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MASTERARBEIT

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„Synthesis and validation of novel chemical probes for the investigation of nonribosomal peptide biosynthesis“

verfasst von

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Contents

Abbreviations

$[\alpha]_{589}^{25}$	Specific rotation at 25 °C and a wavelength of 589 nm
Å	**A**ngström
Ac	**Ac**etate
ACP	**A**cyl **c**arrier **p**rotein
AMP	**A**denosine **m**ono**p**hosphate
aq.	**Aq**ueous solutionn
ATP	**A**denosine **t**ri**p**hosphate
Boc	*tert*-**B**utyl**o**xy**c**arbonyl (protecting group)
br s	**Br**oad **s**inglet
Bu	**Bu**tyl
cat.	**Cat**alyst or **cat**alytic amount
Cbz	**C**arboxy**b**en**z**yl (protecting group)
CoA	**Co**enzyme **A**
d	**D**uplet or **d**euterated
DCM	**Dic**hloro**m**ethane
DMF	**Dim**ethyl**f**ormamide
DNA	**D**eoxyribo**n**ucleic **a**cid
EDC	1-**E**thyl-3-(3-**d**imethylaminopropyl)**c**arbodiimide
eq.	**Eq**uivalents
ESI	**E**lectro**s**pray **i**onisation
Et	**Et**hyl
exc.	**Exc**ess
GlcNAc	***N*-Ac**etyl**gl**u**c**osamine
HR	**H**igh **r**esolution
HWE	**H**orner-**W**adsworth-**E**mmons reaction
IBX	2-**I**odoxy**b**enzoic acid
J	Coupling constant
LC	**L**iquid **c**hromatography
m	**M**ultiplet
m/z	Mass divided by charge
M79	Name of the medium for the preculture
Me	**Me**thyl

MHz	**M**ega**h**ert**z**
MS	**M**ass **s**pectrometry
MurNAc	***N*-Ac**etyl**mur**amic acid
MW	**M**olecular **w**eight
MYM	Name of the medium for the production culture
n	Primary
NMR	**N**uclear **m**agnetic **r**esonance
NRP	**N**on**r**ibosomal **p**eptide
NRPS	**N**on**r**ibosomal **p**eptide **s**ynthetase
PCP	**P**eptidyl **c**arrier **p**rotein
PKS	**P**oly**k**etide **s**ynthase
PP_i	**P**yro**p**hosphate
ppm	**P**arts **p**er **m**illion
PPTase	**P**hospho**p**antetheinyl**t**ransfer**ase**
QXC	**Q**uino**x**aline-2-**c**arboxylic acid
r.t.	**R**oom **t**emperature
R_f	**R**etardation **f**actor
s	**S**inglet
SAM	**S**-**A**denosyl **m**ethionine
sat.	**Sat**urated
SCX	**S**trong **C**ation E**x**change
t	**T**ertiary
t	**T**riplet
TFA	**T**ri**f**luoro**a**cetic acid
THF	**T**etra**h**ydro**f**uran
TLC	**T**hin **l**ayer **c**hromatography
UPLC	**U**ltra High **P**erformance **L**iquid **C**hromatography
Z	**C**arboxy**b**en**z**yl (protecting group)
δ	Chemical shift

1 Abstract

One of the most important approaches for the development of new drugs is the chemical modification of natural products with known structures and effects. The most elegant way to produce modified natural products is probably the genetic manipulation and cultivation of suitable microorganisms. However, this requires a detailed understanding of the underlying biosynthetic mechanisms. The present work addresses the investigation of the mechanisms of nonribosomal peptide biosynthesis. Nonribosomal peptides (NRPs) are produced by multienzyme complexes, called NRPSs (nonribosomal peptide synthetases), in some fungi and bacteria, and some of them show unique biological activities of particular importance. These include, inter alia, antibiotic, cytostatic and immunosuppressive effects. The best known example is probably the β-lactam antibiotic penicillin.

The last decades brought an enormous progress in the understanding of NRPS assembly line operations and in special cases it was already possible to derive the structure of previously unknown NRPs from the corresponding genetic information. However, some mechanistic details of the biosynthesis of these peptides are often still completely unclear. This makes the development of new methods of investigation necessary. The mechanistic uncertainties are particularly based on the fact that the various intermediates of the NRP biosynthesis are covalently bound to the enzyme complexes throughout the whole assembly process. The aim of this work was to develop a strategy for the synthesis of amino acid derivatives that in feeding experiments are expected to interact with the NRPSs in a way to allow the *in vivo* off-loading of intermediates. After extraction of the bacteria, analysis of the extracts by UPLC-HR-ESI-MS should verify the presence of the expected intermediates. These and similar principles are already applied successfully by the Tosin group for investigations on polyketide synthases (PKSs).

This work presents the first successful synthesis, purification and characterization of various amino acid derivatives. Particular attention is paid to the development of the synthetic strategy which led to compound **14a**. To the best of my knowledge, this substance represents the first described aminoacyl carba(dethia)-*N*-decanoyl cysteamine. It is hoped that these amino acid derivatives are able to trap intermediates of the NRP biosynthesis in near future. However, the tested compounds appear to be relatively toxic for the investigated bacterial strain *S. lasaliensis* ACP12 (S970A). Thus, no intermediate, off-loaded from NRPSs by using these probes, could be detected so far. Further work is currently being made within the Tosin group.

2 Kurzfassung

Eine der bedeutendsten Ansätze für die Entwicklung neuer Wirkstoffe, ist die chemische Abwandlung von Naturstoffen mit bekannten Strukturen und Wirkungen. Der wohl eleganteste Weg modifizierte Naturstoffe zu produzieren, ist die genetische Manipulation und Kultivierung geeigneter Mikroorganismen. Dies setzt allerdings voraus, dass die entsprechenden Mechanismen der Biosynthese dieser Substanzen möglichst genau bekannt sind. Die vorliegende Arbeit befasst sich mit der Erforschung der Mechanismen der nichtribosomalen Peptidbiosynthese. Nichtribosomale Peptide (NRPs) werden von Multienzymkomplexen, genannt NRPSs (nonribosomal peptide synthetases), in einigen Pilzen und Bakterien produziert und besitzen z.T. einzigartige biologische Aktivitäten von besonderer Bedeutung. Zu diesen zählen u.a. antibiotische, zytostatische und immunsuppressive Wirkungen. Das prominenteste Beispiel stellt wohl die Gruppe der antibiotisch wirksamen Penicilline dar.

In den vergangenen Jahrzehnten wurde ein enormer Fortschritt im Verständnis der Arbeitsweise von NRPSs erreicht und mittlerweile konnten bereits die Strukturen einiger bis dahin unbekannter NRPs von der entsprechenden genetischen Information abgeleitet werden. Dennoch sind in vielen Fällen mechanistische Details der Biosynthese der schrittweise aufgebauten Peptide noch vollkommen unklar, was die Entwicklung neuer Untersuchungsmethoden notwendig macht. Die mechanistischen Unklarheiten sind besonders darin begründet, dass die verschiedenen Intermediate der Biosynthese über den gesamten Syntheseprozess kovalent an den Enzymkomplexen gebunden sind. Ziel dieser Arbeit war die Entwicklung einer Strategie für die Synthese von Aminosäurederivaten, die in Fütterungsexperimenten erwartungsgemäß mit den NRPSs wechselwirken und die Intermediate der nichtribosomalen Peptidbiosynthese *in vivo* abfangen. Nach Extraktion der Bakterien, sollte eine Analyse mittels UPLC-HR-ESI-MS die Anwesenheit der erwarteten Intermediate verifizieren. Diese und ähnliche Prinzipien werden von der Tosin Gruppe, für Untersuchungen an Polyketidsynthasen (PKSs) bereits erfolgreich angewandt.

Die vorliegende Arbeit präsentiert die erste erfolgreich Synthese, Reinigung und Charakterisierung verschiedener Aminosäurederivate. Dabei wird besonderes Augenmerk auf die Entwicklung der Synthesestrategie von Verbindung **14a** gelegt, da diese Verbindung nach bestem Wissen und Gewissen den ersten Vertreter der Aminoacyl carba(dethia)-*N*-decanoyl cysteamine darstellt. Von diesen Aminosäurederivaten wird erhofft, dass sie Intermediate der nichtribosomalen Peptidbiosynthese abzufangen vermögen. Allerdings scheinen die getesteten Verbindungen für den untersuchten Bakterienstamm *S. lasaliensis* ACP12 (S970A) relativ toxisch zu sein. Bislang konnte kein abgefangenes Intermediat detektiert werden. Weitere Untersuchungen sind gegenwärtig am Laufen.

3 Introduction

Nonribosomal peptides (NRPs) are a group of short peptides essentially consisting of a variety of different proteinogenic and non-proteinogenic amino acids. These kind of natural products are synthesized by various microorganisms as secondary metabolites and some of them play an important role in medicine as they are used to treat a wide range of different diseases. The biological activities of some NRPs have been known for quite a long time, even when their chemical structure and their biosynthetic origin were still unknown. Probable the most known example of nonribosomal peptide product is the β–lactam penicillin, which was discovered in the 1920s and revolutionized medicine due to its antibiotic activity. Some other antibiotics but also anticancer and immunosuppressant drugs belong to the nonribosomal peptideclass and are some of the most important drugs in use nowadays.[1]

Today we know that the biosynthesis of nonribosomal peptides takes place in various fungi and bacteria by a wide variety of different biochemical reactions catalysed by a group of large enzyme complexes called nonribosomal peptide synthetases (NRPSs). The conventional biosynthesis of peptides and proteins proceeds by direct translation of the nucleic acid sequence of mRNA to the amino acid sequence of the final peptide or protein and takes place on the ribosomes. In contrast to this ribosomal biosynthesis of peptides and proteins the utilisation of NRPS multienzyme complexes allows the incorporation of unusual amino acids beside the 20 standard proteinogenic amino acids. Furthermore, during the assembly of the peptide chain the amino acids can be modified by methylation, isomerisation, acetylation, glycosylation, hydroxylation, halogenation and many other kinds of transformations. Moreover the final peptides can ultimately have a branched structure or even be cyclized. A huge structural diversity is achieved in NRPs and this is responsible for the wide range of their biological activities that we can observe.

