gi|17133699
11	all5091	1	5	98	34,824	gi|17134229
12	cystathionine beta-synthase	2	10	119	34,713	gi|17133552
13	oxidoreductase	6	26	391	30,971	gi|17134321
13	pyruvate dehydrogenase E1 beta subunit	3	11	154	36,410	gi|17135100
13	all3378	1	4	85	35,069	gi|17132473
14	transketolase	20	36	1215	72,355	gi|17132439
15	carbon dioxide concentrating mechanism protein	17	38	1113	59,863	gi|17130210
16	ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	14	31	854	53,444	gi|17135344
16	all4388	2	4	127	51,579	gi|17133524
17	ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	18	38	994	53,444	gi|17135344
17	all4388	3	8	225	51,579	gi|17133524
17	ferredoxin--NADP(+) reductase	1	3	110	49,009	gi|17133256
18	translation elongation factor EF-Tu	8	25	452	44,868	gi|17133473
18	ribulose 1,5-bisphosphate carboxylase/oxygenase activase	8	21	441	46,896	gi|17135353
19	all5091	4	14	206	34,824	gi|17134229
19	fructose-1,6-bisphosphate aldolase	2	7	172	38,787	gi|17133699
19	alr0893	2	6	151	39,791	gi|17130239
20	glyceraldehyde-3-phosphate dehydrogenase	14	46	997	37,138	gi|17134200
20	carbon dioxide concentrating mechanism protein	2	7	163	59,863	gi|17130210
21	all3585	5	21	269	29,592	gi|17132718
22	light-repressed protein	8	51	504	24,233	gi|17132279
22	phycobilisome rod-core linker protein	3	14	156	28,651	gi|17129880
22	phycobilisome rod-core linker protein	2	9	147	29,322	gi|17129882
23	not identified					
24	phycobilisome rod-core linker protein	14	54	790	31,934	gi|17129879
24	phycoerythrocyanin-associated rod linker					

	protein	15	50	734	31,409	gi|17129870
24	alr7345	5	21	317	33,492	gi|17134544
24	all3585	2	11	144	29,592	gi|17132718
24	phycobilisome rod-core linker protein	2	9	100	29,322	gi|17129882
25	phycobilisome rod-core linker protein	8	36	501	29,322	gi|17129882
25	alr3276	9	30	477	34,131	gi|17132371
26	phycobilisome rod-core linker protein	12	52	764	29,322	gi|17129882
26	50S ribosomal protein L1	6	27	369	25,975	gi|17134440
26	phycobilisome rod-core linker protein	7	30	319	28,651	gi|17129880
26	all2425	2	8	113	28,039	gi|17131517

III.12.3 Protein analysis by two -dimensional gel electrophoresis with IPG

About 500 µg of extracted proteins was separated in each IPG strip by the first dimension. In second dimension, proteins were separated by 12.5% SDS gel. Master gels were created automatically by the Delta 2D software via an overlay combination of several two-dimensional gels from both treatments. Differential master gels were visualized in orange for one and blue for the other treatment in order to identify differentiated spots easily. Thereby, 30 modulated protein spots were identified. Spots numbered with 29, 30, and 32 were not recognized by computer program; therefore, they were handled manually. There were total 33 modulated spots which were cut out, digested with trysin, and analyzed by nano-LC-MS/MS system. Fig. III-18 shows the positions and numbers of detected protein spots on the second dimension gel. Some of them are not clearly seen on the gel and zoomed in as in Fig. III-19.

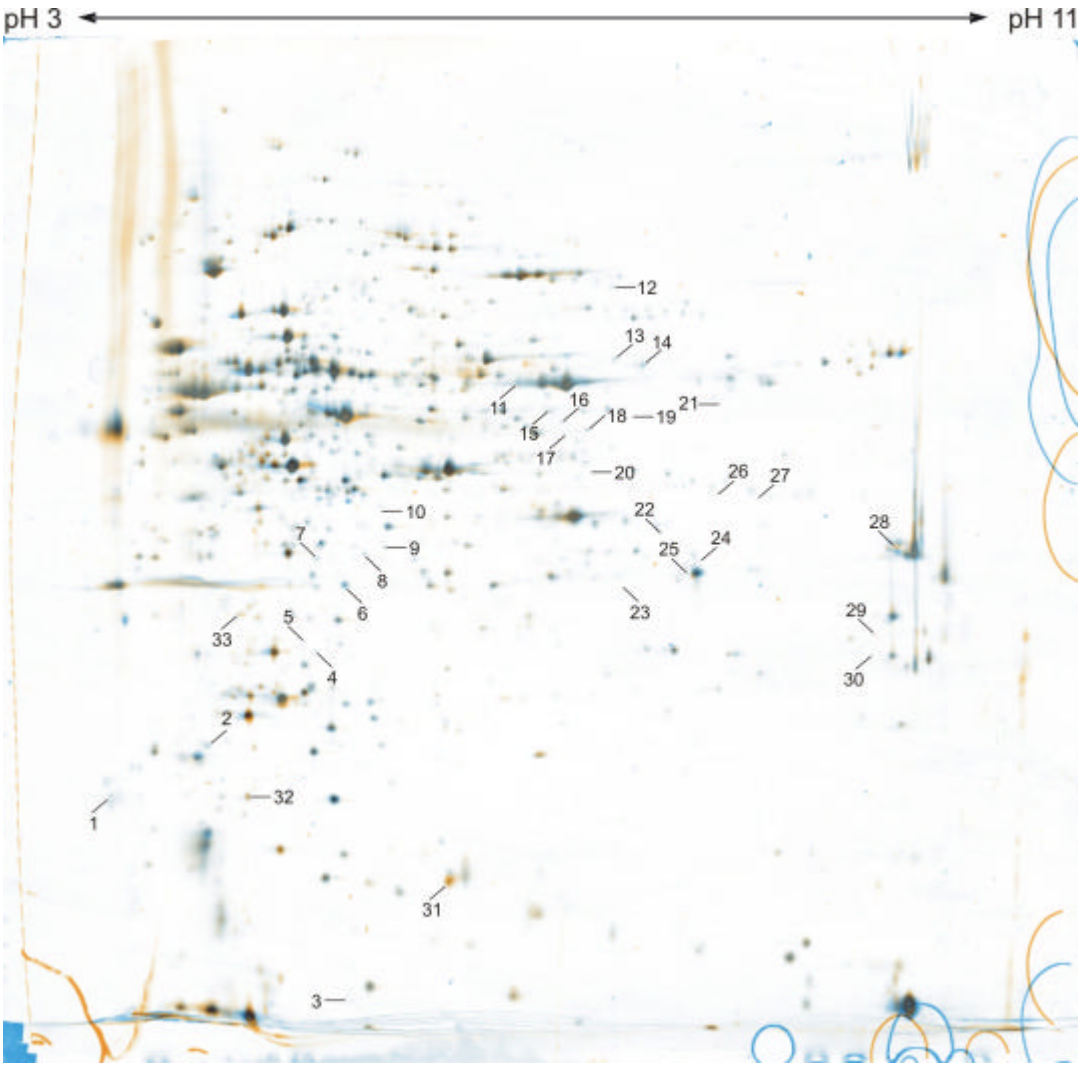


Figure III-18. Master gels from methanol (blue) and 100 µM PPF_1 (yellow) treatments were overlaid and differentiated protein spots were identified by software called Delta 2D (Decodon). Spots with numbers were selected for protein analysis.

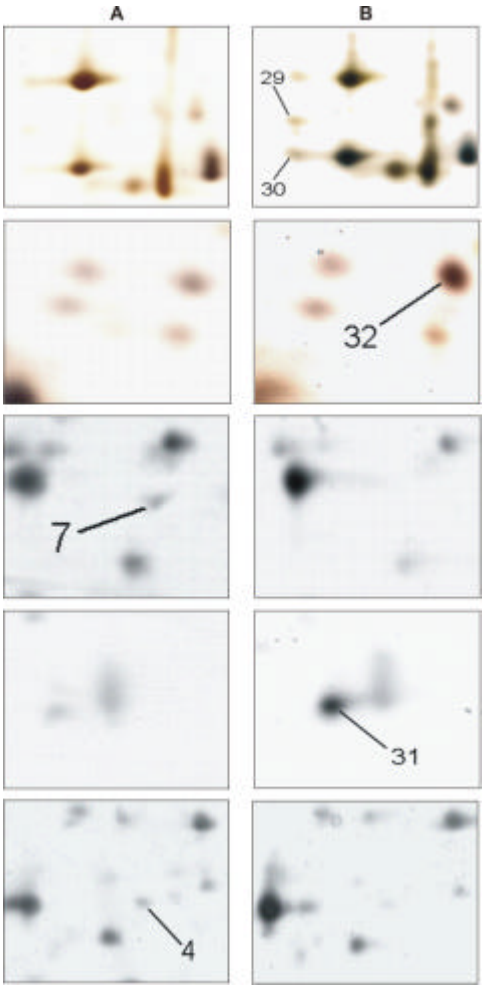


Figure III-19. Zoom-in of some modulated spots from two dimensional gel electrophoresis. Spots on column A were from cells after methanol treatment (0.5%) as control and those on column B were from cells after 100 µM PPF_1 treatment.

Identified proteins in selected spots on second dimension gel are presented in table-6. Most of the identified proteins are related to photosynthesis. As shown in table-6, some spots contain more than one protein. In this case it is uncertain which of the two protein is regulated. However, fewer proteins were found in each spot in comparison with each band of the 2D-gel electrophoresis with IEF experiment. Therefore, electrophoresis with a second dimension with an immobile pH gradient is much better suited for protein separation.

Table-6. List of identified proteins in two dimensional gel electrophoresis.

Spot number	Protein name	Matched peptides	Sequence coverage (%)	Mascot Score	Molecular weight (kDa)	Code number
1	Phycocyanin beta chain					
2	all4779	4	32	264	19.857	gi|17133916
3	ribosome binding factor A	6	48	350	14.665	gi|17135041
4	alr3297	4	21	169	22.927	gi|17132392

5	dihydrodipicolinate synthase	2	8	87	31.445	gi|17132812
6	dihydrodipicolinate reductase	8	47	578	29.636	gi|17131634
7	all3378	1	3	61	35.069	gi|17132473
8	all3953	10	47	518	37.573	gi|17133087
9	oxidoreductase	9	39	555	30.971	gi|17134321
10	glucose-1-phosphate thymidylyltransferase	8	27	543	39.414	gi|17133627
11	ribulose 1,5-bisphosphate carboxylase/oxyge-nase large subunit	10	25	564	53.444	gi|17135344
12	ferredoxin-sulfite reductase	2	6	91	73.587	gi|17130695
13	cobyric acid synthase	7	14	352	54.591	gi|17131469
14	zeta-carotene desaturase	4	10	220	56.829	gi|17135793
15	biotin carboxylase	4	13	229	49.446	gi|17130285
16	bicarbonate transport bicarbonate-binding protein	2	29	117	46.896	gi|17135353
17	ribulose 1,5-bisphosphate carboxylase/oxyge-nase activase	4	11	254	46.896	gi|17135353
18	ribulose 1,5-bisphosphate carboxylase/oxyge-nase activase	9	22	457	46.896	gi|17135353
19	serine hydroxymethyltrans-ferase	3	10	183	46.822	gi|17133943
19	glucose-inhibited division protein	1	3	75	48.458	gi|17133707
20	alr0893	4	15	265	39.791	gi|17130239
20	carbon dioxide concentrating mechanism protein	1	3	60	59.863	gi|17130210
21	alr1324	4	11	183	49.287	gi|17130671
22	ABC transporter ATP-binding protein	5	14	360	36.756	gi|17131464
22	alr0474	2	5	108	66.510	gi|17129819
23	alr0882	4	21	276	30.894	gi|17130227
24	alr0882	2	9	167	30.894	gi|17130227
24	all1225	2	7	78	34.764	gi|17130572
25	alr0882	7	36	473	30.894	gi|17130227
26	carbon dioxide concentrating mechanism protein	2	7	169	59.863	gi|17130210
27	carbon dioxide concentrating mechanism protein	14	29	913	59.863	gi|17130210
28	protochlorophyllide oxido-reductase	7	24	395	36.903	gi|17130833
29	phycobilisome rod-core linker protein	9	32	359	28.651	gi|17129880
30	phycobilisome rod-core linker protein	11	42%	606	28.651	gi|17129880
31	all3324	9	51%	413	16.439	gi|17132419
31	50S ribosomal protein	4	25%	176	19.495	gi|17134441
31	phycoerythrocyanin alpha chain	1	9%	74	17.625	gi|17129869
32	alr1133	3	25%	197	16.924	gi|17130479
33	diaminopimelate epimerase	1	6%	71	27.019	gi|17131139

Differential factors in modulated protein spots are displayed in Table-7. Treated *Anabaena* cells with 100 µl PPF_1 induced only 5 spots but 3 of them were ignored by computer software (spot 29, 30 and 32). Only 2 spots, 31 and 33, were recorded by computer program and their induction factors are quite low, 3.1 and 4.0, respectively. Most differential proteins in *Anabaena* proteome were down-regulated by PPF_1 and some inhibiting degrees are much higher than induction levels (Table-7).

Table-7. Detail in protein modulation between treatments. Sequence program detected only two spots which were up-regulated by PPF_1. All others were down-regulated. MeOH: methanol and PPF_1: 100 µM.

Spot number	Regulation factor	Treatment	Spot number	Regulation factor	Treatment
1	9.5	MeOH	16	10.75	MeOH
2	6.8	MeOH	17	5.4	MeOH
3	7.25	MeOH	18	3.4	MeOH
4	> 20	MeOH	19	9.01	MeOH
5	6.9	MeOH	20	3.6	MeOH
6	3.4	MeOH	21	> 20	MeOH
7	> 20.0	MeOH	22	> 20	MeOH
8	3.2	MeOH	23	> 20	MeOH
9	5.7	MeOH	24	8.69	MeOH
10	3.4	MeOH	25	4.6	MeOH
11	4.0	MeOH	26	6.67	MeOH
12	6.8	MeOH	27	11.36	MeOH
13	3.7	MeOH	28	4.5	MeOH
14	6.6	MeOH	31	3.1	PPF_1
15	4.9	MeOH	33	4.0	PPF_1

Two-dimensional gel electrophoresis with IEF revealed no up-regulated protein. The other method with IPG displayed 3 up-regulated protein in *Anabaena* proteome by 100 µM PPF_1 after 6 h of incubation. For both methods, most proteins with identified functions were down-regulated (Table-8). The lower levels of some proteins participating in photosynthesis apparatus such as carbonate binding or concentrating might result in reduction of photosynthetic activity.

Table-8. List of significant modulated proteins. Down-regulated (-), up-regulated (+)

Protein	Regulated by F_1-phytoprostanes	Method
Alr4550	-	IEF
Phycobilisome core-membrane linker protein	-	IEF
Tranlation elongation factor EF-G	-	IEF
Bicarbonate transport bicarbonate-binding protein	-	IEF
Glutamate-1-semialdehyde 2,1-aminomutase	-	IEF
Cystathionine beta-synthase	-	IEF
Transketolase	-	IEF
Carbon dioxide concentrating mechanism protein	-	IEF
All3585	-	IEF
Phycocyanin beta chain	-	IPG
All4779	-	IPG
Ribosome binding factor A	-	IPG
Alr3297	-	IPG
Dihydrodipicolinate synthase	-	IPG
Dihydrodipicolinate reductase	-	IPG
All3378	-	IPG
All3953	-	IPG
Oxidoreductase	-	IPG
Glucose-1-phosphate thymidylyltransferase	-	IPG
Ribulose-1,5-biphosphate carboxylase/oxygenase large subunit	-	IPG
Ferredoxin-sulfite reductase	-	IPG
Cobyric acid synthase	-	IPG
Zeta-carotene desaturase	-	IPG
Biotin carboxylase	-	IPG
Bicarbonate transport bicarbonate-binding protein	-	IPG
Ribulose 1,5-biphosphate carboxylase/oxygenase activase	-	IPG
Alr1324	-	IPG
Alr0882	-	IPG
Carbon dioxide concentrating mechanism protein	-	IPG
Protochlorophyllide oxido-reductase	-	IPG
Phycobilisome rod-core linker protein	+	IPG
Alr1133	+	IPG
Diaminopimelate epimerase	+	IPG

The numbers of identified proteins that are down-regulated by PPF_1 in the IEF separation method is fewer than the IPG method, 9 and 21, respectively. There are two proteins, bicarbonate transport bicarbonate-binding protein and carbon dioxide concentrating mechanism protein, which were identified by both separation method (Table-8). Apparently, many of these proteins are involved in photosynthetic processes. Hence, application of exogenous phytoprostanes may downregulate photosynthesis in *Anabaena*, thereby decreasing the formation of singlet oxygen and superoxide anion radicals. However, it remains to be clarified if this mechanism is involved in the priming of *Anabaena* cells and the protection against lethal oxidative stress.