As further shown in section 4.2 an NRP is built up ‘step by step’ and every single amino acid is attached to the growing peptide chain by a so called ‘module’ consisting of at least three domains. The A domain (adenylation domain) is responsible for both the selection of the right amino acid and the ATP-dependent activation of its carboxylate group. Furthermore it catalyses the transfer of the activated amino acid to a cofactor of the T domain (thiolation domain) called 4’-phosphopantetheinyl cofactor. This cofactor is basically a long chain carrier that purposely delivers the amino acid to the different catalytic sites within an NRPS and eventually shuttles it to the T domain of the next module. Here the next amino acid waits for its connection with the peptide chain. This condensation is carried out by the third domain, the condensation (C) domain. Repeating these steps the entire NRP is built up. As well as these three basic domains, a module can also contain a

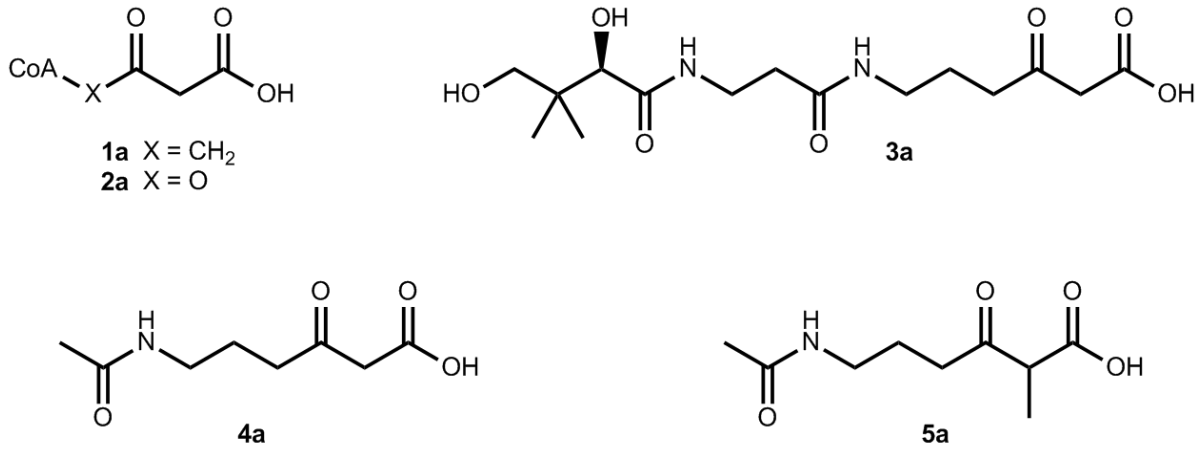


Figure 1: *Examples for different coenzyme A mimics and analogues used for investigations on the polyketide biosynthesis. The CoA derivatives* ***1a*** *and* ***2a*** *were used for* in vitro *studies on the type III PKS stilbene synthase,*[2] *the malonyl carba(dethia)-pantetheine* ***3a*** *and the malonyl carba(dethia)-cystamine derivatives* ***4-5a*** *were used for the investigation of modular polyketide synthases* in vitro *and* in vivo.[2, 3, 5]

variety of additional domains which modify the amino acids before or after its incorporation into the peptide. The last module usually contains a thioesterase domain (Te domain), which causes the cleavage of the peptide from the NRPS by hydrolysis or cyclisation. Although the last decades brought an enormous progress in understanding the mode of action of NRPSs, many important questions in this area remain unanswered and further work needs to be done to clarify important mechanistic details. One of the most challenging problems for the investigation of these details is the fact that the growing peptide chain remains covalently tethered to the NRPS during the entire assembly process. This makes it difficult to investigate the various intermediates of the biosynthesis and limits in some ways the elucidation of the corresponding mechanisms.

Recent work in the Tosin group led to the development of a new chemical method that enables the off-loading of biosynthetic intermediates from polyketide synthases (PKSs), a class of large enzyme complexes which work in a similar fashion to NRPSs to build up polyketide natural products. For PKSs the off-loading was achieved by the use of different nonhydrolysable acyl carrier protein (ACP) mimics. In early approaches dethia-coenzyme A derivatives where used to trap intermediates of a type III PKS *in vitro*; for this type of PKSs, malonyl CoA is required for the extension of the polyketide chain. The decarboxylated malonyl moiety attaches the enzyme-bond polyketide intermediate whereby it is extended by two carbon atoms. Subsequently the thioester bond is cleaved and the extended polyketide chain is simultaneously transferred back to enzyme to be ready for another cycle. This cleavage could be prevented by addition of a malonyl CoA derivative where the sulphur atom is exchanged for instance with a nonhydrolysable methylene group (Figure 1, **1a**). This resulted in different stable trapped intermediates unable to undergo further chain extension. Instead, they could be enriched in the reaction mixture and be detected by LC-ESI-MS/MS.[2]

Further improvement of this concept led to the development of similar probes of smaller size suitable for investigations of more complex PKSs, including type I, *in vitro* and *in vivo*. Examples of these probes are shown in Figure 1. Although the use of the malonyl carba(dethia)-pantetheine derivative **3a** didn't result in a detectable off-loading of any intermediate, it has been observed that in presence of **3a** the formation of the final product decreased. Therefore, a certain type of interaction with the tested PKS was obvious, which inspired the group to the use of even smaller probes derived from carba(dethia)-cysteamine, such as compounds **4a** or **5a**. Using these probes an off-loading of several intermediates could be achieved.[3]

Later on it was discovered that this methodology is not only applicable for investigations *in vitro* but also suitable for studies *in vivo,* which is a great advantage due to the limited number of available and fully functional PKSs. These *in vivo* studies led to the trapping, extraction and detection of various intermediates of the biosynthesis of erythromycin A[4] and lasalocid A.[5] With the newly gained knowledge about the intermediates of the lasalocid A biosynthesis, the Tosin group could gain new insights into the underlying mechanisms, particularly in relation to the timing of the epoxidation, cyclization, aromatization and the release of lasalocid A from its PKS.[5]

Inter alia, due to the applicability *in vivo*, this kind of chemical chain termination became a powerful methodology for the investigation of the functioning of polyketide synthases, especially with regard to the timing of successive enzyme catalyzed steps. Inspired by this success the idea was born to use the same principle for investigations on NRPSs. This required the synthesis and validation of novel chemical probes capable to compete with the natural substrates of these enzymes. This should ultimately lead to a connection between the probe and an intermediate and thereby to its off-loading from the corresponding NRPS. Once a certain predicted, stable and soluble intermediate is off-loaded from the enzyme, it should be possible to extract it with ethyl acetate or butanol and subsequently to confirm its mass and estimate its retention time via LC-MS analysis.

This master thesis presents the design of such novel potential probes and the development of a strategy to synthesize them as well as first attempts of bacteria culturing and feeding experiments, which finally should lead to the aimed off-loading of intermediates. The latter was particularly challenging due to the unknown effects of these substances on the fed bacteria in terms of toxicity. To keep the bacteria alive but still be able to isolate and detect eventually off-loaded intermediates, the exact adjustment of the right dose of the respective probe seems to be crucial. A success could therefore not be achieved so far. More time is required to handle such problems and the necessary future work will be discussed in this context. However, if further work will lead to a successful off-loading of intermediates,

this method can ultimately help to clarify important mechanistic details about NRPSs and hence provide valuable guides for the development of new drugs by manipulating the nonribosomal peptide biosynthesis.

4 Background and Aims

4.1 Nonribosomal Peptide (NRP) Natural Products

Many NRPs show remarkable biological activities. This section presents some representative compounds of this natural product class, which are of great biological and medical interest. Further the mechanisms of action as well as the structural features responsible for the observed effects are presented.

An interesting nonribosomal peptide is echinomycin **6a** (Figure 2 (A)), which belongs to the quinoxaline family of natural products and is probably the prime example for a bis-intercalator antitumor antibiotic. It is highly active against Gram-positive bacteria and was first isolated and obtained as pure substance in 1957 from *Streptomyces echinatus n. sp.*[6]

Echinomycin is capable to bind DNA double-strands by intercalation of the quinoxaline moiety into the base-pairs, while the cyclic peptide chain is placed in the minor groove. It is believed that this results in an inhibition of transcription due to a blockage of the respective enzymes. The application of echinomycin as antibiotic for humans is limited due to its high toxicity on the entire organism. Indeed there are no candidates from this class of substances which are in clinical use at the moment, nevertheless there are some medically highly interesting representatives. Among them special mention should be made of thiocoraline (Figure 2 (A)) and the luzopeptines (not shown), which show a wide range of activities against human cancers and anti-viral potential against HIV respectively. The latter is based on the nearly complete inhibition of the respective reverse transcriptase at concentrations of 10 to 40 µg/mL. Both peptides show potent anti-microbial activities against different bacteria as well.[7]

Many NRPs are 'decorated' with sugars; amongst these are the so-called glycopeptide antibiotics. The most important and clinically relevant representatives are undoubtedly teicoplanin and vancomycin, which are last resort antibiotics. Both target the cell wall of Gram-positive bacteria and are used to tread corresponding infections in the case of resistance to other antibiotics. Typical is the use of these antibiotics against methicillin-resistance *Staphylococcus aureus* in the case of serious infections; vancomycin is the cheaper option whereas teicoplanin appears to be less toxic for the kidneys.[8] Their common mechanism of action is based on the formation of several H-bonds between specific amide moieties of the peptides (Figure 2 (B)) and the D-Ala-D-Ala portion of the growing peptidoglycan in the cell wall of Gram-positive bacteria (Figure 3). This binding causes a steric blockage of the transglycosylases and/or transpeptidases, the enzymes responsible for

the polymerisation and cross-linking of the peptidoglycan, which gives the cell wall the required stability. Losing this stability results in a leaky cell wall and finally in the lysis of the cell which is no longer able to resist the osmotic pressure.[9]