III.13 Novel phytoprostanes in *Synechocystis* PCC 6803

In this work it has been shown that the filamentous cyanobacterium *Anabaena* PCC 7120 endogenously produces plant-like phytoprostanes. In contrast, the unicellular cyanobacterium *Synechocystis* PCC 6803 is not to be expected to form plant-like phytoprostanes (type I and II) since this bacterium does not utilize α-linolenate. Instead, *Synechocystis* was found to use γ-linolenate which is potentially a precursor for dinor isoprostanes (or phytoprostanes of the type III and IV), which represent isomers of plant phytoprostanes of the type I and II. Dinor isoprostanes (or phytoprostanes) have previously been identified as biologically active oxylipins (Liu *et al.*, 2007) in animals. In animals, dinor isoprostanes are derived from ß-oxidation of C20 isoprostanes (autoxidation products of arachidonic acid) or directly from autoxidation of endogenous γ-linolenate (Roberts *et al.*, 1996; Burke *et al.*, 2000). In order to initially characterize and identify phytoprostanes of the type III and IV, a model oxidation of γ-linolenate *in vitro* was performed.

III.14 Preparation and analysis of phytoprostanes type III and IV derived from autoxidation of ?-linolenic acid

Methods for preparation of phytoprostanes type I and II by autoxidation of α-linolenate were established some years ago (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000b). As already proposed by Thoma *et al.* (2003), phytoprotanes type III and IV can be formed from γ-linolenic acid. In this part, these reported GC-MS methods were applied and new HPLC-MS methods were introduced for production, identification, and

quantification of phytoprotanes type III and IV. These methods were then applied to analyse phytoprostanes in *Synechocystis*.

III.14.1 Quantification of phytoprostane F_1 type III and IV from Borage oil autoxidation

Since γ-linolenic acid is an relatively expensive fatty acid, γ-linolenic acid was not used for the first autoxidation experiments. Instead a fatty oil, Borage oil, was autoxidised. Borage oil contains about 25% γ-linolenate. After autoxidation of the Borage oil by stirring the oil for 12 days in an open flask at room temperature, the oil was hydrolized and the liberated oxidised fatty acids/phytoprostanes were analysed as described in the Material and Method section. Alternatively, Borage oil was hydrolised and fatty acids were isolated and autoxidized *in vitro* (see Material and Methods for details). Phytoprostanes were analysed by GC-MS and HPLC-MS. Details about the methods are described in II.21.2. Although both analytic systems show incomplete separation of several peaks, the GC-MS method gives fewer peaks in comparison to that of HPLC-MS. One reason is that the number of isomers is reduced by hydrogenation during the sample preparation and derivatization before GC-MS analysis. Hydrogenation reduces the number of isomers of the PPF_1 mixture from 32 to 16 resulting in fewer peaks (compare Fig. III-20 with Fig. III-21). In addition, reversed-phase HPLC has a better capacity to separate the diastereomers of PPF_1. As internal standard for GC-MS analysis, $[^{18}O]_3$-PPF_1 (mixture of type I and II compounds) was used.

Isotopically labelled PPF_1 (type I and II) gave 4 separate peaks in the GC-NCI-MS chromatogram but PPF_1 type III and IV yielded in several incompletely resolved peaks (Fig. III-20). After 12 days of autoxidation of Borage oil, the PPF_1 (type III and IV) level in Borage oil was 112.71 ± 1.93 µg/g Borage oil (GC-MS quantification), PPF_1 of the type I and II were not detected (Fig. III-20). In contrast by HPLC-MS analysis, the PPF_1 concentration was determined to be 75.87 ± 2.67 µg/g Borage oil. The difference may be due to the integration of several non-resolved peaks which may contain contaminants other than PPF_1 with the molecular anion mass of *m/z* 545 (Fig. III-20).

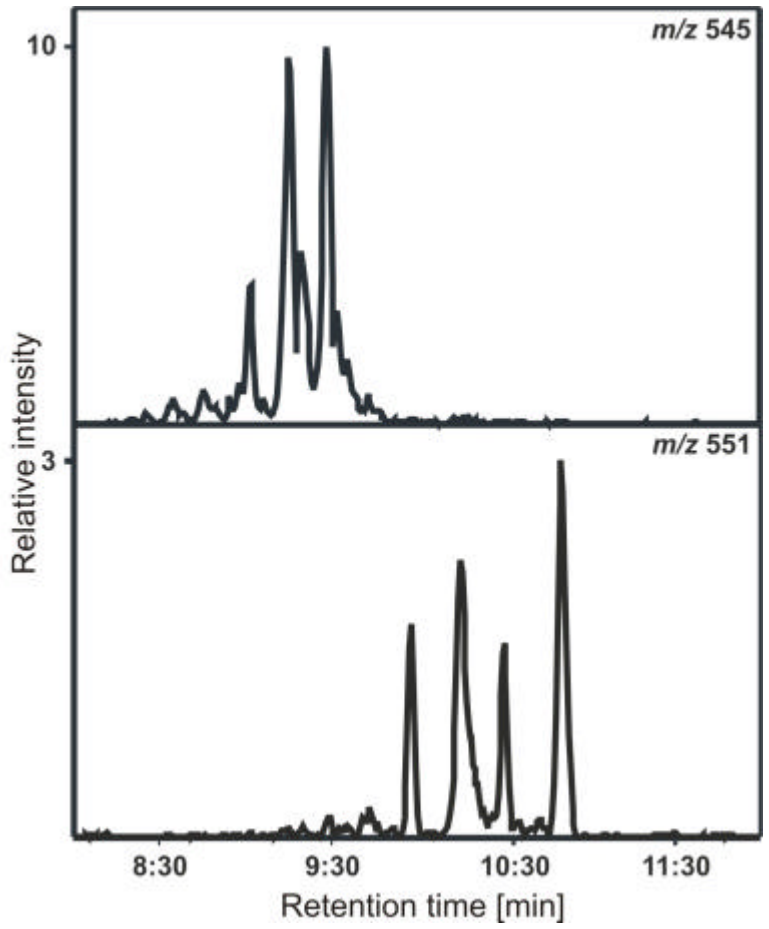


Figure III-20. GC-NCI-MS chromatogram of PPF_1 type III and IV (16 isomers) isolated from autoxidized Borage oil. PPF_1 were analysed after hydrogenation as pentafliorobenzyl ester, trimethylsilyether derivatives in the negative chemical ionization mode.

Since the highly sensitive GC-NCI-MS method only detects the intact molecular anions of all isomers, type III and IV isomers can not be distinguished by the mass spectrum (no fragmentation of the molecular anion observed). In contrast, the HPLC-MS system first isolates the intact molecular anion in the first quadrupole which is then fragmented in the second quadrupole. Fragments produced in quadrupole 2 are then analysed with the third quadrupole in the multiple reaction monitoring (MRM) mode. Since type III and IV isomers yield different and specific fragments, both isomers can be distinguished by LC-MS (data not shown). Unfortunately, this – highly specific – technique is only usefull for structure identification. For quantification, an internal standard ($[^{18}O]_3$-PPF_1 type I and II, as used for GC-MS analysis) has to be employed. Since this internal standard contains structurally different isomers, each isomer will yield specific fragments. However, the fragmentation efficiency of all specific fragments from each isomers is different, i.e. the detector response for each isomer is different and not known. Fortunately, all PPF_1 isomers (regardless of the type I to IV) produce besides isomer specific fragments also one fragment that is

characteristic for all PPF_1 isomers. Cleavage of the PPF_1 cyclopentane ring yields a fragment $[M-44]^-$ that can easily be detected in all isomers. Since it can be assumed that the fragmentation efficiency is similar for all PPF_1 isomers (type I to IV including the labelled PPF_1) the response factor for these fragment ions is one (Fig. III-21). As shown in Fig. III-21, PPF_1 type III and IV elute slightly later from the HPLC column than the oxygen-18 labelled PPF_1 (type I and II) internal standard. Note that the molecular anion of PPF_1-III/IV at *m/z* 327 is selected and fragmented (loss of 44 d). The corresponding product ion is recorded at *m/z* 283. In the case of the $[^{18}O]_3$-PPF_1-I/II standard, the isotopically labelled molecular anion at *m/z* 333 is selected. Since loss of the fragment includes one oxygen-18 atom the product ion is recorder at *m/z* 287.

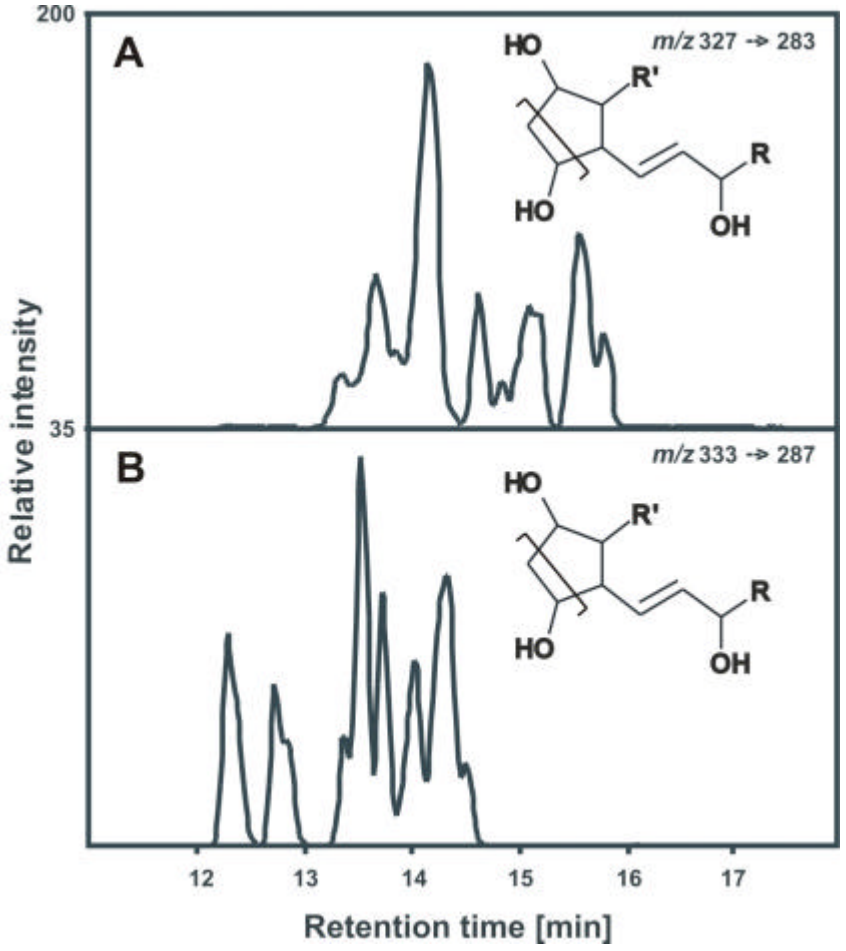


Figure III-21. HPLC-MS chromatogram of PPF_1 type III and IV (32 isomers) isolated from autoxidised Borage oil. A: PPF_1 type III and IV, B: internal standard, $[^{18}O]_3$-PPF_1 type I and II. See text for details.

In order to establish the identity of PPF_1 type III and IV and also to characterise the fragmentation pattern of the two isomeric PPF_1, isomers were isolated from HPLC and derivatized for GC-EI-MS analysis as described in the Materials and Methods section. For

electron impact ionisation (EI) in the positive mode, PPF_1 were methylated and silylated. The fragmentation pattern of the isomers is shown in Fig. III-22.

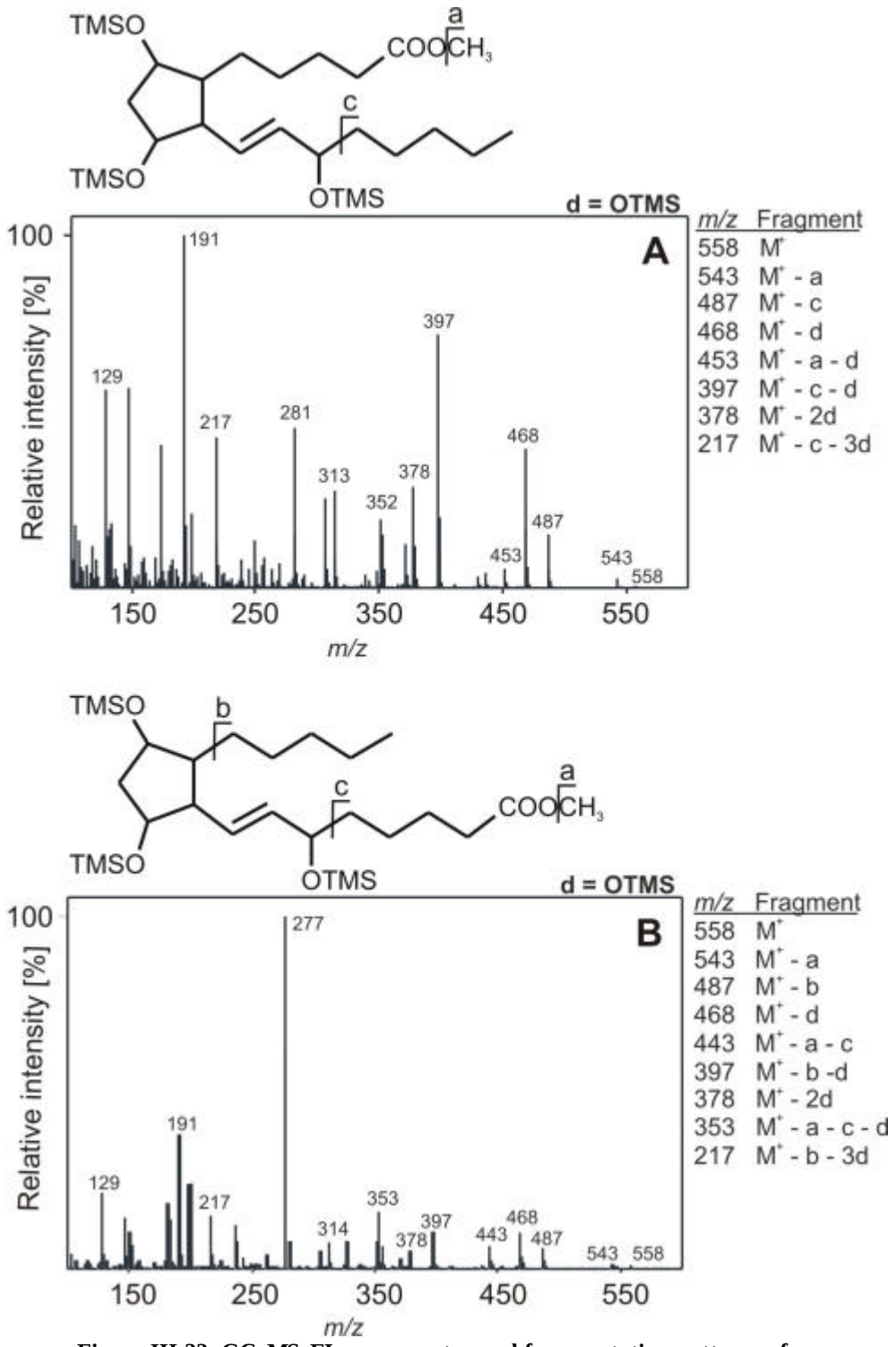


Figure III-22. GC-MS-EI-mass spectra and fragmentation patterns of methylated and silylated PPF_1 type III (A) and IV (B).

EI-MS-data of PPF_1-*O*-TMS-methyl-ester:

PPF_1 type III: EI-MS (70 eV); *m/z* (relative intensity [%]): 558 (1) $[M^+]$, 543 (4) $[M - CH_3]^+$, 487 (16) $[M - C_5H_{11}]^+$, 468 (40) $[M - HOTMS]^+$, 453 (6) $[M - CH_3 - HOTMS]^+$, 397 (72) $[M - C_5H_{11} - HOTMS]^+$, 378 (29) $[M - 2HOTMS]^+$, 217 (43) $[M - C_5H_{11} - 3HOTMS]^+$.