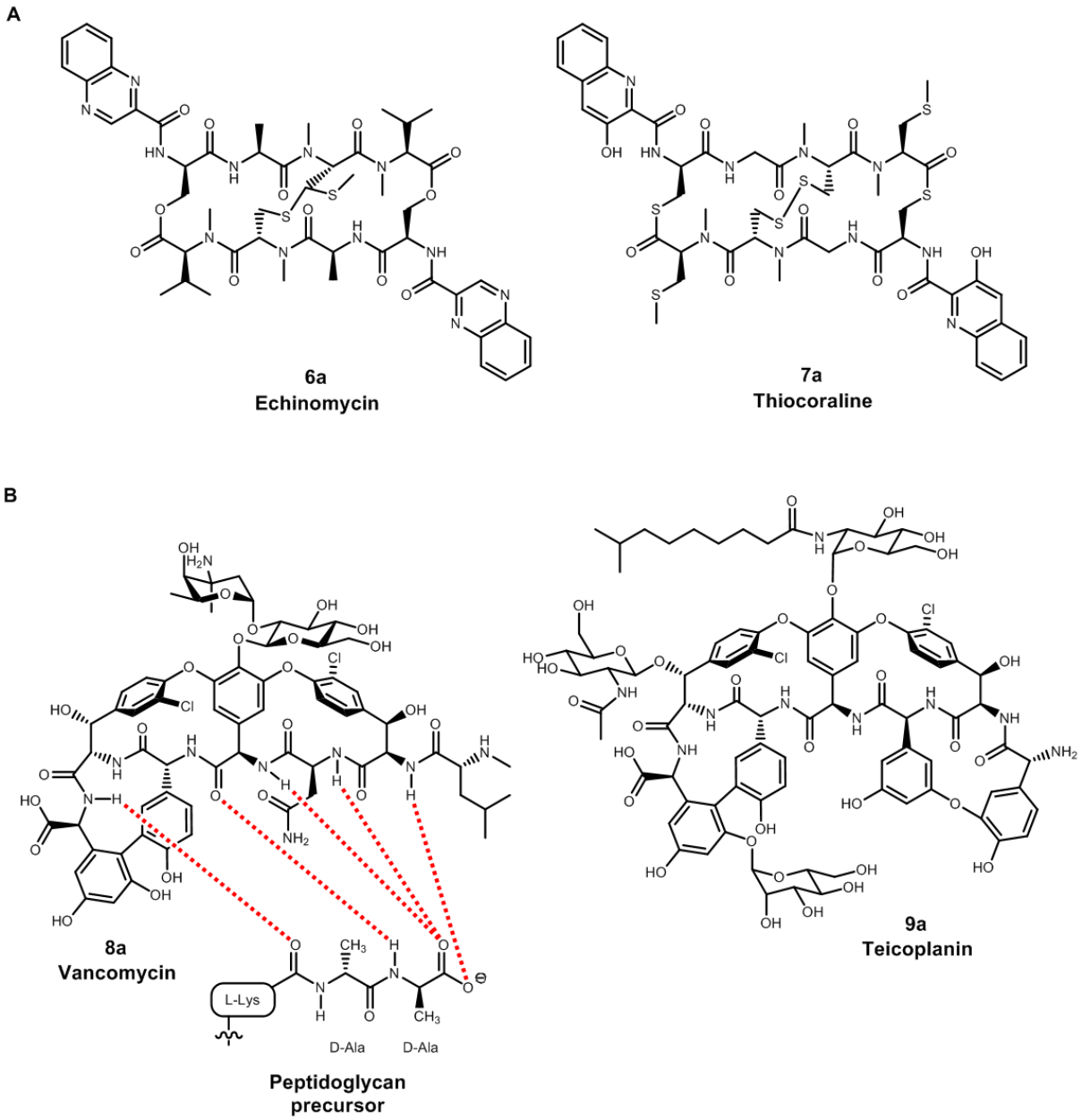


Figure 2: *Examples for biological active and medically relevant NRPs. (A) The quinoxalin antibiotics echinomycin and thiocoraline which target the DNA.[1] (B) The glycopeptide antibiotics vancomycin and teicoplanin, two last resort antibiotics in clinical use. Functional groups responsible for the H-bond formation to the terminal D-Ala-D-Ala portion of the growing peptidoglycan chain in Gram-positive bacteria, are highlighted in vancomycin.[9]*

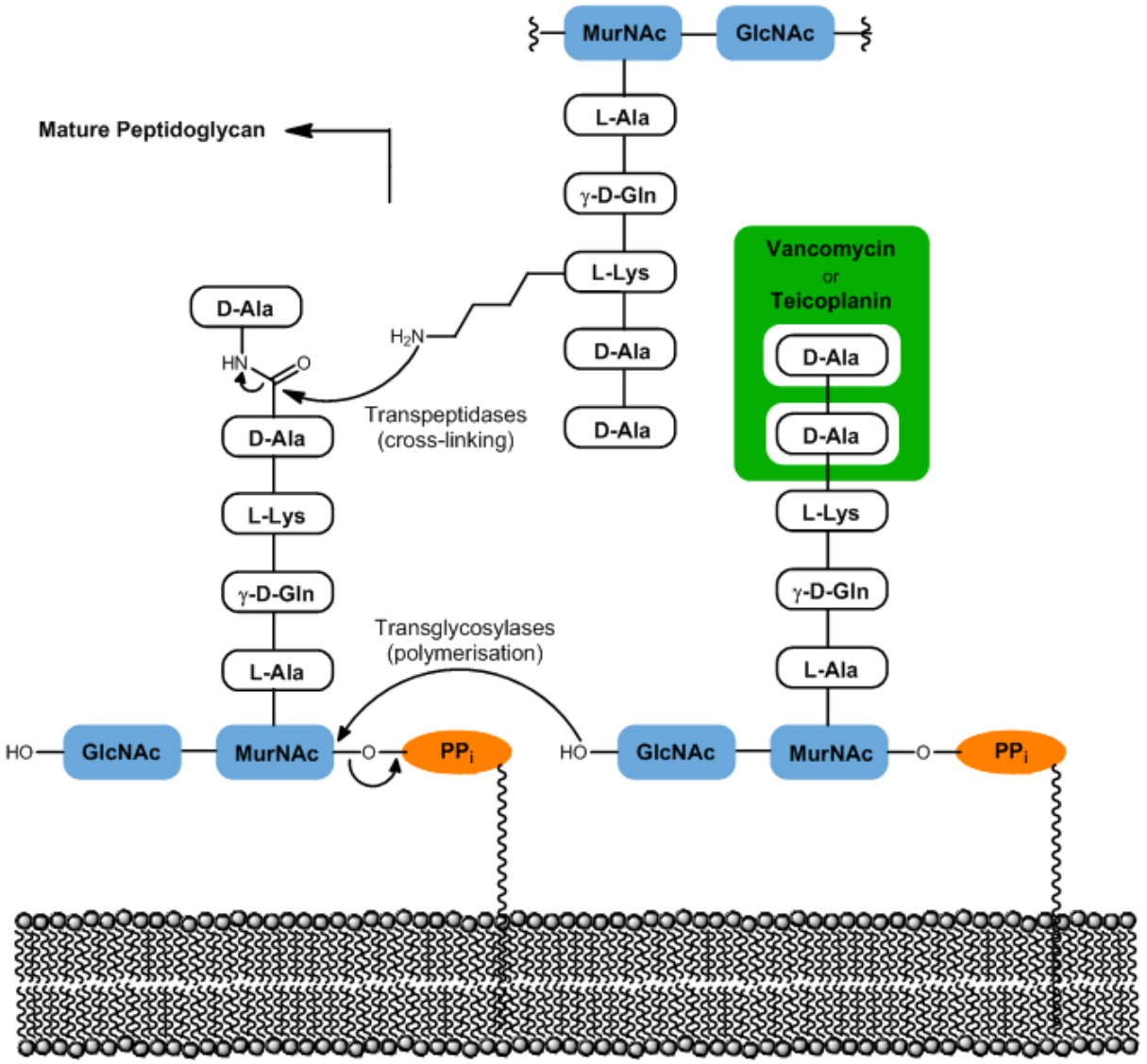


Figure 3: *Synthesis of the cell wall stabilizing peptidoglycan in Gram-positive bacteria. In the absence of antibiotics a transglycosylase mediated polymerisation of the disaccharides (blue) takes place. The subsequent cross-linking between L-Lys and D-Ala is catalysed by transpeptidases. However, vancomycin and teicoplanin are able to bind to the D-Ala-D-Ala moiety of the growing peptidoglycan which prevents this cross-linking. It is further believed that the steric hindrance caused by these antibiotics leads also to an inhibition of the transglycosylases and prevents thereby even the polymerisation itself. The loss of cell wall stability causes ultimately the lysis of the cell.*[8]

4.2 Nonribosomal Peptide Synthetases (NRPSs)

The mechanisms of protein biosynthesis started to be elucidated in the 1950s and 1960s. From then on, it soon became clear that not all peptides are built up *via* the same mechanistic pathways. The formation of certain peptides appeared to be independent of the ribosome. This could be shown by Mach *et. al.* in 1963 on the example of the polypeptide tyrocidine, an antibiotic produced by *Bacillus brevis.*[10] The group studied the production of proteins and peptides in the presence of antibiotics that inhibit protein synthesis by targeting the ribosome. While the production of proteins decreased in the presence of these antibiotics, the formation of tyrocidine was initially unaffected and started to decrease slowly only after several minutes. The knowledge that peptides do not necessarily have to be synthesized on ribosomes, led to further investigations of the biosynthesis of these unusual nonribosomal peptides. In the late 60s it was discovered that the incorporation of an amino acid into a nonribosomal peptide includes an ATP-dependent enzymatic reaction which leads to a covalent connection between the amino acid and the enzyme. This ATP-dependent activation is the same for every amino acid of the growing peptide chain which was observed to be covalently tethered to the NRPS throughout the entire process of NRP assembly.

Guided by the knowledge about the fatty acid biosynthesis, it was soon discovered that the site where intermediates are bound to the enzyme is the thiol group of a 4'-phosphopantetheinyl cofactor (4'-Ppant; see Figure 4). Initially only one molecule of this cofactor was thought to be present per NRPS and this led to the assumption of the so called "thiotemplate" mechanism. This model assumes that the cofactor which is tethered to the enzyme bears the first amino acid. All the other amino acids of the final peptide are initially bound to a thiol group, most likely from a cysteine residue of the corresponding module, a catalytically active subunit of the NRPS responsible for the incorporation of one amino acid. The amino group of the first accepting cysteine-bound aminoacylthioester would than attach the carbonyl group of the cofactor-bound donor amino acid. Subsequently the dipeptide could be transferred back to the liberated thiol group of the cofactor by a transthiolation reaction and the same procedure follows for the next amino acids until the entire peptide chain is built up. However, even providing a plausible model for the modularity of a NRPS, it turned out to be not entirely true. Further improvements in the field of DNA sequencing allowed more detailed investigations and led to the discovery of the currently valid "multiple carrier model". This model implies one molecule of the 4'-Ppant cofactor for every module responsible for the incorporation of an amino acid and the order of the modules, determines the sequence of the amino acids in the final peptide. However, this statement applies only for the type A, linear form of NRPSs. There are two other types with minor deviations from this rule. In type B, iterative NRPSs all the modules are used more than once for the

assembly of the polypeptide and in type C, nonlinear NRPSs single domains (further subunits) are used repeatedly. Furthermore, NRPSs and PKSs can cooperate to build up the final product.[1] Nevertheless, the main principles of NRP assembly are the same in all of the three types and are discussed in the following sections.