PPF_1 type IV: EI-MS (70 eV); *m/z* (relative intensity [%]): 558 (2) $[M^+]$, 543 (2) $[M - CH_3]^+$, 487 (6) $[M - C_5H_{11}]^+$, 468 (10) $[M - HOTMS]^+$, 443 (6) $[M - CH_3 - C_4H_8COO]^+$, 397 (10) $[M - C_5H_{11} - HOTMS]^+$, 378 (5) $[M - 2HOTMS]^+$, 353 (17) $[M - CH_3 - C_5H_{11} - HOTMS]^+$, 217 (15) $[M - C_5H_{11} - 3HOTMS]^+$.

III.14.2 Quantification of phytoprostane E_1/B_1 type III and IV from autoxidized Borage oil

During autoxidation of Borage oil, esterified γ-linolenate is in part oxidized to PPE_1 type III and IV. In addition, oxidised oil will also contain esterified degradation products of PPE_1 such as PPA_1 and PPB_1. During alkaline hydrolysis of the oil, all PPE_1 and PPA_1 isomers will be converted into PPB_1 (type III and IV) that can easily be measured by HPLC with UV detection at 279 nm (Parchmann and Mueller, 1998). PPB_1 isomers of the type III and IV can be easily resolved and quantified against the internal standard PGB_1 (Fig. III-23).

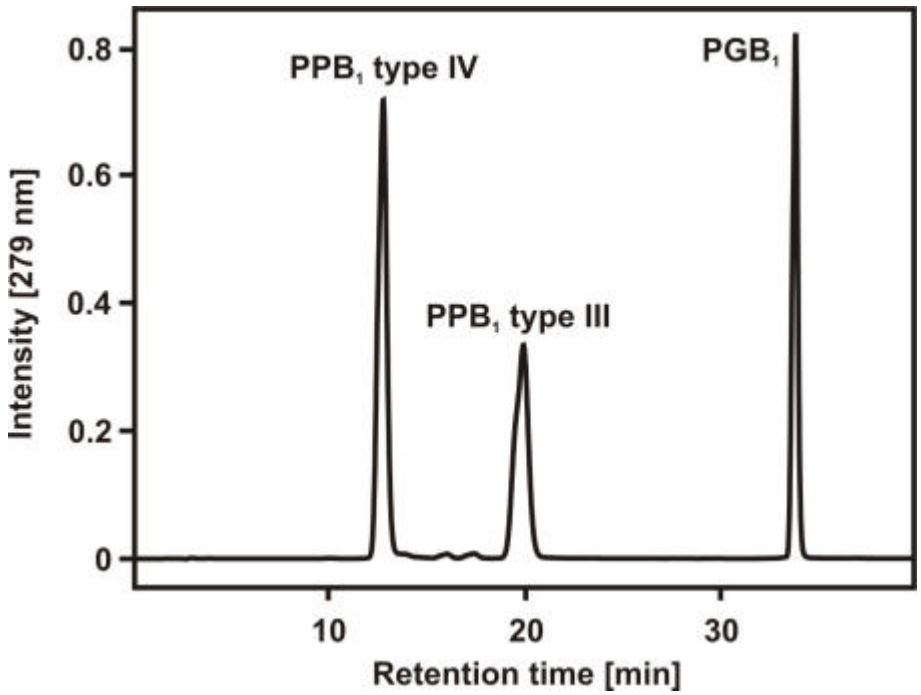


Figure III-23. HPLC chromatogram of PPB_1 type III and IV quantification from autoxidized and hydrolized Borage oil using PGB_1 as internal standard.

In order to verify the PPB_1 structures (type III or IV), the two PPB_1 containing peaks were collected separately and analysed by GC-EI-MS. Samples were dried in a vacuum centrifuge at 60°C, methylated with TMS-Diazomethane, silylated with BSTFA, and analysed by GC-MS. Based on the fragmentation patterns shown in Fig. III-24, the peaks of PPB_1 type III and IV isomers in the HPLC chromatogram could be verified.

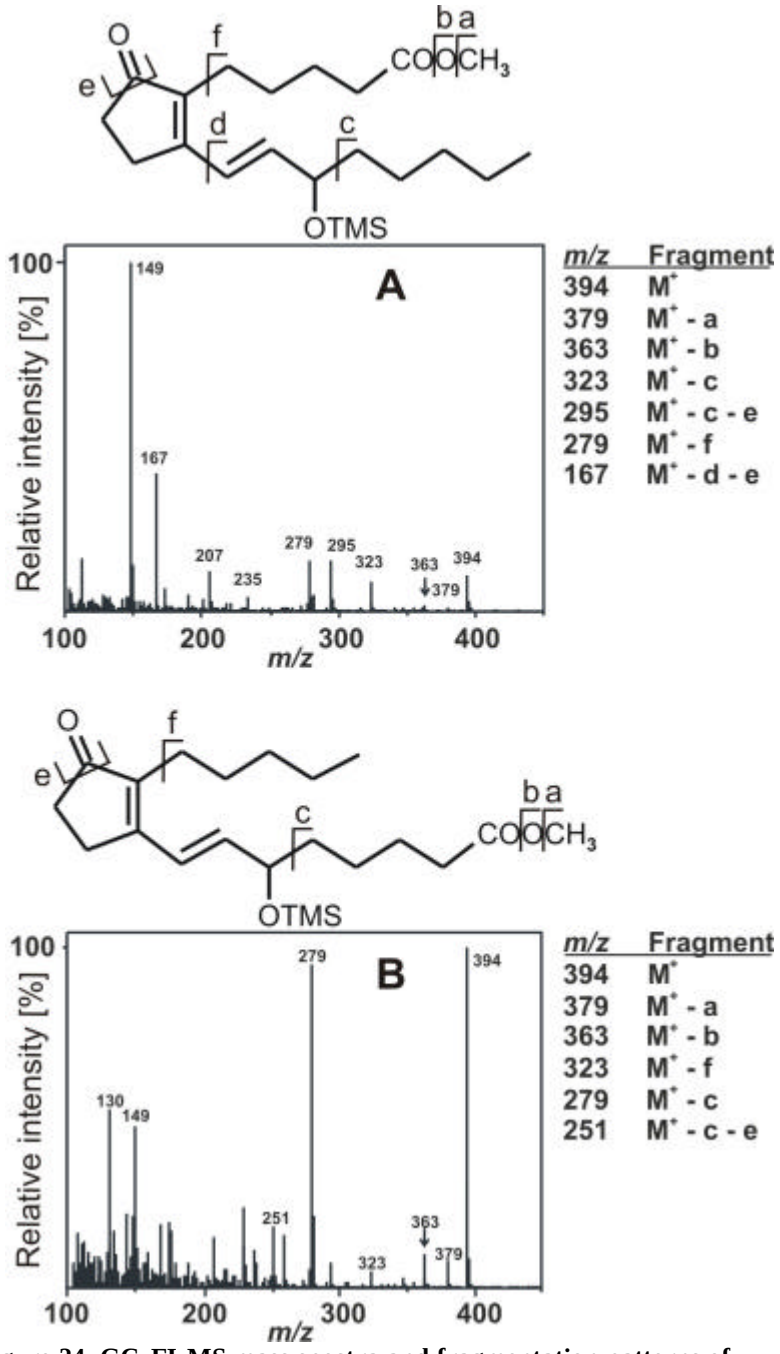


Figure-24. GC-EI-MS mass spectra and fragmentation patterns of methylated and silylated PPB_1 type III (A) and IV (B).

EI-MS-data of PPB_1-*O*-TMS-methyl-ester:

PPB_1 type III: EI-MS (70 eV); *m/z* (relative intensity [%]): 394 (14) $[M]^{\cdot+}$, 379 (1) [M – CH_3]$^+$, 323 (9) [M – C_5H_{11}]$^+$, 295 (14) [M - C_5H_{11} – CO]$^+$, 279 (15) [M – $C_6H_{11}O_2$]$^+$, 167 (39) [M – C_8H_{14}OTMS - CO]$^+$.

PPB_1 type IV: EI-MS (70 eV); *m/z* (relative intensity [%]): 394 (100) $[M]^{\cdot+}$, 379 (8) [M – CH_3]$^+$, 363 (10) [M – OCH_3]$^+$, 323 (5) [M – C_5H_{11}]$^+$, 279 (100) [M – $C_7H_{14}O_2$]$^+$, 251 (18) [M - $C_7H_{14}O_2$ - CO]$^+$.

The peaks of PPB_1-III/IV in the HPLC chromatogram represent the sum of all PPE_1, PPA_1, and PPB_1 isomers that were originally present in the Borage oil. After 12 days autoxidation of 1 g Borage oil, 1.71 ± 0.04 mg PPE_1/PPA_1/PPB_1 type III and 2.09 ± 0.12 mg PPE_1/PPA_1/PPB_1 type IV were obtained.

For analysis of complex biological samples, the HPLC-UV method is not sensitive and specific enough to allow proper quantification. Therefore, the isolated reference compounds were derivatzed with PFB-Br and BSTFA to their corresponding PPB_1-PFB ester, TMS ether derivatives and analysed by GC-NCI-MS using PGB_1 as internal standard (Fig. III-25).

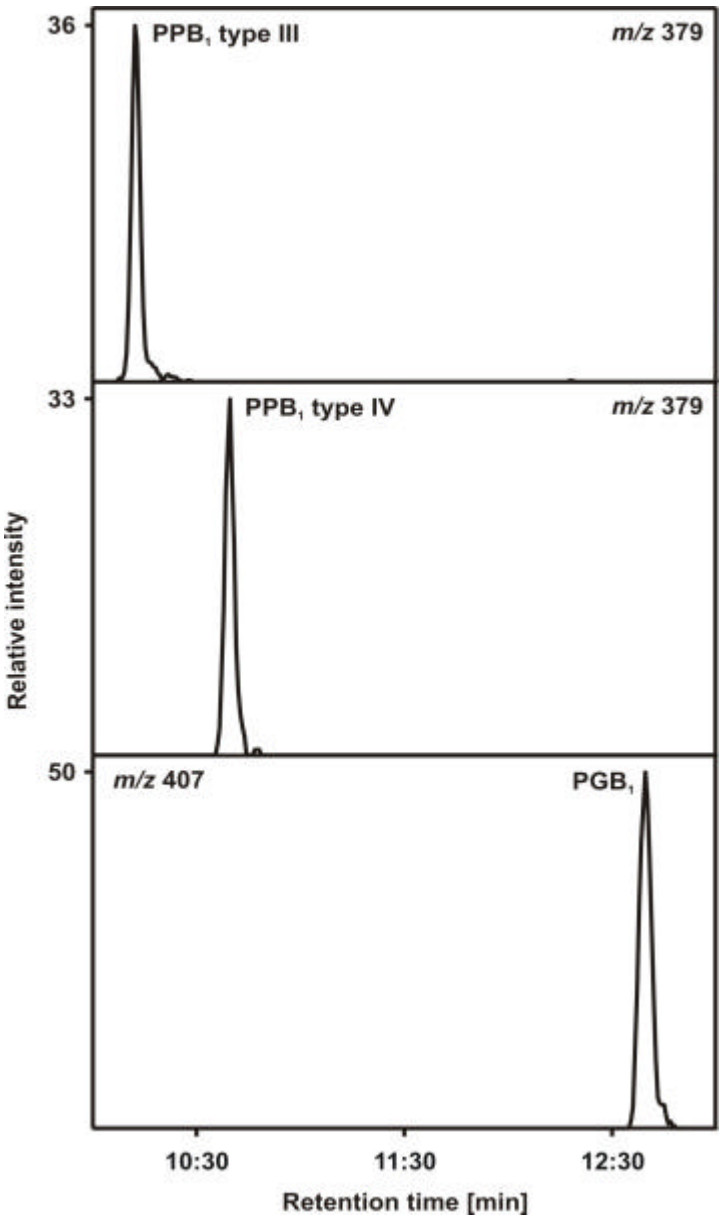


Figure III-25. GC-NCI-MS chromatograms of PPB_1 type III and IV as well as PGB_1. After esterification with PFB-Br and silylation with BSTFA, PPB_1 type III and IV give the mass/charge ratio of 379 and that of PGB_1 is 407.

In order to quantify PPE_1 directly, γ-linolenate (isolated from Borage oil) was autoxidized. Degradation of PPE_1 to PPB_1 can be prevented by methoximation of PPE_1 and PPB_1 with methoxyamine-HCl followed by methylation with TMS-diazomethane and silylation with BSTFA. In the EI mode of GC-MS instrument, PPB_1 and PPE_1 type III and IV can clearly be distinguished by their mass spectra (Fig. III-26 and III-27).

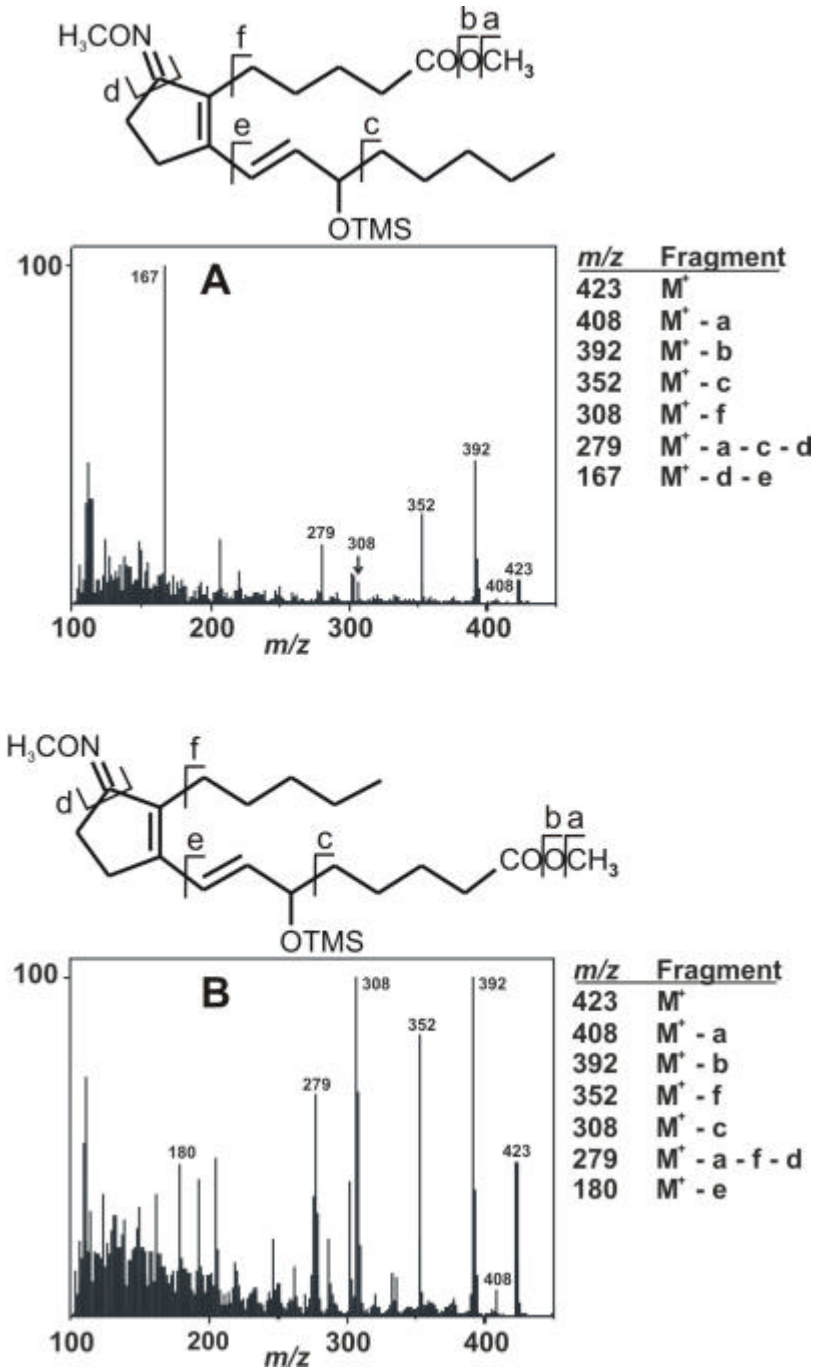


Figure-26. GC-EI-MS mass spectra and fragmentation patterns of methoximated, methylated und silylated PPB_1 type III (A) and IV (B).

EI-MS-data of PPB_1-methoxim, TMS-ether, methyl-ester derivatives:

PPB_1 type III: EI-MS (70 eV); *m/z* (relative Intensität [%]): 423 (7) $[M]^{\cdot+}$, 408 (2) $[M - CH_3]^+$, 392 (42) $[M - OCH_3]^+$, 352 (26) $[M - C_5H_{11}]^+$, 250 (6) $[M - C_6H_{12}OTMS]^+$.