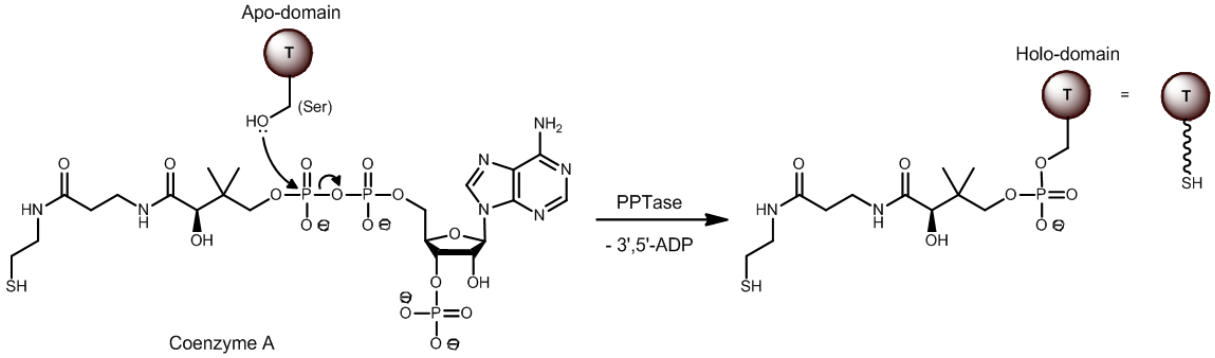


***Figure 4**: Mg^{2+} dependent and PPTase mediated transfer of the 4'-Ppant moiety from CoA to a serine residue of the inactive apo-T domain to form the active holo-T domain.[11, 12]*

4.2.1 Structural and Functional Features of NRPSs

NRPSs are large enzyme complexes of variable size and some of them can reach masses in the order of megadaltons, as shown by the example of the tyrocidine synthase.[13] This NRPS is split into three proteins while some other NRPSs consist of one single protein. However, a common feature of all NRPSs is their division into modules, which can be considered as subunits of these enzymes. As previously mentioned, each module's job is to incorporate (and further elaborate) a single amino acid moiety into the growing peptide chain. Each module consists of several subunits (domains) exhibiting different catalytic functionalities.

The general peptide chain extension demands three basic requirements: (i) the activation of the amino acid monomer, (ii) its spatial proximity to the growing chain, and finally (iii) its condensation with the peptide chain. For this reason the minimal chain extending NRPS module consists of three domains, each one responsible for one of these requirements. Additionally a module can contain further domains responsible for various transformations of the amino acids like methylation, isomerization and many other modifications as well as cyclisation and chain termination. Individual adjacent domains are connected by linker regions, which enable their correct spacial arrangement to allow interdomain interactions to take place. The crystal structure of an entire NRPS module, presented by Marahiel and co-workers in 2008, gives insights into this structural

arrangement and provides more mechanistic details in how individual domains co-operate in NRP elongation.[14] Figure 5 shows the 3D structure of individual domains and gives an idea about their approximate size and structure. The function of these domains will be discussed stepwise in the following sections.

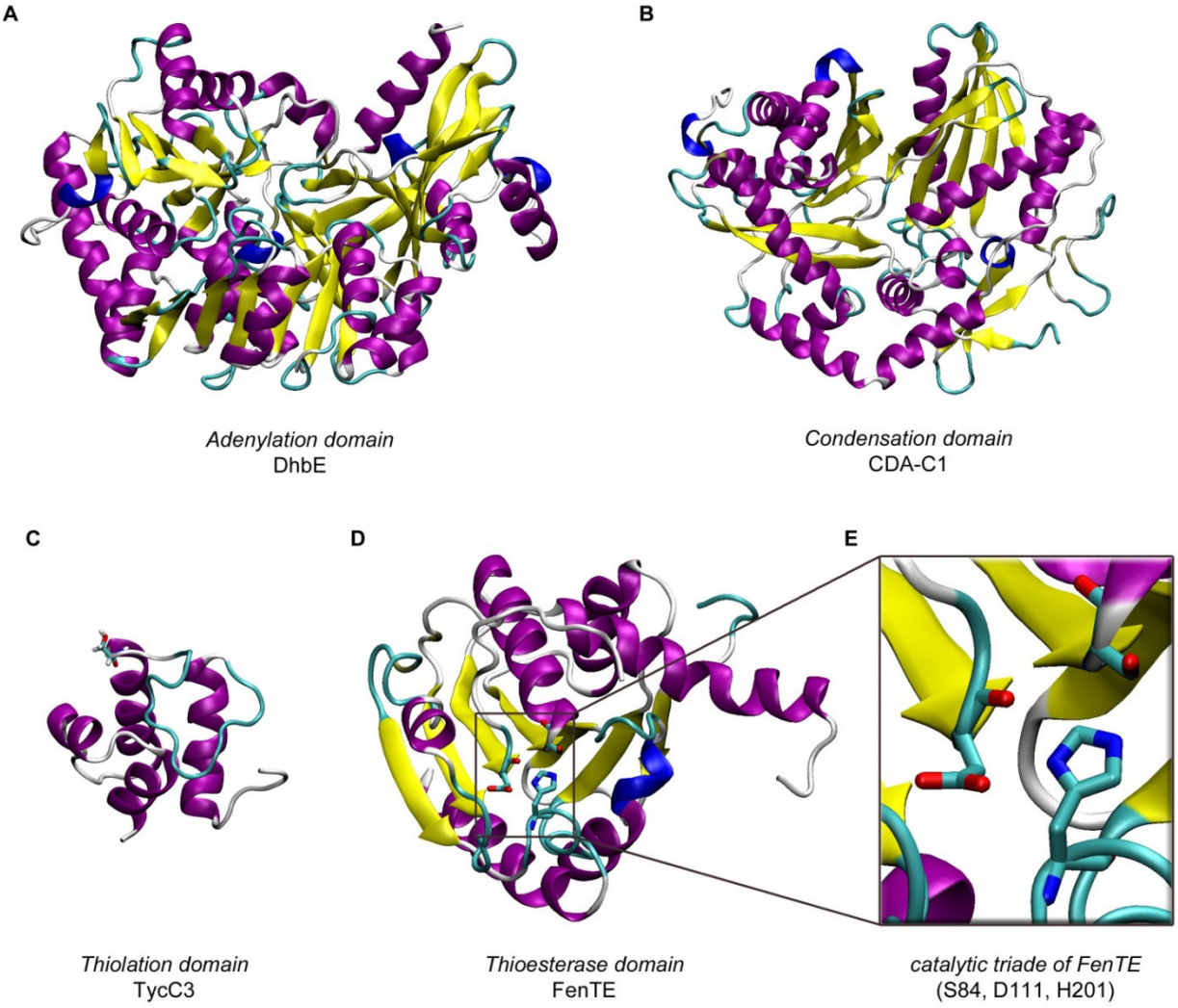


Figure 5: *3D structures of various NRPS domains. (A) Crystal structure of DhbE, the first A domain of the Bacillibactin NRPS cluster responsible for the activation of 2,3-dihydroxybenzoate (DHB) in the biosynthesis of Bacillibactin in* Bacillus subtilis.[15] *(B) Crystal structure of CDA-C1, the first C domain of the CDA (calcium-dependent antibiotic) synthetase which is responsible for the link between the 2,3-epoxyhexanoyl group and the serine residue in the CDA synthesized by* Streptomyces coelicolor.[16] *(C) Solution structure of TycC3, the thiolation domain located in the 3rd module of the 3rd synthetase TycC of the tyrocidine synthetase cluster, an NRPS produced by* Bacillus brevis. *The 4'-Ppant cofactor binding serine residue is shown in Licorice representation.[17] (D) Crystal structure of FenTE, the thioesterase domain of the fengycin synthetase cluster. This domain catalyses the cyclisation and release of fengycin, an antibiotic produced by* Bacillus subtilis. *Responsible for this process is a catalytic triad.[18] (E) Enlarged view of the catalytic triad. The corresponding residues serine 84, aspartic acid 111 and Histidine 201 are shown in Licorice representation. (PDB codes: (A) 1MDF; (B) 4JN3; (C) 1DNY; (D) 2CB9)*

4.2.2 Amino acid Selection and Activation

The first step of chain initiation or extension is the activation of the corresponding amino acid monomers. This step occurs thanks to the catalytic activity of adenylation (A) domains. As the name already suggests, these domains are able to catalyse a reaction where the AMP moiety of ATP is transferred to the carboxyl group of the amino acid, which results in its activation (Figure 6). Moreover this reaction is a substrate selective process wherein it is ensured that the correct amino acid is activated for the incorporation into the peptide chain. Subsequently the A domain catalyzes the transfer of the activated amino acid to the 4'-Ppant cofactor of the corresponding thiolation domain. It is known that adenylation domains can adopt different conformations, which depend on whether the domain catalyzes the adenylation reaction or the transfer of the activated amino acid to the thiolation domain. In fact an A domain can be divided into a N-terminal and a C-terminal subdomain: these can rotate relative to each other whereby the binding-pocket for the substrate is placed in between. [19] Based on known structures of different adenylation domains Yonus *et al.* suggested a model for the reaction cycle of the stand-alone adenylation domain DltA.[20]

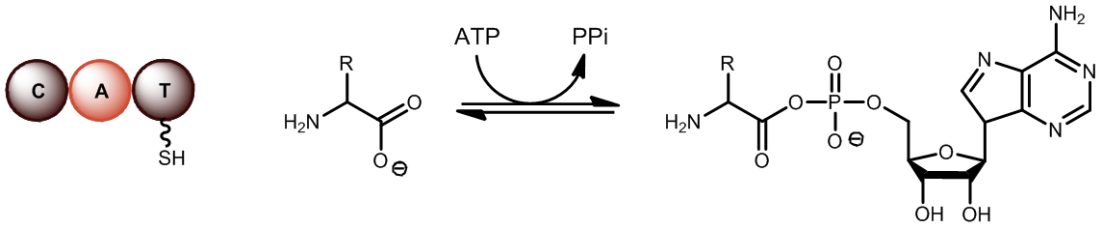


***Figure 6**: The A domain mediates the transfer of the AMP moiety of ATP to the carboxylate group of a specifically selected amino acid.*[1]