PPB_1 type IV: EI-MS (70 eV); *m/z* (relative Intensität [%]): 423 (46) $[M]^{\cdot+}$, 408 (8) $[M - CH_3]^+$, 392 (100) $[M - OCH_3]^+$, 352 (83) $[M - C_5H_{11}]^+$, 308 (100) $[M - C_6H_{11}O_2]^+$, 277 (66) $[M - C_6H_{11}O_2 - OCH_3]^+$, 206 (47) $[M - C_7H_{12}O_2OTSM]^+$, 180 (44) $[M - C_9H_{14}O_2OTSM]^+$.

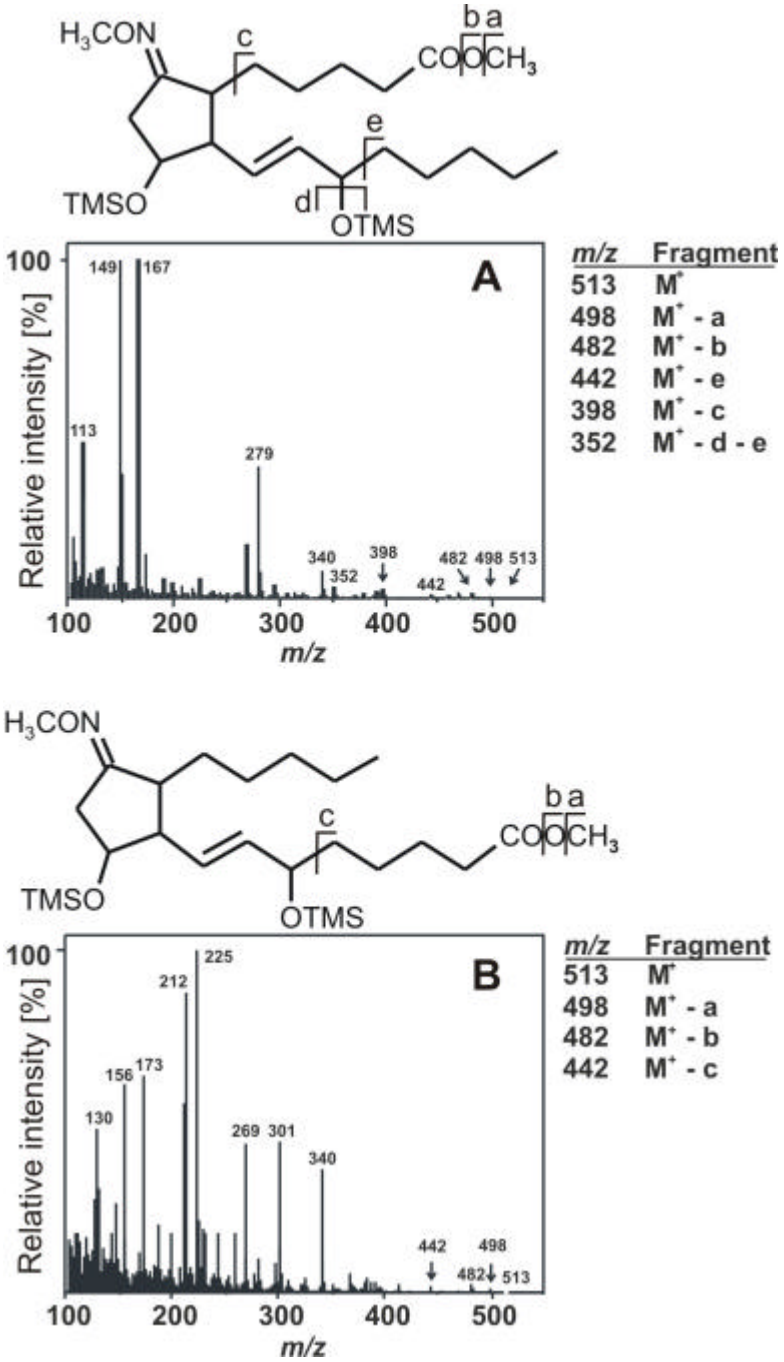


Figure-27. GC-EI-MS mass spectra and fragmentation patterns of methoximated, methylated and silylated PPE_1 type III (A) and IV (B).

EI-MS-data of PPE_1-methoxim, TMS-ether, methyl-ester derivatives:

PPE_1 type III: EI-MS (70 eV); *m/z* (relative intensity [%]): 513 (0,2) $[M]^{\cdot+}$, 498 (0,5) $[M - CH_3]^+$, 482 (1) $[M - CH_3O]^+$, 442 (1) $[M - C_5H_{11}]^+$, 352 (3) $[M - C_5H_{11} - HOTMS]^+$, 340 (8) $[M - C_5H_{11} - CHOTMS]^+$, 167 (100) $[M - C_5H_{11} - C_2H_2CHOTMS - HOTMS - CNOCH_3]^+$.

PPE_1 type IV: EI-MS (70 eV); *m/z* (relative intensity [%]): 513 (0,5) $[M]^{\cdot+}$, 498 (1) [M – CH_3]$^+$, 482 (2) [M – CH_3O]$^+$, 442 (1,6) [M – C_5H_{11}]$^+$, 412 (2) [M – $C_5H_9O_2$]$^+$, 340 (36) [M – $CHOTMSCH_2CNOCH_3$]$^+$ 269 (44) [M -]$^+$, 173 (63) $[CHOTMSCH_2CNOCH_3]^+$.

Sample preparation and analysis of phytoprostanes by GC-MS is time consuming. For this reason, fast HPLC-MS/MS analysis methods were developed. There are many advantages of using HPLC-MS for analysis. Samples can be analysed directly since there is no need for repeated SPE purifications and derivatisations. Hence, there is only a minimal loss of analytes prior analysis. Quantification of PPB_1/PPE_1 type III and IV can be run overnight by auto-sampler in the HPLC-MS system. In addition, $[^{18}O]_3$-PPE_1 type I and II could be used as an approriate internal standard. To facilitate analysis, PPE_1, PPA_1 and PPB_1 were analysed together as PPB_1 after treatment of the samples with KOH. By this treatment, the internal standard $[^{18}O]_3$-PPE_1 looses two oxygen-18 atoms (Fig. III-28).

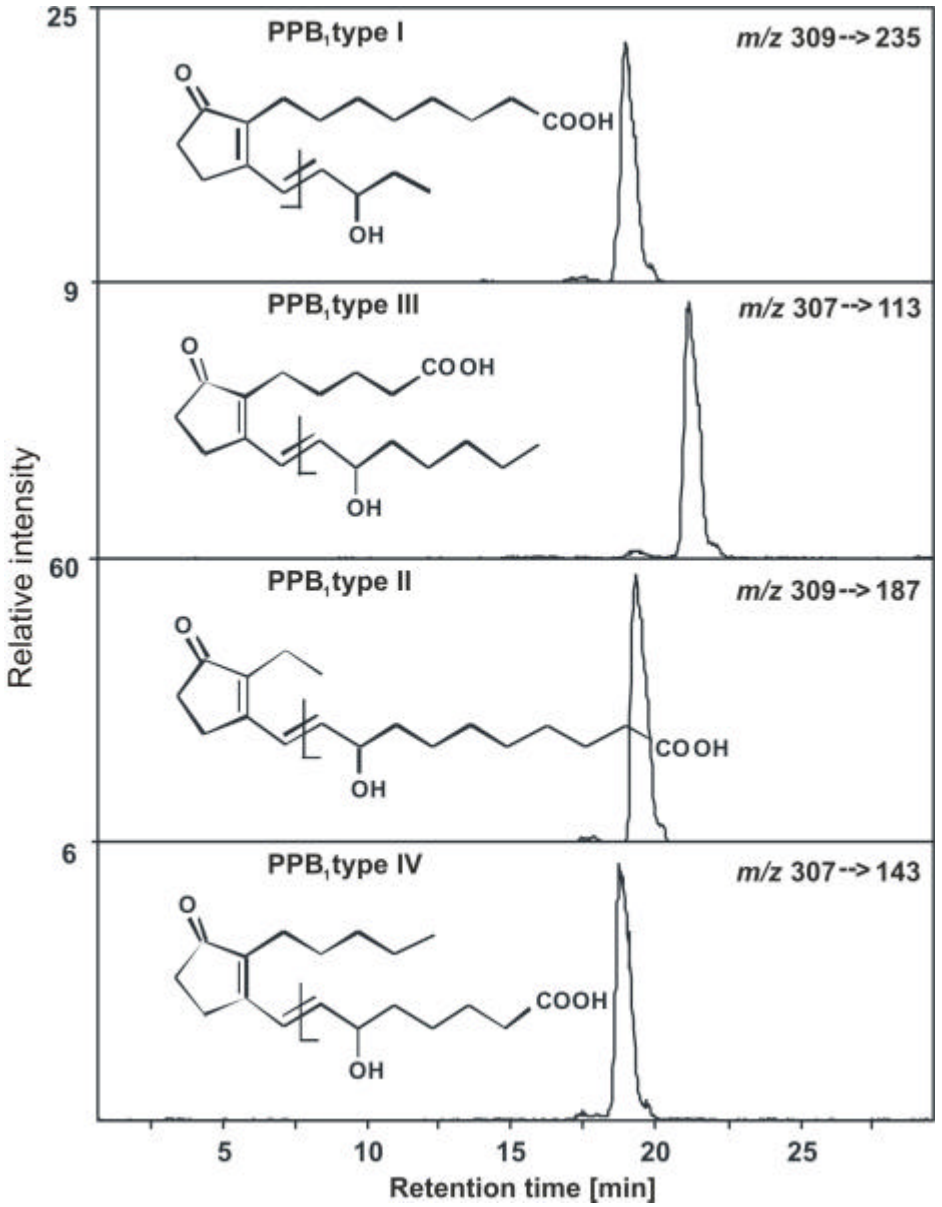


Figure III-28. HPLC-MS chromatograms of PPE_1 type III and IV isomers after conversion into the corresponding PPB_1 type III and IV derivatives. Brackets show the detected fragments. In amonium acetate buffer, [^{18}O]-PPB_1 type I and II is ionized to M^- = 309 and that of PPB_1 type III and IV is M^- = 307.

III.14.3 Identification and measurement of A_1 phytoprostanes type III and IV

Typically, PPA_1 are minor products of fatty acid autoxidation. In order to obtain enough compounds for characterisation of the compounds, PPE_1 were isolated from oxidised γ-linolenate and chemically converted into PPA_1 of the type III and IV (see Material and Methods for details). PPA_1 were first analysed by HPLC equipped with an UV-detector at 217 nm. However, PPA_1 type III and IV could not be resolved and eluted together in one peak (Fig. III-29). The peak was collected and analyzed by GC-EI-MS. However, type III and IV isomers displayed an almost identical fragmentation pattern i.e. no isomer specific fragments could be detected (Fig. III-30). Hence, the isomers can not be distinguished by GC-EI-MS.

Purified PPA_1 type III and IV were then converted to the corresponding PPB_1 compounds by alkali treatment and quantified by HPLC.

In order to quantify, PPA_1 in the peak (in Fig. III-29) was collected. PPA_1 were isomerized to coresponding PPB_1 by treating with KOH. Quantification of PPA_1 was done indirectly by quantifying coresponding PPB_1 using PGB_1 as internal standard. After 12 days auto-oxidation, 1g Borage oil yielded 18.56 ± 0.62 µg PPA_1 including 8.38 ± 0.35 µg type III and 10.18 ± 0.30 µg type IV. This PPA_1 yield is much lower than PPB_1 and PPE_1 (in mg/g range).

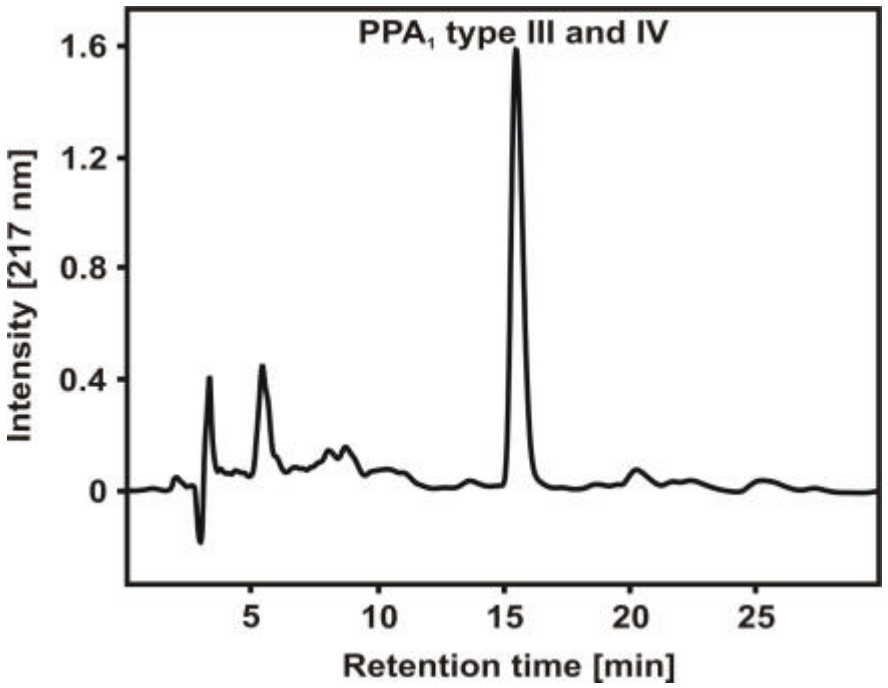


Figure III-29. HPLC chromatogram of PPA_1 type III and IV separation.

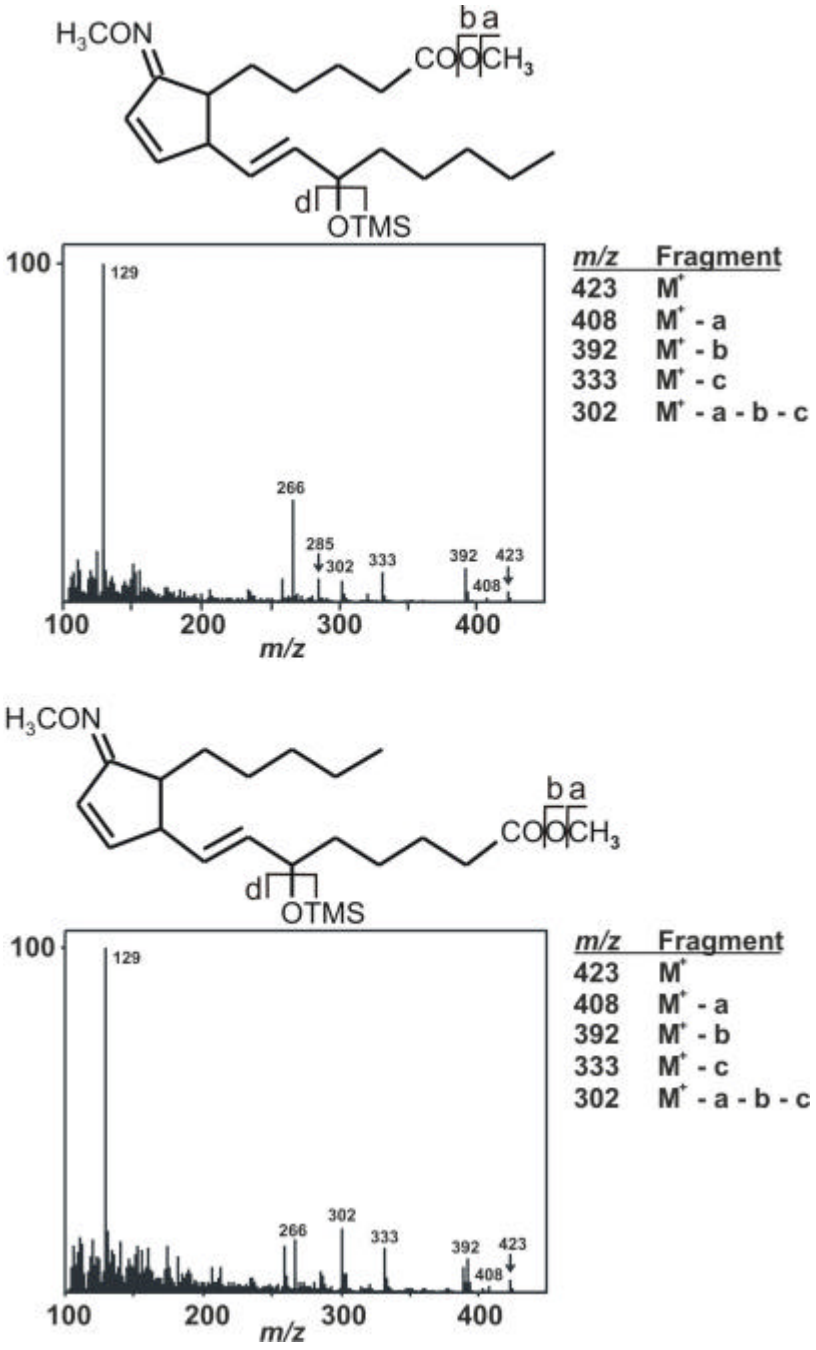


Figure III-30. GC-EI-MS mass spectra and fragmentation patterns of methoximated, methylated and silylated PPA_1 type III and IV. Due to the similar fragmentation pattern, both isomers cannot be distinguished by their mass spectra.

EI-MS-data of PPA_1-methoxim, TMS-ether, methyl-ester derivatives:

PPA_1 type III and IV: EI-MS (70 eV); *m/z* (relative intensity [%]): 423 (4) $[M]^{\cdot +}$, 408 (1) $[M - CH_3]^+$, 392 (10) $[M - CH_3O]^+$, 333 (9) [M - HOTSM]$^+$, 302 (7) [M - CH_3O - HOTSM]$^+$.

III.15 Quantification of F_1- and E_1- phytoprostanes type III and IV in *Synechocystis* PCC 6803

After phytoprostanes type III and IV have been identified and quantified *in vitro*, it is interesting to investigate if they are present *in vivo.* As mentioned, *Synechocystis* possesses ?-linolenic acid, the precusor of phytoprostanes type III and IV. Therefore, it was tested if the two main phytoprostanes, F_1 and E_1 type III and IV can be identified and quantified in this cyanobacterium.

PPF_1 type III and IV in *Synechocystis* can be detected and quantified by both GC-MS and HPLC-MS (Fig. III-31 and Fig. III-32). As shown in the GC-NCI-MS chromatogram (Fig. III-31), the peak pattern of endogenous PPF_1 type III and IV detected at *m/z* 545 is identical to the peak pattern of PPF_1 produced by autoxidation of γ-linolenic acid *in vitro* (compare Fig. III-31 with Fig. III-20). The internal standard, $[^{18}O]_3$-PPF_1 type I and II was detected at *m/z* 551 and used for quantification. If endogenous, unlabelled PPF_1 of the type I and II would have been present in *Synechocystis*, this peak pattern should have been detected at *m/z* 545 with the same retention time. As can be seen, PPF_1 type I and II are not present or below the limit of detection in *Synechocystis*.

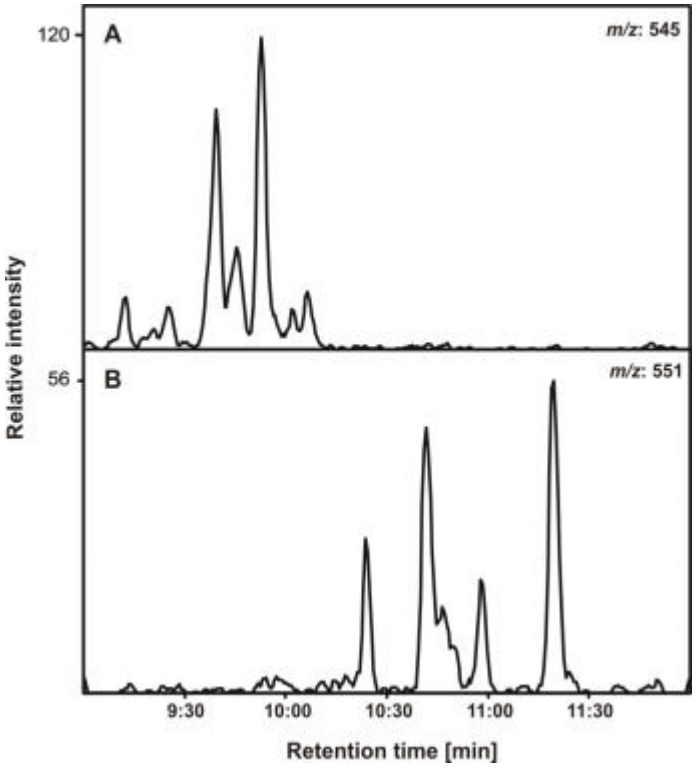


Figure III-31. GC-NCI-MS chromatogram of PPF_1 type III and IV derivatives from *Synechocystis*. A: endogenous PPF_1-III/IV but not PPF_1-I/II can be detected at *m/z* 545. B: The internal standard derivative of $[^{18}O]_3$-PPF_1 type I and II can be detected at *m/z* 551.

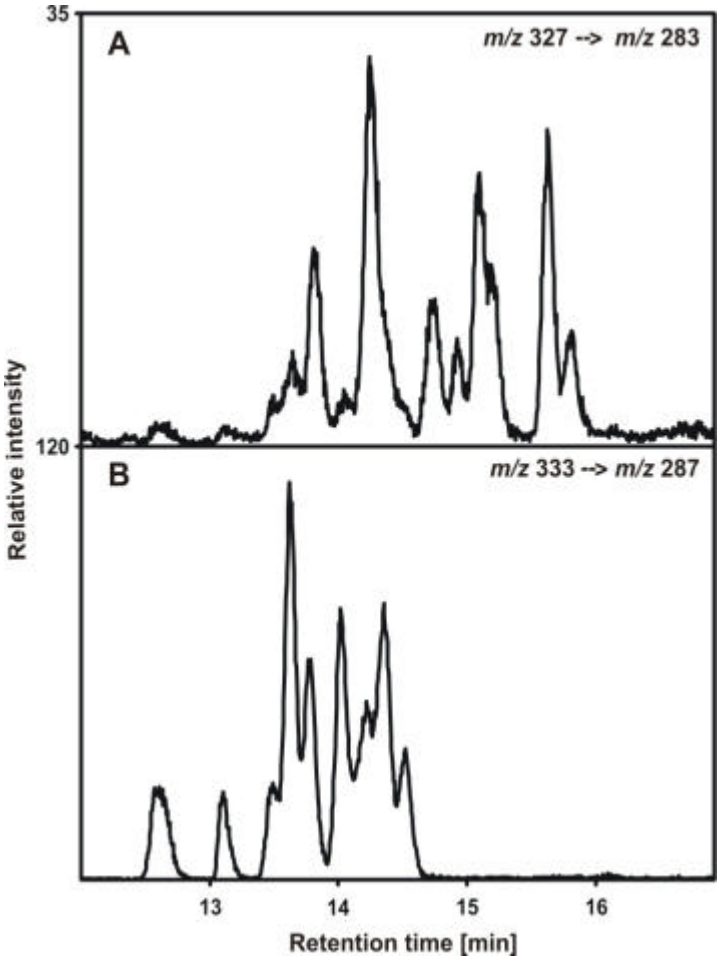


Figure III-32. HPLC-MS-MRM chromatogram of PPF_1 type III and IV from *Synechocystis*. A: endogenous PPF_1-III/IV. B: internal standard, $[^{18}O]_3$-PPF_1-I/II.

Endogenous PPE_1 type III and IV in *Synechocystis* were also easily identified by both GC-MS and HPLC-MS analysis (Fig. III-33 and III-34). By GC-MS analysis, endogenous PPF_1 type III and IV in *Synechocystis* showed incompletely separated peaks in comparison with PPF_1 type I and II standard. For routine analysis, HPLC-MS/MS is prefered over GC-MS due to the faster processing and analysis of samples. Data from HPLC-MS of PPF_1 and PPE_1 type III and IV analysis was used for quantification.

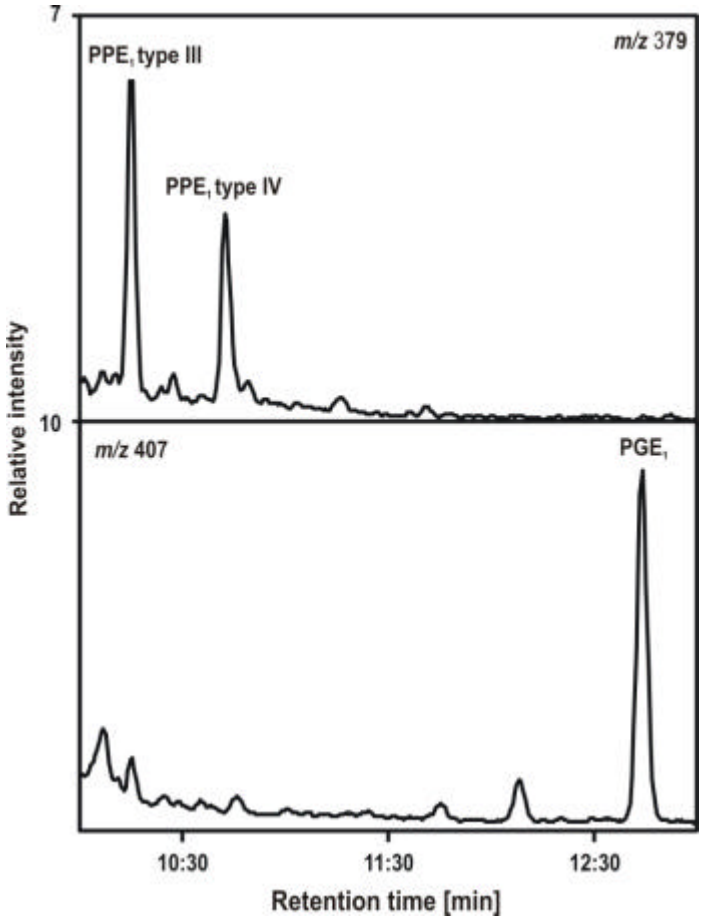


Figure III-33. GC-NCI-MS chromatogram of PPE_1 type III and IV analysis in *Synechocystis*. PPE_1 type III and IV was isomerized to corresponding PPB_1 type III and IV and they were detected as PPB_1 type III and IV. Internal standard PGE_1 was identified as PGB_1 in the same condition. After esterification with PFB-Br and silylation with BSTFA, PPB_1 type III and IV give the mass/charge ratio of 379 and that of PGB_1 is 407.

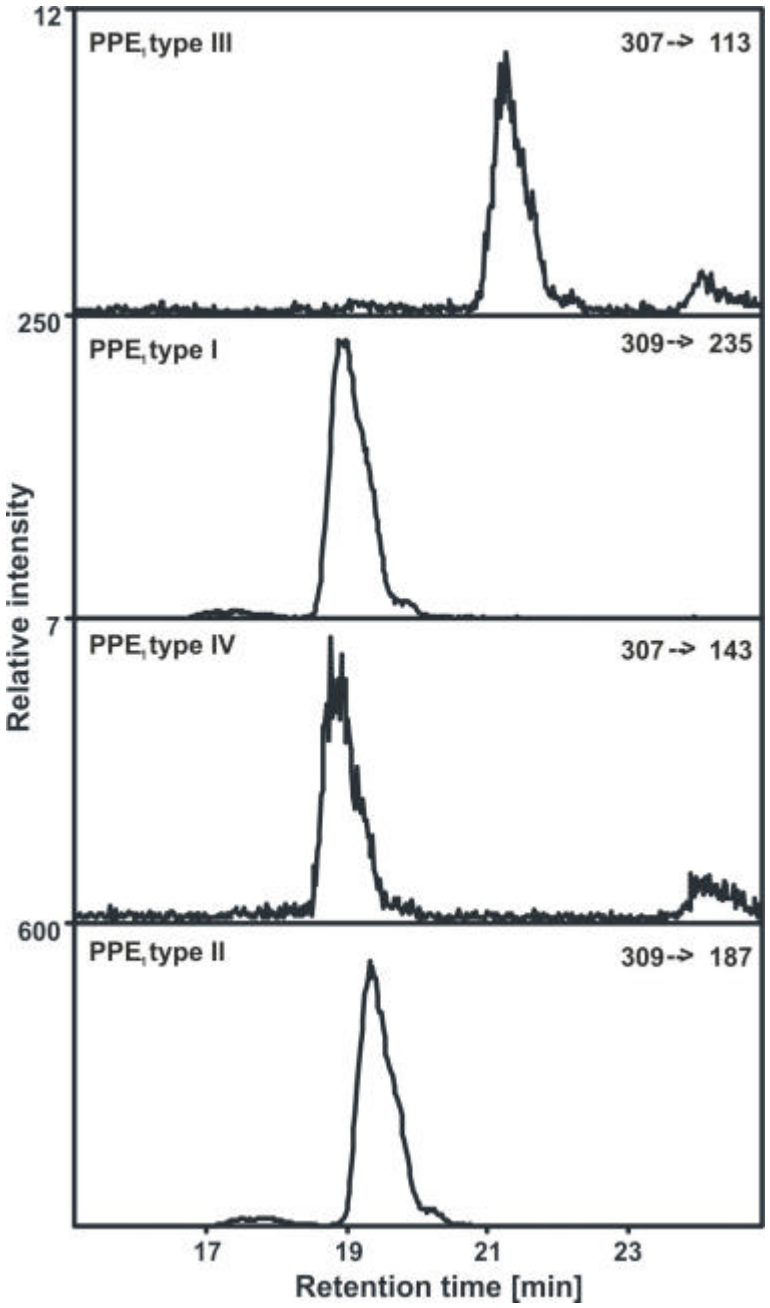


Figure III-34. HPLC-MS-MRM chromatograms of PPE_1 type III and IV analysis in *Synechocystis*. PPE_1 was isomerized to corresponding PPB_1 and detected as PPB_1. For fragmentation, see figure III-28.

Table-9 shows that phytoprostanes in *Synechocystis* are present both in free and esterified forms. PPE_1 are more abundant than PPF_1. In comparison with phytoprostanes type I and II in *Anabaena*, levels of phytoprostanes type III and IV in *Synechocystis* are much higher, both in medium and in the cells (compare Table-3 with Table-9). Together with the identification and quantification of phytoprostanes type III and IV from Borage oil, this result confirms that phytoprostanes type III and IV can be produced from γ-linolenic acid both *in vitro* and *in vivo.*

Table-9. Phytoprostanes level in *Synechocystis* by HPLC-MS analysis.

Phytoprostane	Medium (ng/l)	Free (ng/g DW)	Total (ng/g DW)
E_1	1003.3 ± 365.2	2331.0 ± 87.7	2738.3 ± 208.4
F_1	231.8 ± 36.2	164.9 ± 15.2	1067.1 ± 209.0

Notably, *Synechocystis* type III and IV phytoprostanes typically occur not in higher plants but have been identified as biologically active prostanoids in humans. Although type III and IV phytoprostanes can be formed by endogenous, chemical oxidation of γ-linolenate in humans, it is assumed that most of these phytoprostanes are formed by reduction and ß-oxidation of isoprostanes (Burke *et al.*, 2000). Since they are two methylene groups shorter than C20-isoprostanes they have also been termed dinor isoprostanes. In *Synechocystis*, isoprostanes can not be formed due to the lack of appropriate fatty acid precursor (arachidonate). Hence, formation of phytoprostanes type III and IV in *Synechocystis* is by autoxidation of γ-linolenate while the same products are mainly formed in humans by ß-oxidation of arachidonate autoxidation products (isoprostanes).

IV. DISCUSSION

The goal of this work was to elucidate the presence of different oxylipins in cyanobacteria, to compare the levels with those found in plants and to investigate the biological activity of the oxylipins identified.

IV.1 Discovery of phytoprostanes in *Anabaena* and *Synechocystis*

At the beginning of this study, there was no evidence for the presence of oxylipins in the bacterial kingdom including cyanobacteria. Typically oxylipins are produced from multicellular organisms that synthesize oxylipins from polyunsaturated fatty acids esterified in membrane lipids. After release from the membrane by lipases, the fatty acid is oxygenated by lipoxygenases or cyclooxygenases to lipid peroxides that serve as substrates for several lipid modifying enzymes (Mueller, 1998). The best studied pathways are the jasmonate pathway in higher plants and the prostaglandin and leukotriene pathway in animals. In plants and animals, these oxylipins have essential signalling functions in development, reproduction and host defense. Jasmonates and prostaglandins have occasionally also been detected in algae, fungi and mosses, however, the occurrence is limited to a few species only (Mueller, 2004). Thus far, essential functions of oxylipins in these lower organisms have never been described and in most cases their biological activities and functions remain to be clarified.