4.2.3 Thiolation

The size and fold of T (thiolation) domains, which are also known as peptidyl carrier proteins (PCPs), are similar to the acyl carrier proteins (ACPs), which play an important role in the biosynthesis of PKSs and fatty acids. In fact, the function is very similar too. It is essential that these proteins are posttranslational activated by the transfer of the 4'-Ppant cofactor from CoA to a specific serine residue (see Figure 5 (C)). This Mg^{2+} dependent transfer is catalyzed by a phosphopantetheinyltransferase (PPTase) and converts the PCP from the inactive *apo*-state to the active *holo*-state (Figure 4).[21, 11, 12] Only in this state the PCP is able to work in conjunction with the A domain and to accept the activated amino acid. The latter is achieved by an attack of the 4'-Ppant thiol group on the carboxyl group of the activated amino acid which results in the release of AMP and the formation of the thioesterified amino

acid (Figure 7). As shown by Marahiel *et. al.,* the T domain can now switch its conformation, which results in a repositioning of the cofactor; this is necessary for the relocation of the thioesterified amino acid to the catalytic center of the condensation domain.[22]

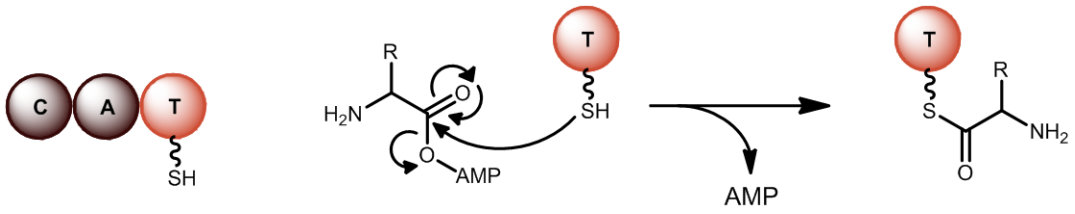


Figure 7: *Transfer of the activated amino acid to the 4'-Ppant cofactor of the thiolation (T) domain, to form the corresponding aminoacyl thioester.*[1]

4.2.4 Condensation

Condensation domains or C domains catalyse the peptide bond formation between the downstream amino acid and the upstream intermediate and are therefore responsible for the actual chain extension. These domains have two binding sites, one for each substrate. The binding site for the single 4'-Ppant-bound amino acid of the same module is referred to as the acceptor site and the donor site is the binding site for the growing peptide chain. These two binding sites are located one on each end of a tunnel, which goes through the C domain: the proposed catalytic centre (His157) is located in the middle of this tunnel. Like A domains, also a C domain can be divided into an *N*-terminal and a *C*-terminal subdomain and the active site is located in between these two subunits. A specific segment of the *C*-terminal subdomain crosses over the catalytic centre and interacts with the *N*-terminal subdomain forming the aforementioned tunnel. Despite inadequate evidence, it seems obvious that this covering segment must be removed from the *N*-terminal subdomain after the condensation reaction to allow the release of the extended chain.[16]

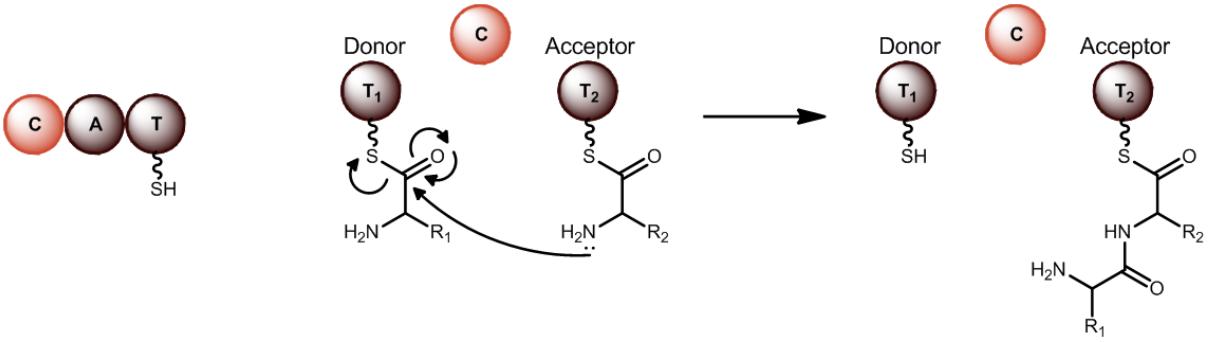


Figure 8: *C domain-mediated condensation of two amino acids bound on adjacent T domains. The product is a thioesterified dipeptide which is ready for further modifications or the condensation with the next amino acid.*[1]

4.2.5 Further Enzymatic Modifications

The wide range of structural diversity of NRPSs is in large part based on the selectivity of A domains, but is further expanded by the presence of additional domains which work in conjunction with the three basic domains mentioned above to modify single amino acids before or after their incorporation into the peptide chain. A frequently occurring representative of such an additional domain is, for instance, the epimerisation domain or E domain. E domains are generally located after thiolation domains in NRPS modules to catalyse the inversion of configuration of the α-carbon of amino acids after their incorporation into the PCP-bound growing peptide chain. According to the mechanism of epimerisation postulated by Stachelhaus and Walsh in 2000, a basic group of the E domain abstracts the proton in α position of the amino acid or peptide bound to the 4'-Ppant cofactor of the adjacent T domain, which results in an enolate intermediate. In the subsequent step this intermediate is reprotonated forming a mixture of D and L substrate (Figure 10).[23] Due to the stereoselectivity of the donor site of the following C domain, only the right stereoisomer will finally be connected with the next amino acid. However, not every C domain shows the same level of stereoselectivity.[24] Based on the first crystal structure of an epimerisation domain elucidated by Samel *et. al.*, a model of the transition state of the epimerisation was elaborated recently which suggests a stabilization of the enolate intermediate due to electrostatic interactions between the negative charged enolate O atom and the positive charge of the protons from an Aspartic acid amide group and a Histidine NH as well as the dipol moment of an α-helix. The epimerisation itself is thought to be carried out by a glutamate residue.[25]

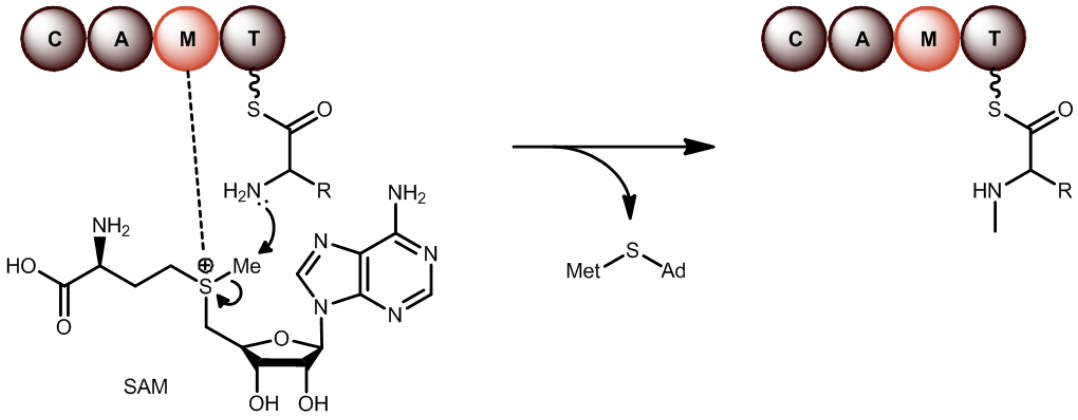


Figure 9: *M domain catalysed methylation of an amino acid with S-adenosyl methionine (SAM) being the methyl donor.*[26]

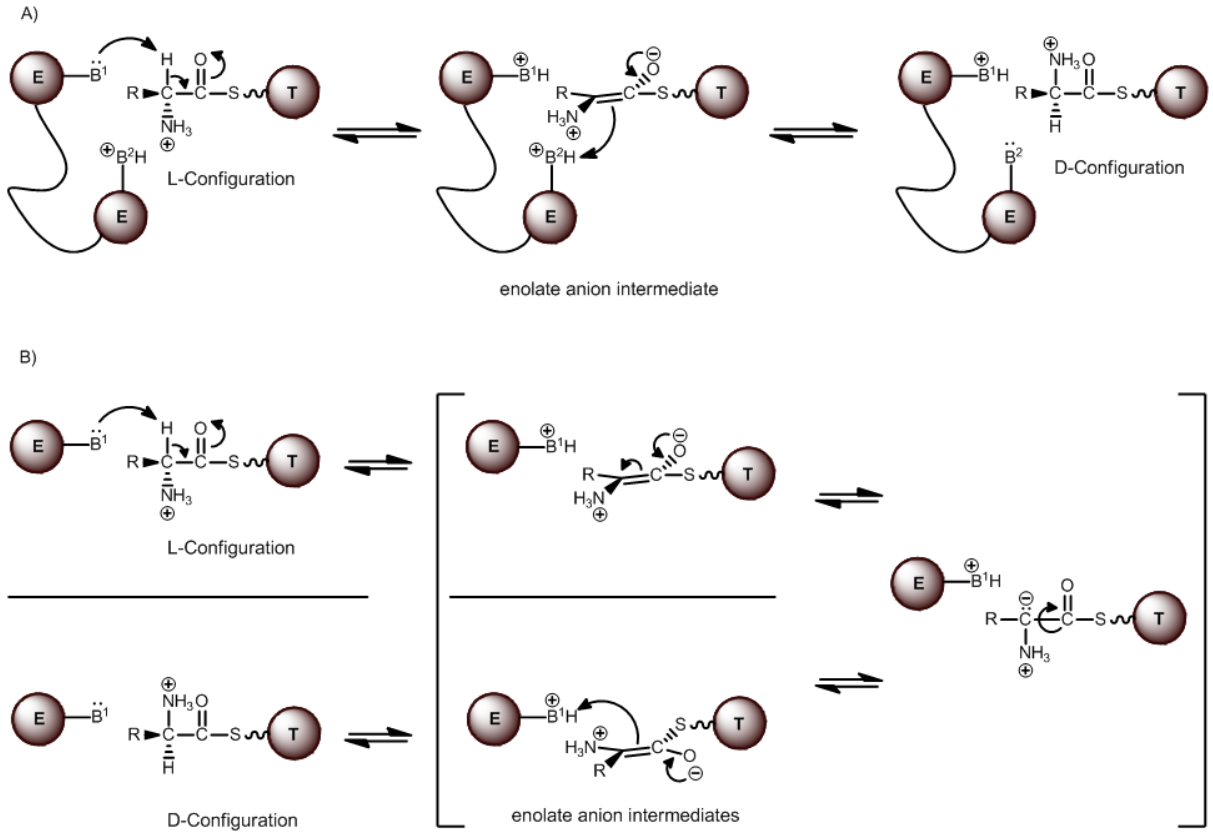


Figure 10: *Two possible mechanisms for the epimerisation of amino acids by the epimerisation domain (E) of NRPSs as postulated by Stachelhaus and Walsh in 2000.[23] A) 'Two base' mechanism: a basic group of the epimerisation domain deprotonates the α-carbon of an L-amino acid to form an enolate anion intermediate. The conjugate acid of a second basic group inside the same domain reprotonates the intermediate from the other site which leads to the formation of the corresponding D-amino acid. B) 'one base' mechanism: here the deprotonation and the reprotonation of the α-carbon is done by the same basic group. In both cases the result is a mixture of L- and D-amino acid.*

Other modifying domains are methyltransferase (M) domains. These are found in some NRPS modules between the A and T domain; they increase NRP structural diversity by methylating amide nitrogens.[27] This reaction is dependent on the presence of S-adenosyl methionine (SAM), which proved to be the cofactor donor of the methyl group; indeed *in vitro* studies showed that the absence of SAM results in the formation of nonmethylated peptides with a significantly lower reaction rate.[28] The methylation occurs after the transfer of the activated amino acid to the 4'-Ppant cofactor of the thiolation domain and prior to its condensation with the peptidyl chain from the upstream T domain (Figure 9).[26]

As well as from *N*-methylation, also *C*-methylation and *O*-methylation are possible; besides various other domains able to modify the final NRPs or single monomers during NRP biosynthesis have been discovered. Noteworthy are, for example, glycosyltransferase enzymes or oxidative domains, reductase domains, aminotransferase domains and

cyclisation domains.[11] The cyclisation of NRPs is often related with their release from the enzyme complex, which is catalysed by thioesterase (Te) domains; however, alternative release mechanisms have been observed, such as briefly discussed below.

4.2.6 Release of Products

Te domains are often located at the end of a NRPS, where they catalyse the hydrolysis or cyclisation of the final NRP. To enable this, the peptide is first transferred to a serine residue of the Te domain which takes part of a catalytic triad (Figure 5 (E)). Subsequently the Te domain catalyses either the nucleophilic attack of a water molecule on the carbonyl group of the serine ester which results in hydrolysis or the nucleophilic attack comes from an intramolecular NH_2 or OH group which leads to a cyclisation via peptide or ester bond formation respectively. In any case the release of the final peptide from the NRPS is achieved (Figure **11** (A)). Another rather rarely occurring mechanism of chain release was described by Read and Walsh in 2007; it passes along the reduction with NADPH. An unusual reductase domain (Red) is responsible for this four-electron reduction of the thioester which results in the formation of the corresponding alcohol (Figure **11** (B)).[29]

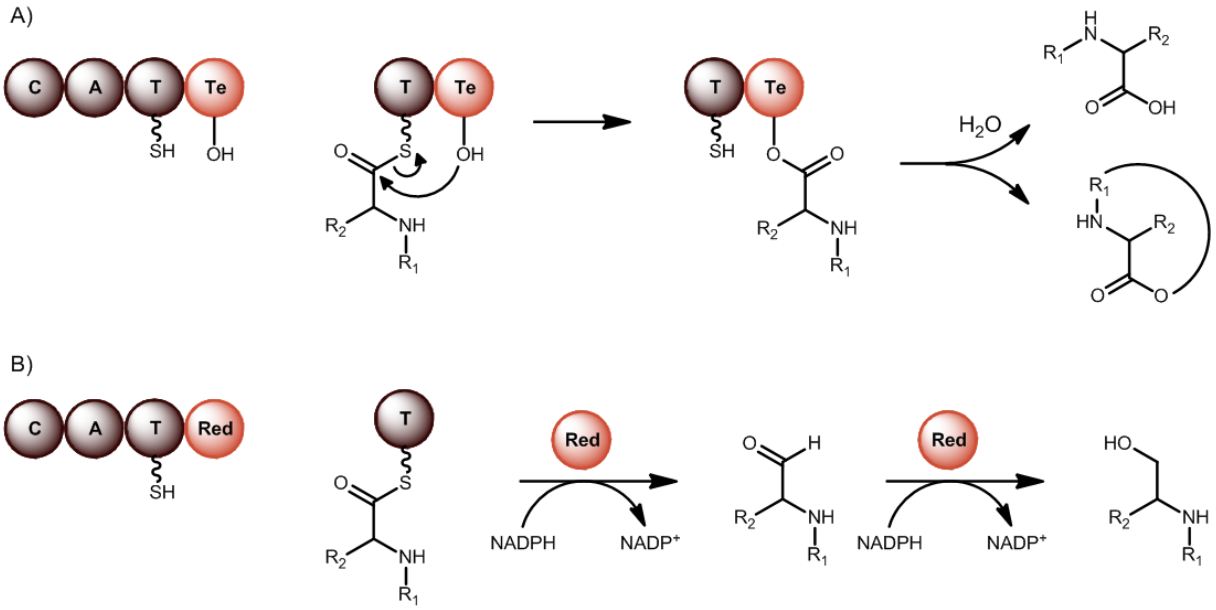


Figure 11: *Release of the final peptide product. A) The oxygen of a serine residue from the Te domain attacks the carbonyl group of the thioester and accepts hereby the peptide chain. Due to the activity of a catalytic triad the chain is released from the enzyme either by hydrolysis or cyclisation.[1] B) Example for a rarely occurring mechanism of chain release involving a reductase domain (Red) and NADPH as reducing agent.[29]*

4.2.7 Biosynthesis of Echinomycin

To give an example for the complete biosynthesis of a nonribosomal peptide, this section refers to the assembly of echinomycin, a NRP produced by a type B iterative NRPS. The latter was investigated in the course of the practical work of this master thesis. In 2006 Watanabe et. al. presented the biosynthetic gene cluster which is responsible for the biosynthesis of echinomycin in *Streptomyces lasaliensis.*[30] According to this model, the quinoxaline antibiotic is produced on the basis of 19 different genes, 18 of them take part of the echinomycin gene cluster itself and one gene is located outside of the cluster. Eight genes are involved in the conversion of L-tryptophane into the quinoxaline-2-carboxylic acid (QXC), which is the first acid incorporated into the final depsipeptide. Four genes, including the one outside the cluster, are responsible for the formation of the peptide and two genes cause the final thioacetal formation. Furthermore three regulating genes, one resistance gene and one inactive gene of unknown function are present in the biosynthetic gene cluster. A clear graphical representation which shows the arrangement of these genes is provided in Figure 12.

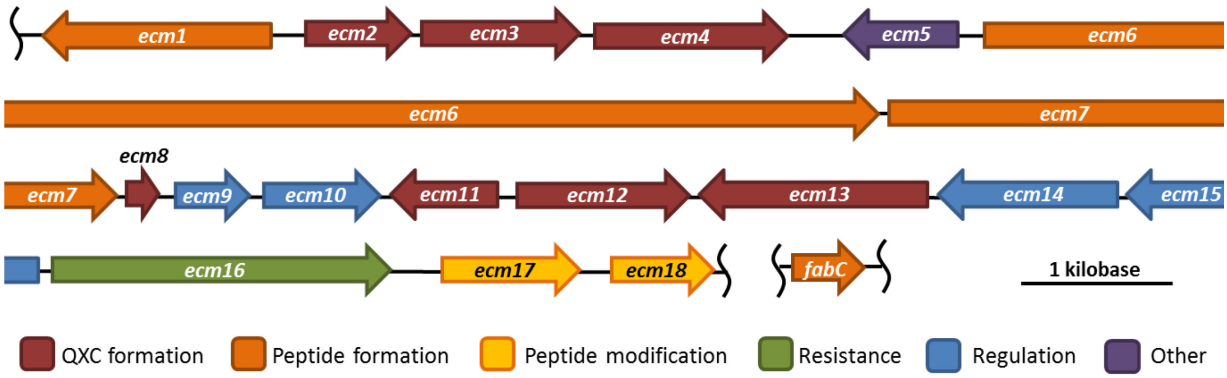
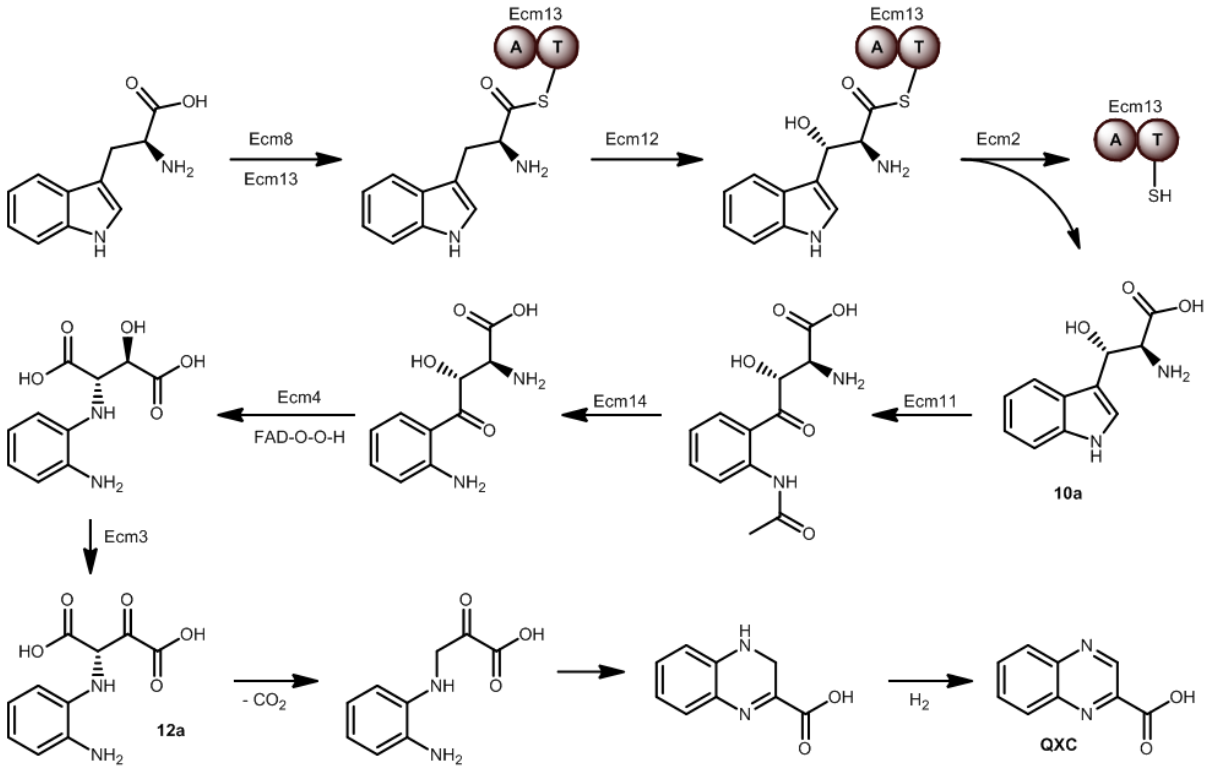