Generally, bacteria use only monounsaturated fatty acids (mostly oleic acid) in their membranes and lack the ability to synthesise polyunsaturated fatty acids. Since polyunsaturated fatty acids are the substrates for oxylipin pathways, bacteria do not synthesize oxylipins. Notable exceptions within the bacterial kingdom, at least with respect to the occurrence of polyunsaturated fatty acids, are cyanobacteria. These bacteria are believed to be ancestors of plant chloroplasts and are able to perform photosynthesis. Like chloroplast membranes, cyanobacterial membranes contain high levels of polyunsaturated fatty acids i.e. linoleic (18:2) and linolenic acid (18:3). Linolenate-deficient *Arabidopsis* plants grow normally at room temperature, however, high membrane unsaturation becomes essential to perform photosynthesis at low temperatures (Routaboul *et al.*, 2000). While a high linolenate content in thylakoids supports growth under extreme temperatures (Vijayan and Browse, 2002), there are also inherent problems associated with the use of linolenate in thylakoids. This is because the photosynthetic apparatus produces reactive oxygen species (ROS) as side products (even under optimal conditions) that can readily oxidize linolenate and linoleate in

the membrane. In the light, chlorophyll is a photosensitizer that can excite normal, unreactive oxygen to singlet oxygen. Singlet oxygen can be quenched and thereby detoxified by carotenoids and tocopherols, both found in close vicinity of chlorophyll in thylakoids of both chloroplasts and cyanobacteria (Sattler *et al.*, 2006). However, some singlet oxygen escapes detoxification and oxidizes linoleate/linolenate to hydroperoxy fatty acids (Mueller *et al.*, 2006). In addition, superoxide anion radicals can be formed during photosynthesis by transfer of a single electron only on molecular oxygen. Neither superoxide nor its metabolite hydrogen peroxide can oxidize polyunsaturated fatty acids directly. However, hydrogen peroxide can be converted non-enzymatically to the highly reactive hydroxyl radical through the iron catalysed Fenton reaction. Hydroxyl radicals in turn readily attack polyunsaturated fatty acids thereby initiating a radical chain reaction ultimately leading to the formation of hydroperoxy fatty acids in the presence of oxygen. Non-enzymatically formed fatty acid hydroperoxides comprise a variety of structural isomers including all those that can also be synthesized enzymatically in plants and animals. Moreover, lipid peroxy radicals can undergo secondary oxidation and cyclization reactions that lead to prostaglandin-like compounds termed isoprostanes (from 20:4 fatty acids i.e. arachidonate) in animals and phytoprostanes (from 18:3 fatty acids i.e. linolenate) in plants (Thoma *et al.*, 2004). Isoprostanes represent a mixture of isomers that, however, also comprise the isomers that can be synthesized enzymatically in animals (Roberts and Morrow, 1997).

Hence, even in the absence of lipoxygenases and cyclooxygenases, hydroxy fatty acids and prostaglandins can be formed by chemical oxidation of endogenous fatty acids. Therefore, it is to be expected that all aerobic organisms synthesizing the necessary substrates for lipoxygenase and cyclooxygenase pathways contain hydroxy fatty acids and prostaglandin-like compounds (Mueller, 2004). *Anabaena* synthesizes α-linolenate and *Synechocystis* produces γ-linolenate. Both fatty acids are precursors for a series of hydroxy fatty acids and phytoprostanes which differ in the position of oxygenation and the location of the double bonds. While non-enzymatic oxidation of polyunsaturated fatty acids cannot be avoided completely as it is associated with aerobic metabolism, the degree of membrane and fatty acid oxidation may differ in different organisms because of the variability of the antioxidative capacity as well of the environment. However, it was found that *Anabaena* not only produces the same hydroxy fatty acids and phytoprostanes as higher plants but also that the levels of these compounds are in the same range. For example, about 70 ng PPE_1 and 150 ng PPF_1 were found in 1 g of dry weight in tomato leaves (Thoma *et al.*, 2004) which compares well to the levels (on dry weight basis) found in *Anabaena* (150 ng/g PPE_1 and 200

ng/g PPF_1). Since phytoprostanes are excellent markers of oxidative stress, this result indicates that cells from *Anabaena* and higher plants are exposed to a comparable (endogenous) oxidative stress under normal physiological conditions.

In vitro, in planta as well as in *Anabaena* chemical oxidation of α-linolenate yields two regioisomeric phytoprostanes with a prostaglandin G-like endoperoxy cyclopentane ring system termed PPG_1. The two regioisomers are classified as type I and type II compounds. PPG_1 are highly unstable compounds that rapidly rearrange to the hydroxy cyclopentanone compounds PPE_1 or are reduced to the dihydroxy cyclopentane compounds PPF_1. Both PPE_1 and PPF_1 were unequivocally identified by GC-MS and quantified by using an isotopically labelled internal standard.

Since *Synechocystis* lacks α-linolenic acid, phytoprostanes of the type I and II can not be formed and were therefore absent in *Synechocystis* extracts. Fatty acid analysis of *Synechocystis* revealed that α-linolenate is substituted by γ-linolenic acid (about 24% of all fatty acids) in this cyanobacterium. By chemical oxidation of γ-linolenate, phytoprostanes of the type III and IV can be obtained. The type III F_1-phytoprostanes has been first described as a metabolite of a F_2-isoprostane in humans (Hou *et al.*, 2001). In humans, arachidonate (a C20:4 fatty acid) oxidizes to F_2-isoprostanes that can be metabolized by ß-oxidation (loss of two carbons) and reduction (loss of one side chain double bond) to F_1-phytoprostanes type III. In the original literature this metabolite is termed 2,3-dinor-5,6-dihydro-15-F_2-isoprostane and has been identified as one of the major metabolites excreted into urine (Hou *et al.*, 2001). Moreover, the metabolite was shown to retain biologic activity and is a potent vasoconstrictor. Although the authors discuss the possibility, that the compound could also be formed by direct oxidation of γ-linolenate *in vivo,* this is unlikely because γ-linolenate is only a minor fatty acid in humans. Moreover, the type IV compound which should be formed in equal amount by chemical oxidation of γ-linolenate has never been described or identified.

In order to identify the postulated type III and IV phytoprostanes in *Synechocystis*, reference compounds were prepared by autoxidation of γ-linolenate *in vitro*, isolated and characterized by GC-MS and HPLC-Tandem-MS. Analytical methods were developed to identify and quantify both regioisomers in *Synechocystis*. Analysis revealed that *Synechocystis* contained comparably high levels of PPE_1 type III and IV (2.7 µg/g of dry weight) as well as PPF_1 type III and IV (1.1 µg/g of dry weight).

Results indicate that both the unicellular cyanobacterium *Synechocystis* and the filamentous cyanobacterium *Anabaena* contain phytoprostane isomers in the same or even higher concentration range as leaves of higher plants.

IV.2 Oxidative stress increases phytoprostane levels only under high light condition in *Anabaena*

Previously, it has been shown that plant cell cultures and intact plants accumulate phytoprostanes in their membranes as well as in free form (after release from membranes) when they are treated with sublethal concentrations of heavy metals or hydrogen peroxide. However, in *Anabaena* cells phytoprostane levels did not significantly increase after both treatments. Hence, *Anabaena* appears to be well protected from non-enzymatic lipid peroxidation even under severe oxidative stress conditions. We observed, however, an increase of phytoprostane levels when either stress occurred under high light conditions. Under saturating high light conditions, the photosynthetic apparatus produces both more singlet oxygen and superoxide which in turn may increase chemical oxidation of lipids. However, high light alone is not sufficient to increase phytoprostane formation indicating again that *Anabaena* is remarkably resistant towards lipid peroxidation.

IV.3 Enzymatic oxylipin pathways are absent in *Anabaena*

As mentioned previously, bacteria typically produce no oxylipins due to the lack of the required polyunsaturated fatty acid substrates. Since almost all cyanobacteria contain high levels of linolenate, likely required for the proper function of the photosynthetic apparatus (Vijayan and Browse, 2002), and also produce the second required substrate, molecular oxygen, cyanobacteria potentially could synthesize enzymatically oxidized lipids. *Synechocystis*, however, can neither produce true jasmonates (due to the lack of α-linolenate) nor prostaglandins (lack of arachidonate). Moreover, the *Synechocystis* genome lacks also genes similar to lipoxygenases, cyclooxygenases or any other enzyme/receptor involved in plant or animal oxylipin biosynthesis or recognition (http://bacteria.kazusa.or.jp/cyanobase/).

In contrast, the *Anabaena* genome contains a putative lipoxygenase – allene oxide synthase fusion protein. Both enzymes represent the first two enzymes of the jasmonate pathway in plant chloroplasts. Incubation of the two plant enzymes with α-linolenic acid *in vitro* yields a highly unstable allene oxide that rapidly decomposes non-enzymatically to

racemic (cis and trans) 12-oxo-phytodienoic acid and two acyclic ketol compounds (Mueller, 1997). In plant chloroplasts, the unstable allene oxide is immediately converted to cis 12-oxo-phytodienoic acid only by allene oxide cyclase (Delker *et al.*, 2006). Genes homologous to allene oxide cyclise and other enzymes of the jasmonate pathway could not be identified in the *Anabaena* genome (http://bacteria.kazusa.or.jp/cyanobase/).

Careful analysis of *Anabaena* extracts revealed that 12-oxo-phytodienoic acid (or isomers thereof) as well as jasmonic acid do not occur in *Anabaena* or are at least under the limit of detection (< 100 pg/g of dry weight). In addition, analysis of hydroxy fatty acids suggests that oxygenation of α-linolenic acid occurred at all theoretically possible positions in a random fashion as it is expected after non-enzymatic oxidation of linolenate. In contrast, lipoxygenases from animals and plants selectively oxygenate α-linolenate at the carbon 9 and/or 13.

From the other plant hormones analyzed in *Anabaena*, only salicylic acid (63.1 ± 22.9 ng/g DW) and indole acetic acid (67.1 ± 6.6 ng/g DW) were found. IAA has previously been identified in cyanobacteria (Sergeeva *et al.*, 2002).

IV.4 Oxidized lipids protect *Anabaena* from lethal oxidative stress

Results indicate that there is no evidence for the presence of a jasmonate or prostaglandin pathway in the two representative cyanobacteria analysed. Jasmonates and prostaglandins are sporadically found in some algae and fungi but their occurrence in only very few species suggests that these oxylipins have no general or essential function in algae or fungi. In animals, the "classical" prostaglandins $PGF_{2\alpha}$, PGE_2, PGD_2, PGI_2 and TXA_2 are rapidly released from the producer cells into the extracellular environment. Exit of prostaglandins from producer cells appears to be solely by passive diffusion along the electrochemical gradient (Kanai *et al.*, 1995). Since isoprostanes have the same physico-chemical properties as prostaglandins, it is to be expected that free isoprostanes are also rapidly released from cells. Typically, prostaglandins act as auto- and paracrine messengers i.e. the signal is limited to the producer and other cells in the local area. Extracellular prostaglandins can be recognized by specific G-protein coupled receptors located in the cell membrane of target cells (Wright *et al.*, 2001). Activation of prostaglandin receptors triggers in most target cells an increase or decrease of cAMP, an intracellular messenger found in bacteria including cyanobacteria, fungi and animals. The prostaglandin signal is short lived since prostaglandins are rapidly taken up by cells in the local area and degraded. Uptake of

prostaglandins is an active transport mechanism (Schuster, 1998). The prostaglandin transport proteins have a low substrate selectivity and transport not only prostaglandins but also isoprostanes (Itoh *et al.*, 1996). Thus far, genes homologous to prostaglandin biosynthesis genes, prostaglandin transporters and prostaglandin receptors have not been identified in the sequenced genomes of bacteria (including cyanobacteria) and higher plants. Therefore it appears that not only the substrate but also the complete signal transduction machinery of the mammalian prostaglandin pathway is missing in bacteria and plants.

In addition to the classical prostaglandins, cyclopentenone prostaglandins and isoprostanes have been found in animals. Cyclopentenone prostaglandins such as PGA_2, PGB_2, PGJ_2 and deoxy-PGJ_2 are derived from PGE_2 and PGD_2 by non-enzymatic dehydration and isomerization (Straus and Glass, 2001). An analogous series of cyclopentenone isoprostanes has also been found in animals (Chen *et al.*, 1999). Cyclopentenone prostanoids are thought to act as intracellular messengers. One common target of these compounds are transcription factors of the PPAR (peroxisome proliferator-activated receptor) family that are activated by reversible binding of the PPARs. Other targets include transcription factors (such as NFκB and others) and regulatory enzymes (such as thioredoxin reductase, IκB kinase and others) that display exposed thiols on their surface (Straus and Glass, 2001). In this case, cyclopentenone compounds may bind and irreversibly inactivate the target proteins (Levonen *et al.*, 2004). Cyclopentenone prostaglandins and isoprostanes are thought to antagonize the pro-inflammatory action of prostaglandins during resolution of inflammation (Willoughby *et al.*, 2000) and to protect cells from the detrimental activities of endogenous stress mediators (Kawamoto *et al.*, 2000). Previously, it has been shown that phytoprostanes (contained in the plant diet) also occur in humans (Karg *et al.*, 2007). Since animals and humans do not accumulate α-linolenate, phytoprostanes can not be formed endogenously in animals. However, cyclopentenone phytoprostanes have been shown to display potent anti-inflammatory and anti-apoptotic activities in the same concentration range as their prostaglandin counterparts in a variety of standard in vitro bioassays (Karg *et al.*, 2007). Moreover, E_1-phytoprostanes display PGE_2-like activities on human dendritic immune cells and these effects are most likely mediated by cognate PGE receptors (Gutermuth *et al.*, 2007; Mariani *et al.*, 2007; Traidl-Hoffmann *et al.*, 2005). Although phytoprostanes are xenobiotics in humans, these compounds display powerful biological activities. The traditional view is that isoprostanoids interfere with the prostaglandin signaling pathway because they are structurally highly similar to prostaglandins (Roberts and Morrow, 1997).

A related scenario apparently exists in plants. The best studied oxylipins in plants are 12-oxo-phytodienoic acid (OPDA) and its metabolite jasmonic acid (JA). One of the first biological activities displayed by these compounds collectively termed jasmonates are inhibition of root and seedling growth, induction of senescence and induction of biosynthesis of secondary metabolites (Mueller *et al.*, 1993; Wasternack *et al.*, 1998). Interestingly, these effects are also displayed by a variety of structurally different oxylipins including phytoprostanes (Birkett *et al.*, 2000; Iqbal *et al.*, 2005; Loeffler *et al.*, 2005; Thoma *et al.*, 2003; Vellosillo *et al.*, 2007). Notably, several types of phytoprostanes have been shown to induce secondary metabolites with similar potency in a variety of plant families (Iqbal *et al.*, 2005; Loeffler *et al.*, 2005; Loeffler *et al.*, 2003; Thoma *et al.*, 2003).

However, one of the oxylipins, JA, has been shown to be essential for fertility in *Arabidopsis* and can not be substituted by OPDA and other oxylipins (Stintzi and Browse, 2000). Most biological activities of JA involve the signaling component *COI1* (CORONATINE INSENSITIVE 1). *COI1* encodes an F-box motif (Xie *et al.*, 1998) and forms an integral part of an SCF-type E3 ubiquitin ligase that is predicted to target repressors of JA signaling to the proteasome for degradation (Devoto *et al.*, 2002). That has similarity to F-box proteins involved in targeting proteins for removal by ubiquitination. Recently it has been shown that about 84% of the genes induced by JA and 53% of genes repressed by JA are *COI1* dependent (Devoto *et al.*, 2005). Genes involved in secondary metabolism are induced in *Arabidopsis* in a *COI1* dependent fashion (Devoto *et al.*, 2005), suggesting that phytoprostanes signals at least in part through *COI1*. However, a receptor for JA or phytoprostanes has not yet been identified and, therefore, it remains to be clarified weather or not JA and phytoprostanes share the same receptor.

While most OPDA and JA effects are mediated through *COI1*, *COI1* independent gene induction by OPDA has been well characterized. Most of these genes are not activated by JA (Stintzi *et al.*, 2001). Interestingly, gene expression induced by phytoprostanes resembles that of OPDA but not of JA. A significant proportion (about 40%) of the *COI1*-independent genes that are induced by phytoprostanes are also induced by OPDA (Stefan Mueller and Susanne Berger, personal communication). Remarkably, the expression of many OPDA and phytoprostane inducible genes is also regulated by oxidative stress (Taki *et al.*, 2005). Recent microarray analysis of phytoprostane induced gene expression revealed that – similar to OPDA – a large group of genes involved in detoxification and xenobiotic metabolism is regulated by phytoprostanes (Loeffler *et al.*, 2005). Notably, cyclopentenone prostanoids also induce detoxification mechanisms in mammalian cells (Kawamoto *et al.*, 2000). Moreover,

pre-incubation of tobacco cell cultures with phytoprostanes protected the cells from subsequent oxidative injury and cell death suggesting that the phytoprostane triggered protein expression increased the cells resistance towards oxidative stress (Loeffler *et al.*, 2005).