Figure 12: *Arrangement of the genes in the gene cluster for the echinomycin biosynthesis in* Streptomyces lasaliensis.[30]

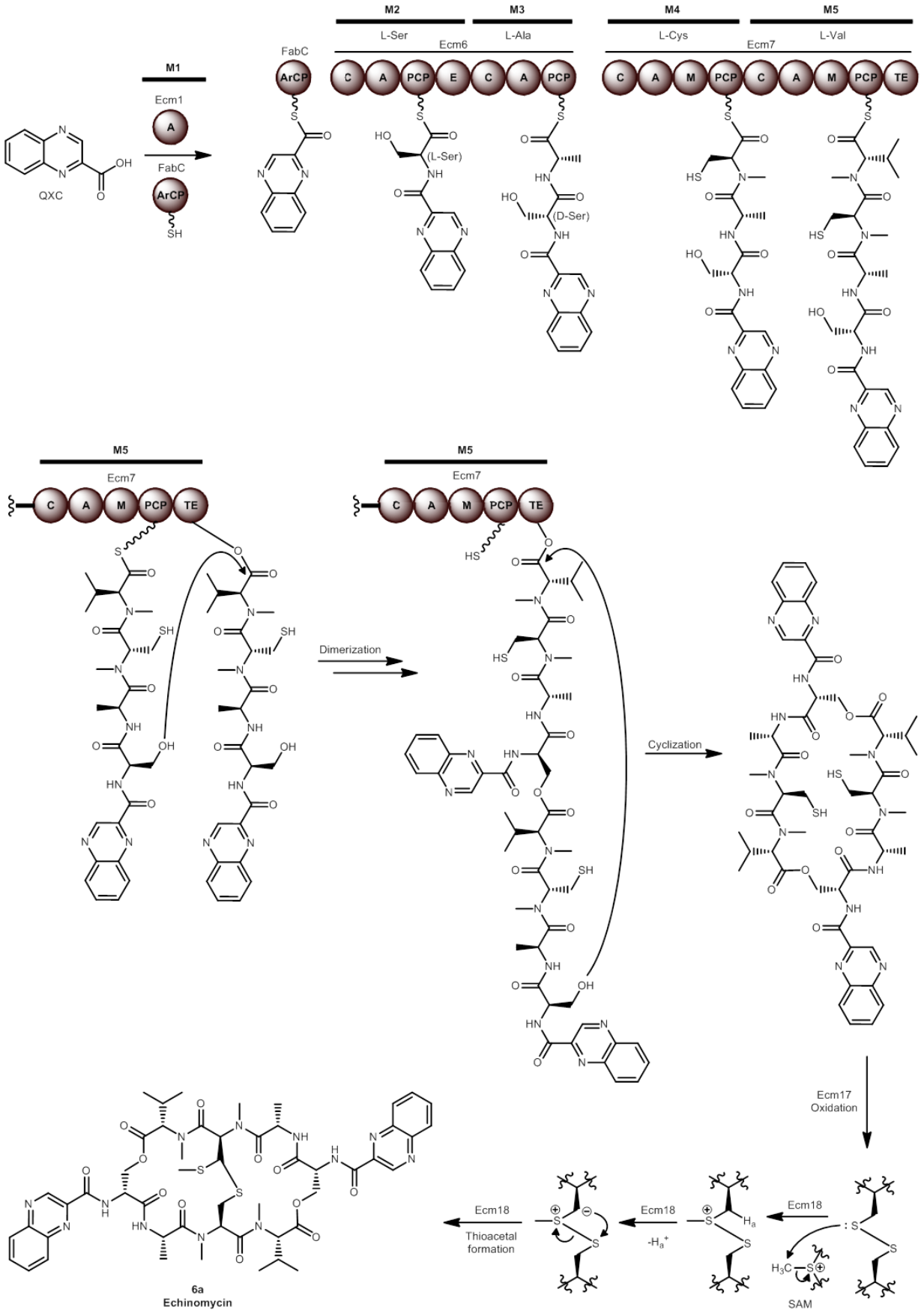
The formation of echinomycin starts with the biosynthesis of quinoxaline-2-carboxylic acid which is derived from L-tryptophane. For this purpose the amino acid is first of all activated by the A domain of an A-T bidoamin (Ecm13) in an ATP dependent reaction and subsequently, as usual, transferred to the corresponding T domain (Scheme 1). In this state the methylene group of tryptophane is oxidized to the alcohol by a Cytochrome P450 oxidase (Ecm12) and the thioester bond is hydrolysed by a type II thioesterase (Ecm2) to release compound **10a**. The tryptophane 2,3-dioxigenase (Ecm11) causes an oxidative cleavage of the double bond in a radical-mediated reaction and the erythromycin esterase (Ecm14) hydrolyzes the amide bond of the generated *N*-formylated intermediate. Further

modifications by a FAD-dependent oxidoreductase (Ecm4) and an Isopropylamate dehyxdogenase (Ecm3) induced oxidation of the OH group to the corresponding α-keto acid leads to compound **12a**. After a spontaneous decarboxylation, imine bond formation and aromatization by a loss of H_2 the formation of quinoxaline-2-carboxylic acid is completed and the NRPS-mediated echinomycin biosynthesis can start.



Scheme 1: *Proposed biosynthesis of the quinoxaline-2-carboxylic acid (QXC). Eight out of eighteen genes from the echinomycin biosynthetic gene cluster are involved in the transformation of L-tryptophane to this single aromatic monomer.*

The acid is activated by an A domain expressed from ecm1 and transferred to an ACP domain, denoted as FabC and expressed from the corresponding gene fabC. The resulting QXC-S-FabC acts as substrate for the NRPS Ecm6 whose C domain from the first module catalyses the condensation of QXC with the next monomer of the assembly line as described in the corresponding section above. The second monomer is in this case an L-serine residue and after its condensation with the QXC its stereocenter is inverted by an epimerisation (E) domain to form the corresponding D-serine derivative (Scheme 2). The next module of Ecm6 incorporates an L-alanine and makes the resulting intermediate available for the next NRPS Ecm7. Also Ecm7 is made up of two modules, both of them incorporate amino acids after their methylation by M domains. In this way *N*-Me-L-Cys and *N*-Me-L-Val are introduced to



Scheme 2: *Representation of the biosynthesis of Echinomycin.* [30, 31]

form a pentapeptide. Since echinomycin is produced by a type B iterative NRPS, a second cycle is required to build up the final decapeptide. Therefore the same pentapeptide is produced again and before the first one is released from the NRPS, it is condensed with the second one by ester bond formation. The decapeptide is subsequently cyclized due to a second ester bond formation catalyzed by the terminal thioesterase (Te) domain. The cyclisation finally results in the release of the peptide from the multyenzyme. At this stage, two additional modifications are still necessary to yield the final product, namely the formation of the disulfide bridge between the two cysteine residues and the subsequent methylation of the sulfur which finally results in thioacetal formation. The first one is catalyzed by the Thioredoxin reductase Ecm17 and the latter is probably initiated by a Ecm18 catalyzed transfer of a SAM-methyl group to one sulfur atom of the disulfide bridge. Subsequent abstraction of a proton in α position to the sulfonium cation enables a rearrangement to the thioacetal which finally leads to echinomycin.[31]

4.3 Methods to Investigate NRP Biosynthesis

A major problem for the elucidation of nonribosomal peptide biosynthesis is the fact that all the intermediates are covalently tethered to the associated NRPS over the entire assembly process. This complicates in some ways the investigation of the single intermediates, especially with respect to their short lifetime. However, accurate knowledge about the exact functioning is crucial for future combinatorial manipulation of this biosynthetic machinery aimed at generating modified natural products with improved biological activities. Deeper understanding of the underlining mechanistic details, particularly in relation to the selectivity of A and C domains, is therefore highly desirable.