In this work, we speculated that protection from oxidative stress could be an evolutionary old function of phytoprostanes. Results from Fig. III-10 and Table III-4 show that all phytoprostanes protect *Anabaena* cells from copper sulphate and hydrogen peroxide stress except for PPE_1. The phytoprostane pre-incubation effect is much more dramatic as that has been observed in tobacco cell cultures previously. Without phytoprostane pre-treatment, more than 90% (in most experiments more than 97%) of all cells turned yellow and died within 5 h after treatment with hydrogen peroxide or copper sulphate. Pre-incubation with phytoprostanes protected up to 77-84% of the cells from cell death. Interestingly, the efficiency by which pre-incubation with different oxylipins protected against cell death triggered by hydrogen peroxide and copper ions were strikingly similar. The most effective compounds are the cyclopentenone phytoprostanes and in particular a mixture of type I and type II PPA_1. However, non-cyclopentenone phytoprostanes such as PPF_1 displayed also a protective effect of about 36 to 48% while PPE_1 was almost inactive. The only difference between the two phytoprostane classes is a change from a hydroxy group to a keto group in the prostane ring (Fig. IV-1).

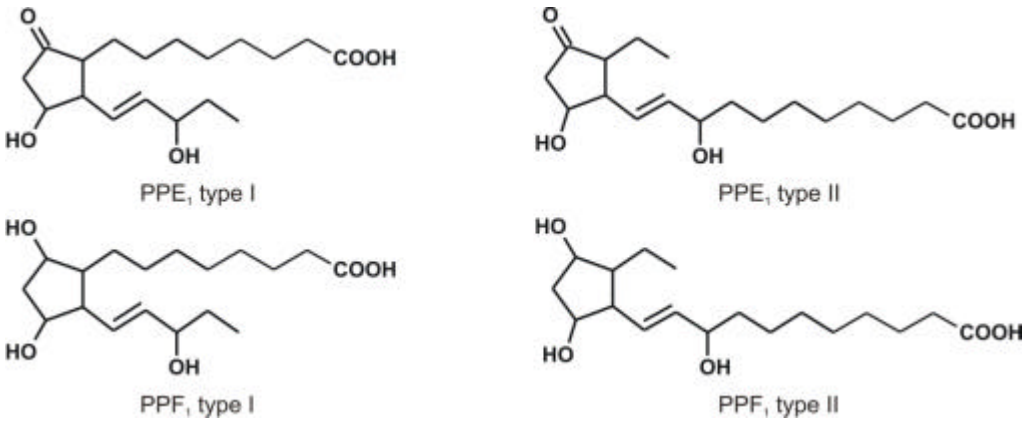


Figure IV-1. Structures of PPE_1 and PPF_1 in *Anabaena*. The small change in the structure has a dramatic effect on the biological activity.

Interestingly, when oxylipins were tested that occur not endogenously in cyanobacteria, we found that most of these oxylipins also had the capacity to protect *Anabaena* cells from cell death albeit with much lower potency. Although, for instance, PPA_1, PGA_1 and OPDA have strikingly similar structures (Fig. IV-2), biological activities differ dramatically. Cell death protection against 50 µM copper sulphate reached 84% after

incubation with PPA_1 while protection provided by PGA_1 (11%) and OPDA (15%) was much lower and was more similar to a synthetic cyclopentenone without lipophilic side chains (8%, Table III-4). Hence, biological activity of different oxylipins depends on the structure.

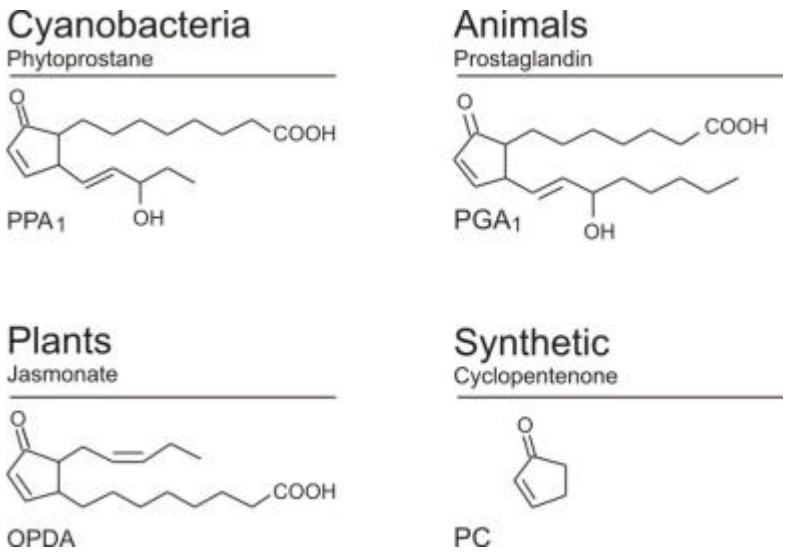


Figure IV-2. Oxylipins structures. Chemically and enzymatically oxidized lipids from different organisms display high structural similarities. A_1-phytoprostanes endogenously formed in *Anabaena* are the most active oxylipins tested.

Interestingly, however, is the notion that most oxylipins, regardless their origin, are biologically active. Phytoprostanes occurring endogenously in *Anabaena* appear to be generally more active. Results suggest that protection from oxidative stress is not limited to phytoprostanes but might be a general biological activity of an array of oxidised lipids. Since at least some oxidised lipids appear to protect also plant (Loeffler *et al.*, 2005) and animal cells from oxidative stress (Levonen *et al.*, 2004) it might be speculated that this is an evolutionary ancient and conserved function of oxidised lipids.

To this end, we investigated wether pre-incubation with oxidised lipids could also protect other bacteria from lethal hydrogen peroxide stress. However, pre-incubation of *Pseudomonas syringae* or *Eschscherichia coli* with oxidised lipids did not have any effect. Apparently, both bacteria can not sense or interpret oxidised lipids as signals of oxidative membrane damage. Since bacteria other than cyanobacteria do not utilize polyunsaturated fatty acids, membrane oxidation and lipid peroxidation does not occur. Therefore, recognition mechanisms for oxidized lipids have never evolved in these bacteria.

It should be noted that the concentrations used for the pre-incubations were rather high in this work (100 µM) as well as in previously published work with tobacco cell cultures (75 µM). Typically, exogenous treatments of mammalian or plant cells with prostaglandins or

jasmonates are performed with much higher concentrations (10 to 100 µM) than those which are expected to occur endogenously in cells even after elicitation. Basal, endogenous levels of prostaglandins, jasmonates and phytoprostanes are estimated to be in the pico- to nanomolar range. However, it is often necessary to use much higher concentrations for exogenous application experiments because oxylipins typically enter the cells slowly by passive diffusion and are rapidly metabolized. Therefore, rather high exogenous oxylipin concentrations are necessary to build up a biologically active pool of oxylipins within the cells. *In vivo*, membrane oxidation leads not only to the formation of phytoprostanes but also (simultaneously) to an array of oxidised lipids. Within the mixture of oxidised lipids, a single class of phytoprostanes i.e. the PPF_1 represents only a small fraction, likely less than 2%, of all oxidized lipids. Results from this work suggest that a large fraction of all oxidized lipids may have a similar effect as phytorostanes. Therefore, each single oxylipin species may contribute to the same overall effect.

In addition to the phytoprostanes that are produced endogenously from *Anabaena* itself, *Anabaena* might also be exposed to phytoprostanes from external sources. For instance, one natural habitat of *Anabaena* are rice paddy fields. These fields may contain much dead plant material which may release significant amounts of oxidized lipids including phytoprostanes into the water. At low concentrations, phytoprostanes are water soluble and are readily extracted from oxidized plant material. For example, an aqueous suspension of hay in water (1 g/ml) was shown to contain phytoprostane concentrations of about 16 µM PPF_1 and 17 µM PPE_1 (Fig. III-12). Normally after rice harvest, most of un-economical parts of rice are left on the field. This remaining material on the field may become a main source of phytoprostanes. However, the significance of external phytoprostanes from dead plant materials remains to be clarified.

IV.5 Biological function of exogenous oxylipins is independent on some known defense mechanisms in cyanobateria

After the observation of dramatic effects of phytoprostanes in protection *Anabaena* cells from lethal oxidative stress, the mechanism of this action was investigated. In filamentous cyanobacteria, shinorine, a well-known secondary metabolite, is induced strongly by UV light (Sinha *et al.*, 1999; 2001). The induction of many secondary metabolites (phytoalexins) in plants by phytoprostanes has also been reported (Thoma *et al.*, 2003; Loeffler *et al.*, 2005). In this research, both jasmonates and phytoprostanes did not induce

shinorine production in *Anabaena* under normal light. UV light alone or in combination with exogenous oxidized lipids enhanced shinorine formation but there was no significant difference between them. Therefore, it can be concluded that oxylipins do not induce shinorine biosynthesis in *Anabaena*. It seems that shinorine formation is specifically induced by UV light.

By the same experimental procedure, UV light did not induce tocopherol production in *Anabaena* even though the exposure time was extended up to 72 h (Fig. III-15). Tocopherols are lipid-soluble antioxidants that protect *Synechocystis* from lipid peroxidation (Maeda *et al.*, 2005). Tocopherol-deficient *Arabidopsis thaliana* showed growth defects and a massive production of PPF_1 early in seedling development (Sattler *et al.*, 2006). However, in this work, exogenous application of 50 µM oxylipins showed no effect on tocopherols levels in *Anabaena*.

In some cyanobacterial species, particular *Synechocystis*, the expression of *isiA* (iron stress induced protein A) gene is clearly seen when they are living in iron free medium (Singh *et al.*, 2005) or under oxidative stress (Havaux *et al.*, 2005). However, *isiA* expression was not observed in *Anabaena* after 90 min exposure to 1.5 mM hydrogen peroxide or 100 µM PPF_1.

IV.6 Down-regulation of proteins in the *Anabaena* proteome by PPF_1 indicates a possible adaptation mechanism to oxidative stress

In order to clarify the mechanism of action and putative functions of PPF_1, it would be highly interesting to study the global impact of phytoprostanes on gene and protein expression. Since the genome of *Anabaena* is known both types of studies are now principally feasible. Unfortunately, microarrays are not available for *Anabaena* yet and therefore it was decided to study protein expression on the proteome level. The results from III.12 show that most proteins in the *Anabaena* proteome were down-regulated 6 h after PPF_1 application. Interestingly, most of modulated proteins are down-regulated and relate to photosynthesis and glycolysis such as carbon dioxide binding protein, bicarbonate binding protein, bicarbonate transport, Rubisco, several phycobilisome proteins, fructose-1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase.

We have observed that *Anabaena* is remarkably resistant towards oxidative stress under low light conditions. Membrane oxidation and phytoprostane formation occurred only under high light conditions. Under high light conditions, the photosynthetic apparatus and pigments produce more superoxide and singlet oxygen that in turn may damage membranes

directly or indirectly. Therefore, PPF_1 may decrease *Anabaena* photosynthetic activity and thereby reduce the endogenous oxidative stress which might help *Anabaena* to survive otherwise lethal, exogenous oxidative stress. Hence, this process might help to maintain the stability of the membrane, especially in the presence of exogenous oxidative stress. The impact of PPF_1 on the *Anabaena* proteome may provide a reasonable explanation for the high ratio of survival of oxylipin primed cells under lethal oxidative stress. In 33 regulated spots, only 5 were up-regulated by PPF_1. However, not all modulated proteins were recorded because only those spots that reproducibly differed more than three times between treatments were considered as regulated proteins. Regulated proteins were also missed when they were not sufficiently resolved from other proteins or when proteins in both treatments were present at high level and saturated stained with silver nitrate. In these cases, the computer software cannot distinguish differences.

Although many photosynthetic proteins were observed to be down-regulated, the main pigments for photosynthesis, chlorophyll a and phycobilins, were not affected by PPF_1 as judged by the UV-absorption spectrum (data not shown). Perhaps, the incubation time of 6 h was not long enough to have an impact on these pigments. Similarly, Liu *et al.* (2005) reported that copper ions have no effect on phycobilisomes but silver ions and hydrogen peroxide can induce a disassembly of the core of phycobilisomes in *Synechocystis chlN* mutant.

In a recent study, Bhargava *et al.* (2006) reported that the proteome of *Anabaena doliolum* is modulated by copper both in short and long term. Also in these experiments, the number of down-regulated proteins was always higher than the up-regulated ones. Therefore, down-regulation of protein synthesis appears to be the survival strategy of *Anabaena* when being exposed to oxidative stress. In comparison with other phytoprostanes, PPA_1 appears to be the best compound for protecting *Anabaena* from oxidative stress. Therefore, it can be expected that PPA_1 might have a stronger modulation of *Anabaena* proteome than PPF_1.

IV.7 Protection from oxidative stress is a putative evolutionary ancient function of oxidized lipids (phytoprostanes)

It has been assumed that phytoprostanes interfere – by accident – with the OPDA and JA signaling machinery because phytoprostanes – by accident – structurally resemble jasmonates. Similarily, it has been claimed that isoprostanes interfere – by accident – with the prostaglandin signal transduction pathway because isoprostanes – accidentially - structurally

resemble prostaglandins. According to these lines of argumentation, it is surprising that phytoprostanes protect *Anabaena* cells from oxidative stress as it has been observed in plants and animals since in *Anabaena* a prostaglandin or jasmonate signaling system has not evolved. Notably, oxidized lipids including phytoprostanes and/or isoprostanes were prevalent early in evolution and present in all aerobic organisms utilizing polyunsaturated fatty acids in their membranes. Signaling of oxidized lipids in the absence of the enzymatic pathways in *Anabaena* indicates that oxidized lipids might be signals in their own right. It also implicates that *Anabaena* has evolved recognition mechanism(s) for oxidized lipids that recognize a broad spectrum of oxylipins. Interestingly, jasmonates and prostaglandins are also recognized by the ancient signaling system although these mediators evolved only in higher and highly differentiated organisms.

Since the first discovery of jasmonates by Vick and Zimmerman, the high structural similarity of jasmonates and prostaglandins has been noted (Vick *et al.*, 1980). However, enzymes involved in oxylipin biosynthesis (lipoxygenases, cyclooxygenases), recognition (receptors) and signaling mechanisms (cAMP, COI1) are completely different albeit these oxylipins are essential in development, reproduction and defense in both organisms. Therefore, prostaglandins and jasmonates biosynthesis pathways are independently evolved and are products of a convergent evolution. In the light of the findings in cyanobacteria, it can be speculated that the first receptors or recognition mechanisms for these oxylipins were already in place when the evolution of the enzymatic oxylipin pathways started out. Evolution of enzymatic biosynthesis of oxylipins that initially targeted the oxidized lipid signaling system conferring oxidative stress resistance might have been a dramatic advantage. The "ancient" system of generating signaling molecules (oxidized lipids) through severe oxidative membrane damage is not only inefficient but also detrimental to the producer cells. Although formation of oxidized lipids through membrane oxidation by reactive oxygen species is a highly reliable and robust process it can not be tuned and regulated for special needs. The enzymatic oxylipin biosynthesis pathway starts out with the release of a polyunsaturated fatty acid that is subsequently enzymatically oxidized by enzymes without release of reactive oxygen species or free radicals. The enzymatic pathway is therefore a regulated and "safe" pathway to produce oxylipins.

Moreover, chemical oxidation of lipids is linked to oxidative stress and therefore the mechanism can likely not be modified for other signaling purposes and needs. In animals and plants, enzymatically formed oxylipins have acquired new and specific functions that are apparently not shared by other oxidized lipids such as essential function in organ development

and reproduction. Therefore it can be speculated that also novel receptors/recognition systems and functions have evolved that are more specific for the enzymatic oxylipins. Hence, recognition of isoprostanes and phytoprostanes by prostaglandin and (putative) jasmonate receptors might not occur by chance but may rather reflects the evolutionary history of the oxylipin signaling systems (Fig. IV-3).

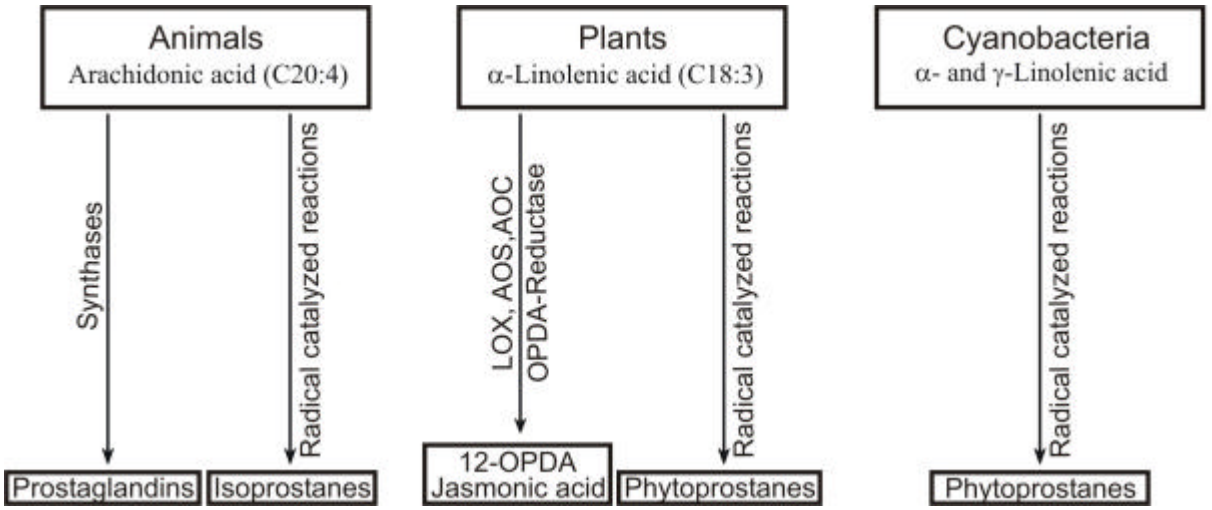


Figure IV-3. Summary of oxylipin pathways in different life kingdoms. Cyanobacteria have only the non-enzymatic isoprostanoid. In higher organisms (plants and animals) more efficient enzymatic pathways have evolved in addition to the ancient isoprostanoid pathway. LOX: lipoxygenase; AOS: allene oxide synthase; AOC: allene oxide cyclase; 12-OPDA: 12-oxo-phytodienoic acid.

V. Summary

Oxylipins are important biological active compounds that play essential roles in defense, growth, development, and reproduction of plants and animals. Oxylipins are formed either by enzymatic pathways or radical catalyzed reaction from polyunsaturated fatty acids. Products of oxidation of arachidonic acid (C20:4) in animals by enzymatic and non-enzymatic pathways are prostaglandins and isoprostanes, respectively. In plants, radical catalyzed reaction of α-linolenic acid (C18:3) forms phytoprostanes and enzymatic oxidation of this fatty acid produces OPDA and jasmonic acid. Like plants, cyanobacterial membranes contain a high ratio of polyunsaturated fatty acid, about 25% of total fatty acids. Oxylipin biosynthesis and function was studied in two model cyanobacteria, *Anabaena* PCC 7120 and *Synechocystis* PCC 6803, for the first time:

1. The filamentous cyanobaterium *Anabaena* PCC 7120 can naturally produce phytoprostanes type I and II as well as hydroxy fatty acids like in plants but lacks the enzymatic capacity to form jasmonates (12-oxo-phytodienoic acid and jasmonic acid) and prostaglandins. Data obtained provide the first evidence for the occurence of phytoprostanes in cyanobacteria as well as in the baterial kingdom.

2. By GC-MS analysis, the E_1- and F_1-phytoprostanes in *Anabaena* PCC 7120 were detected both in free and esterified form. Their levels are comparable with those in plants, in the range of ng/g DW. In one week old cultures, there was no evidence of PPF_1 in the medium but its level accumulated up to 142 ng/l in six weeks old cultures. In contrast, PPE_1 was stable over time, about 20 ng/g DW. Free cellular PPE_1 was found about 4 times higher than that of PPF_1, 80.5 ± 23.6 and 24.1 ± 10.9 ng/g DW, respectively. However, there was no significant difference in the total cellular levels of PPF_1 and PPE_1, ranging from 150 to about 200 ng/g DW.

3. Phytoprostanes are inducible in *Anabaena*. In the combination of oxidative stress (200 µM H_2O_2 or 10 µM $CuSO_4$) with high light intensity (330 $\mu E.m^{-2}.s^{-1}$) for 8 h, levels of total cellular PPE_1 and PPF_1 were increased about 2 to 4 times. Interestingly, unlike in higher plants, application of oxidative stress or high light intensity alone showed no phytoprostaneous induction in this cyanobacterium.

4. When *Anabaena* cells were treated with phytoprostanes, Anabaena cells became remarkably resistant against subsequently applied – otherwise lethal – oxidative stress. All phytoprostanes displayed a high protective effect except for PPE_1. The highest protection level was contributed by a mixture of PPA_1 type I and II. After preincubation of *Anabena* cells with 100 µM PPA_1–type I/II for 16 h followed by application of 1 mM H_2O_2 or 50 µM $CuSO_4$ for 5 h, A_1-phytoprostane pre-treatment protected 84.2% and 77.5% of the cells from cell death, respectively. Without oxylipins pre-treatment, about 98% of the cells were dead. Surprisingly, preincubation of *Anabaena* with other oxylipins derived from enzymatic pathway in plants and animals showed also an effect, however, the protection effect was low and ranged from 10 to 30%. In contrast, phytoprostanes did not protect *Pseudomonas syringae* and *Escherichia coli* from the toxicity of hydrogen peroxide. However, these bacteria do not synthesize polyunsaturated fatty acids and are therefore devoid of and not exposed to endogenously formed oxidized lipids.

5. Exogenous application of 100 µM PPF_1 or 1.5 mM H_2O_2 for 90 min did not activate the expression of isiA in *Anabaena*. Oxylipins also displayed no effect on shinorine and tocopherol levels in *Anabaena*. However, application of 100 µM PPF_1 for 6 h altered the protein expression in *Anabaena*. Most PPF_1-modulated proteins are down-regulated and related to photosynthesis. Since oxidative stress only in combination with high light intensity increased lipid peroxidation, down-regulation of photosynthesis after recognition of oxidised lipids (phytoprostanes) may be a survival strategy of *Anabaena* to avoid damage by peroxidized lipids.

6. Dead plants may be the main source of (exogenous) phytoprostanes in the natural environment of *Anabaena*. Dry hay releases PPE_1 and PPF_1 (11 µg/g DW) into an aqueous environment. *Anabaena* is the typical cyanobacterium in paddy rice fields. After harvesting, most of uneconomical parts of rice plants are abundant on the field, which may release phytoprostanes that in turn might have an impact on cyanobacteria in the rice ecosystems. However, field research is needed to clarify this suspection.

7. A new class of oxylipins, phytoprostanes type III and IV, was identified and quantified *in vitro*. The two main phytoprostanes, PPE_1 and PPF_1 (type III and IV), can be obtained by autoxidation of γ-linolenic acid or Borage oil (containing 25% esterified γ-linolenic acid). After 12 days of autoxidation and subsequent hydrolysis, 1 g of Borage oil yielded 112.71 ± 1.93 µg of PPF_1 and 3.80 ± 0.14 mg of PPE_1. PPB_1 and PPA_1 (type III and IV) were prepared by isomerization and dehydration of PPE_1 (type III and IV). The overall yield of PPB_1 was 1.71 ± 0.04 mg/g oil (type III) and 2.09 ± 0.12 mg/g oil (type IV). Those of PPA_1 were 8.38 ± 0.35 µg/g and 10.18 ± 0.30 µg/oil, respectively.

8. A rapid HPLC-MS/MS method for phytoprostane and phytohormone analysis has been developed. This method was applied to quantify free and esterified E_1- and F_1-phytoprostanes type III and IV in *Synechocystis* PCC 6803. The *in vivo* phytoprostanes type III and IV are present both in free and esterified form. The total cellular level of PPE_1 type III and IV in *Synechocystis* is at least 2 times higher than that of PPF_1. Unlike *Anabaena*, PPE_1 and PPF_1 were detectable in the medium of one week old *Synechocystis* cultures. Free levels of PPF_1 in the medium (231.8 ± 36.2 ng/l) and in the cells (164.9 ± 15.2 ng/g DW) are lower than those of PPE_1 (1003.3 ± 365.2 ng/l and 2331.0 ± 87.7 ng/g DW).

VI. Zusammenfassung

Oxylipine sind wichtige biologisch aktive Verbindungen, die entscheidende Rollen in der Abwehr, dem Wachstum, der Entwicklung und der Reproduktion von Pflanzen und Tieren spielen. Oxylipine können entweder über enzymatische Wege oder eine Radikal-katalysierte Reaktion gebildet werden. Enzymatische und nicht-enzymatische Oxidationsprodukte der Arachidonsäure (C20:4) in Tieren sind Prostaglandine und Isoprostane. In Pflanzen werden ausgehend von der α-Linolensäure (C18:3) über einen enzymatischen Weg OPDA und Jasmonsäure und durch Radikal-katalysierte Reaktion Phytoprostane gebildet. Die Membranen von Cyanobakterien enthalten, ähnlich denen von Pflanzen, einen großen Anteil an mehrfach ungesättigten Fettsäuren, ca. 25% der gesamten Fettsäuren. Biosynthese und Funktionen der Oxylipine wurden an zwei Modell-Cyanobakterien, *Anabaena* PCC 7120 und *Synechocystis* PCC 6803 untersucht:

1. Das fadenförmige Cyanobakterium *Anabaena* PCC 7120 kann Phytoprostane Typ I und II sowie Hydroxyfettsäuren ähnlich wie Pflanzen produzieren aber die enzymatische Ausstattung zur Bildung von Jasmonaten (12-oxo-Phytodiensäure und Jasmonsäure) und Prostaglandinen ist nicht vorhanden. Die erhaltenen Daten stellen den ersten Nachweis für das Vorkommen von Phytoprostanen in Cyanobakterien bzw. bei Prokaryonten dar.

2. Durch GC-MS Analyse wurden E_1- and F_1-Phytoprostane in *Anabaena* PCC 7120 in freier und veresterter Form detektiert. Die Spiegel sind vergleichbar mit denen in Pflanzen und lagen im Bereich von ng/g TG. PPF_1 ließen sich nicht in einwöchigen Kulturen nachweisen, die Spiegel in sechswöchigen Kulturen lagen bei 142 ng/l. Die Spiegel von PPE_1 waren hingegen in ein- und sechswöchigen Kulturen ähnlich und lagen bei ca. 20 ng/g TG. Die Mengen an freien PPE_1 in den Zellen waren mit 80.5 ± 23.6 etwa viermal höher als die von PPF_1 mit 24.1 ± 10.9 ng/g TG. Allerdings gab es keine signifikanten Unterschiede in den Spiegeln an gesamten PPF_1 und PPE_1 in den Zellen, sie lagen im Bereich von 150 bis zu ca. 200 ng/g TG.

3. Die Akkumulation von Phytoprostanen in *Anabaena* ist induzierbar. Nach der Kombination von oxidativem Stress (200 µM H_2O_2 oder 10 µM $CuSO_4$) und hoher Lichtintensität (330 $\mu E.m^{-2}.s^{-1}$) für 8 h stiegen die Spiegel an gesamten PPE_1 und PPF_1 um den Faktor 2 bis 4 an. Interessanterweise führte im Gegensatz zu höheren Pflanzen die Applikation von oxidativem Stress oder hoher Lichtintensität alleine nicht zur Induktion der Phytoprostanakkumulation in diesen Cyanobakterien.

4. Eine Vorbehandlung von *Anabaena* Zellen mit exogenen Phytoprostanen führte zu einer erhöhten Toleranz gegenüber oxidativem Stress. Alle Phytoprostane außer PPE_1 zeigten einen Schutzeffekt. Eine Mischung von PPA_1 Typ I und II ergab den höchsten Schutzeffekt. Eine Vorinkubation von *Anabena* Zellen mit 100 µM PPA_1–type I/II für 16 h schützte 84.2% beziehungsweise 77.5% der Zellen vor einer anschießenden lethalen Applikation von 1 mM H_2O_2 beziehungsweise 50 µM $CuSO_4$ für 5 h. Ohne eine Oxylipin-Vorinkubation starben etwa 98% der Zellen. Überraschenderweise ergab auch die Vorbehandlung mit anderen, enzymatisch gebildeten Oxylipinen aus Tieren und Pflanzen einen Schutzeffekt, der allerdings nur 10 bis 30% betrug. Dagegen schützte eine Phytoprostan-Vorbehandlung nicht *Pseudomonas syringae* und *Escherichia coli* gegen toxische Mengen von Wasserstoffperoxid. Allerdings fehlen in den Membranen dieser Bakterien mehrfach ungesättigte Fettsäuren und deshalb endogen oxydierte Lipide.

5. Eine exogene Applikation von 100 µM PPF_1 oder 1,5 mM H_2O_2 führte in *Anabaena* nicht zu einer Induktion der Expression des *isiA* Gens. Oxylipin-Behandlungen zeigten auch keine Wirkung auf Shinorin- und Tocopherol-Spiegel in *Anabaena*. Die Applikation von 100 µM PPF_1 für 6 h führte aber zu Änderungen im Proteinmuster in *Anabaena*. Der größte Teil der differentiellen Proteine wurde durch PPF_1 herunterreguliert. Bei vielen dieser Proteine handelt es sich um photosynthetische Proteine. Da ein oxidativer Stress nur in der Kombination mit hoher Lichtintensität die Lipidperoxidation erhöht, könnte die negative Regulation der Photosynthese nach Erkennung von oxydierten Lipiden (Phytoprostanen) eine Überlebens-Strategie sein um Schäden durch peroxidierte Lipide zu vermeiden.

6. Tote Pflanzen könnten eine hauptsächliche Quelle der exogenen Phytoprostane in der natürlichen Umgebung von *Anabaena* sein. Trockenes Heu gibt PPE_1 und PPF_1 (11 µg/g TG) an die wässerige Umgebung ab. *Anabaena* ist ein typisches Cyanobakterium in Reisfeldern. Nach der Ernte bleiben meist die nicht genutzten Teile der Reispflanzen auf dem Feld. Diese könnten Phytoprostane abgeben, die wiederum einen Einfluß auf die Cyanobakterien im Reis-Ökosystem haben könnten.

7. Eine neue Kategorie von Oxylipinen, die Phytoprostane Typ III und IV, wurden *in vitro* identifiziert und quantifiziert. Die beiden Haupt-Phytoprostane, PPE_1 und PPF_1 (Typ III und IV), können durch die Autoxidation der γ-Linolensäure oder des Borretschsamenöls (enthält 25% der γ-Linolensäure) gewonnen werden. Nach 12 Tagen Autoxidation und anschließender Hydrolyse wurden aus 1 g Borretschsamenöl 112,71 ± 1,93 µg PPF_1 und 3,80 ± 0,14 mg PPE_1 isoliert. PPB_1 und PPA_1 (Typ III und IV) wurden durch Isomerisierung und Dehydratisierung von PPE_1 hergestellt. Die Ausbeute von PPB_1 lag bei 1,71 ± 0,04 mg/g Öl (Typ III) und 2,09 ± 0,12 mg/g Öl (Typ IV), die von PPA_1 lag bei 8,38 ± 0,35 µg/g und 10,18 ± 0,30 µg/g Öl.

8. Es wurde eine schnelle HPLC-MS/MS Methode für die Analytik der Phytoprostane und Phytohormone entwickelt. Diese Methode wurde für die Quantifizierung von freien und veresterten E_1- and F_1-Phytoprostane Typ III und IV in *Synechocystis* PCC 6803 angewendet. Die Phytoprostane Typ III und IV sind *in vivo* in freier und veresterter Form vorhanden. Die Spiegel der gesamten PPE_1 Typ III und IV in *Synechocystis* sind mindestens doppelt so hoch wie die von PPF_1. Im Gegensatz zu *Anabaena*, waren PPE_1 und PPF_1 in ein- und sechswöchigen Kulturen von *Synechocystis* detektierbar. Die Spiegel an freien PPF_1 im Medium (231,8 ± 36,2 ng/l) und in den Zellen (164,9 ± 15,2 ng/g TG) waren niedriger als die von PPE_1 (1003,3 ± 365,2 ng/l und 2331,0 ± 87,7 ng/g TG).

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2000 – 2002	Master of Science in Management of Natural Resources and Sustainable Agriculture, Noragric Center, The Agricultural University of Norway (now is Norwegian University of Life Sciences)
04 - 09/2004	German language training at Mannheim – Heidelberg Geothe Institute, DSH examination (pass).
from 10/2004	PhD student at department of Pharmaceutical Biology, Würzburg University

Working experience

1998 –2000	research assistant at Plant Physiology Department, College of Agriculture, Cantho University
2002 – 2004	research and teaching assistant at Crop Science Department, College of Agriculture, Cantho University

EHRENWÖRTLICHE VERSICHERUNG

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Diese Arbeit hat weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen.

Ich habe bisher noch keinen akademischen Grad erworben oder zu erwerben versucht.

Würzburg, den

Pham Phuoc Nhan