4.3.1 Isotopic Labeling

In early approaches to elucidate the nonribosomal peptide biosynthesis, radiolabeled amino acids have been applied in the late sixties. Lipmann and collaborators elaborated a strategy to examine the formation of the NRP tyrocidine. They added each amino acid of the tyrocidine biosynthesis step-by-step in the proper order to the corresponding NRPS whereby the first amino acid was labeled with ^{14}C. Precipitation of the enzyme-bound intermediates by addition of trichloroacetic acid and subsequent liberation of the intermediates under basic conditions followed by two-dimentional TLC enabled the group to follow the stepwise assembly of the peptide. Furthermore the results indicated the starting point of chain growth as well as its direction, from amino to carboxyl terminus.[32]

4.3.2 Genetic Approaches

Today isotopic labeling for this purpose is not necessarily the method of choice. Since DNA sequencing became routine and the understanding of NRPS enzymology progressed, a more attractive way of NRPs discovery and characterisation was born. Examples have shown that it is now possible to identify the structure of unknown NRPs on the basis of the corresponding genetic information. This was shown for instance by G. L. Challis and co-workers in 2005. The group was able to predict part of the structure of the tetrapeptide coelichelin by bioinformatics analysis of the corresponding gene cluster found in *S. coelicolor*.[33]

About 10 conserved short amino acid sequences can be found in adenylation domains. These motives are thought to be responsible for the common processes of all adenylate-

forming enzymes, such as the ATP binding and the adenylation of the corresponding amino acids. However, other moieties are quite inhomogeneous throughout the superfamily of A domains and are thought to be involved in the formation of the specific binding pockets which recognize the amino acids to be activated.[34] This hypothesis was further strengthened as E. Conti *et. al.* revealed the crystal structure of the N-terminal subunit of the first A domain (PheA) of gramicidin synthetase, complexed with Phe and AMP.[35] The exact location of the binding pocket forming residues could be discovered and it was assumed that the binding pocket of other A domains is formed by suitable residues at the same defined positions. In fact, comparison of these residues from different A domains shows a correlation between their polarity and the polarity of the corresponding substrate. Furthermore a way could be developed to decrypt the A domain specificity-conferring code.[36] This remarkable finding established a valuable basis for future attempts to alter the substrate selectivity of A domains by targeted mutation in order to create new 'unntatural' natural products of enhanced structural diversity. This strategy was even used to introduce non-natural amino acids into NRPs.[37]

4.3.3 Synthetic Probes

Apart from the selectivity of A domains, also C domains selectivity plays an important role in NRP assembly, especially with respect to the field of biochemical engineering. Both selectivities must be in accordance with each other to guarantee the synthesis of a peptide product. Since C domains have two substrates and even two binding pockets, the question arises on which one is showing the higher selectivity. In fact, both donor and acceptor sites are showing substrate selectivity, even though the acceptor site seems to be more selective. To prove this, Belshaw *et. al.* synthesized different aminoacyl-S-CoA molecules and loaded them *in vitro* independently onto T domains of separate but cooperating modules, with the aid of PPTases.[38] This way the selectivity of the corresponding A domains could be bypassed. Monitoring of dipeptide formation showed higher substrate flexibility for the donor site of the investigated C domain. Searching a way to facilitate the investigation of C domain specificity led to the development of another strategy using aminoacyl-*N*-acetylcysteamine thioester probes, so called aminoacyl (aa)-SNACs instead of enzyme bound substrates (Figure 13 (A)).[39] According to the results of these investigations the acceptor site of the C domain from enterobactin synthetase was proved to be highly selective and the donor site of the C domain from TycB showed a clear preference for the D-isomer of Phe-SNACs. One advantage of these small-molecule substrates is the fact that the products are not tethered to the enzymes and hence easily accessible for analysis. However, a disadvantage is the low stability, e.g. with respect to non-enzymatic hydrolysis.

4.3.4 A New Chemical Approach to the Investigation of NRP Biosynthesis

The subject of this master thesis is to develop and test a new methodology for the investigation of nonribosomal peptide biosynthesis. It involves the synthesis of novel small-molecule probes, which mimic the aminoacyl-4'-Ppant moiety of *holo*-T domains. The main difference to the aa-SNACs previously mentioned is the absence of a thioester group. Instead of sulphur, the probes contain an amino or a methylene group and are therefore referred to as aminoacyl amino(dethia)- and aminoacyl carba(dethia)-*N*-acyl cysteamines respectively. Because of this feature, the probes appear to be stable towards non-enzymatic and enzymatic hydrolysis under physiological conditions. As a result, the probes can be used for investigations *in vivo* and, in contrast to SNAC substrates, they can act only as substrates for the acceptor site of C domains. By doing so, they should condense with the corresponding upstream PCP-bound intermediates and off-load them from the enzyme, as illustrated in Figure 13. The off-loaded intermediates could then be analyzed by mass spectrometry. In the course of the practical work of this master thesis, some examples of

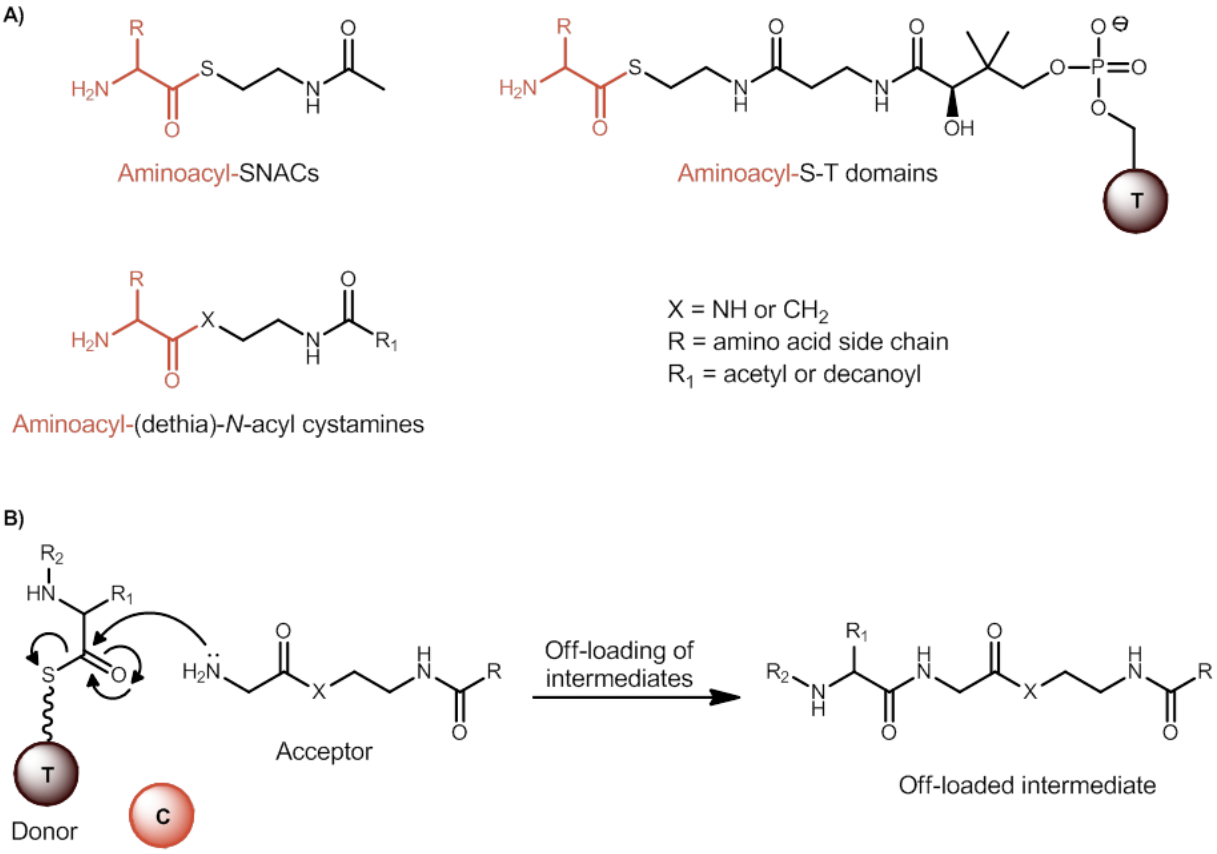


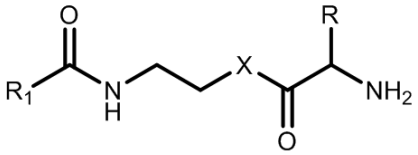
Figure 13: *A) Aminoacyl-SNACs as well as aminoacyl amino(dethia)- and aminoacyl carba(dethia)-N-acyl cysteamines are small molecule mimics of the aminoacyl 4'-Ppant moiety of aminoacyl-S-T domains; B) Proposed off-loading of NRP intermediates due to the action of synthetic probes as acceptors in C domain-mediated condensation reactions.*

this synthetic probes have been synthesized and tested by feeding them to cultures of *Streptomyces lasaliensis*, an echinomycin producing strain. Indeed the biosynthesis of echinomycin is well documented as shown in a previous section. However, the aforementioned method is a new strategy, never tested before for NRPSs and hence it makes sense to test its applicability with the aid of NRPS assembly lines whose mode of action is already well known. Once the method appears to be working, it can be applied even for other NRPSs whose modes of operation are less trivial. This applies e.g. to some type C, nonlinear NRPSs, such as the system assembling the peptide antibiotic capreomycin. As in this case single enzymatic domains are utilised more than once through the NRP assembly, such systems are often not very well understood, especially with respect to the order of amino acid activation and condensation, as well as to the function of additional unusual domains. Moreover there are uncertainties concerning the timing of side chain modification. We therefore hope to gain new insights into important mechanistic details of this nonlinear NRP biosynthesis by the application of the strategy herein described. Furthermore the synthetic probes could possibly be useful for investigations regarding the activity of mutated C domains and E domains, as well as of *N*- and *C*-methylation domains in future studies. This could ultimately provide a valuable contribution to the engineered biosynthesis of new 'unnatural' products.

5 Theoretical Concepts of the Present Work

5.1 NRP Probes Design

The aim of the laboratory work of this master thesis was to develop a strategy to synthesize various compounds which should constitute suitable probes for the off-loading of intermediates from NRPSs. For this purpose, the design of the probes was chosen in a way to represent mimics of the aminoacyl 4'-Ppant moiety of aminoacyl-S-T domains, as shown in Figure 13 (A). The probes are small molecules and not bound to any enzyme, which, according to the plan, should allow the off-loading of intermediates. To confer stability towards hydrolysis, the sulphur of the thioester group in the natural substrate is replaced by an amino or methylene group in the synthetic analogues. Furthermore, the effect of two acyl groups of different length is planned to be tested. The probes with the small acetyl groups could fit better in the acceptor site binding pocket of C domains, whereas the larger decanoyl group could represent a better mimic for the 4'-Ppant cofactor. A successful off-loading of intermediates form PKSs, using long-chain probes could recently be achieved by the Tosin group in attempts to functionalize polyketide intermediates.[40] With respect to the choice of the right amino acid side chain of the probes the original plan was to use that one of glycine. Bearing two hydrogen atoms at the α-carbon and therefore no side chain, probes derived from glycine were thought to be suitable because they should fit in any binding pocket. Furthermore glycine shows no stereochemistry and hence effects regarding the stereoselectivity of C domain acceptor sites are cancelled as well. Hence, this probes should theoretically be able to off-load every NRP intermediate. However, the activity of some C domains may be highly dependent on the interaction between the binding pocket and the side chain of their natural substrates. In the biosynthesis of echinomycin, this may apply to the C domains responsible for the incorporation of L-Ser, L-Ala, *N*-Me-L-Cys and *N*-Me-L-Val. Therefore also the synthesis of appropriate probes bearing the corresponding side chains was highly desirable. This led to the plan of the synthesis of L-Ala and L-Ser probes as well. An overview of all the probes whose synthesis was desired in the course of the laboratory work is provided in Figure 14.



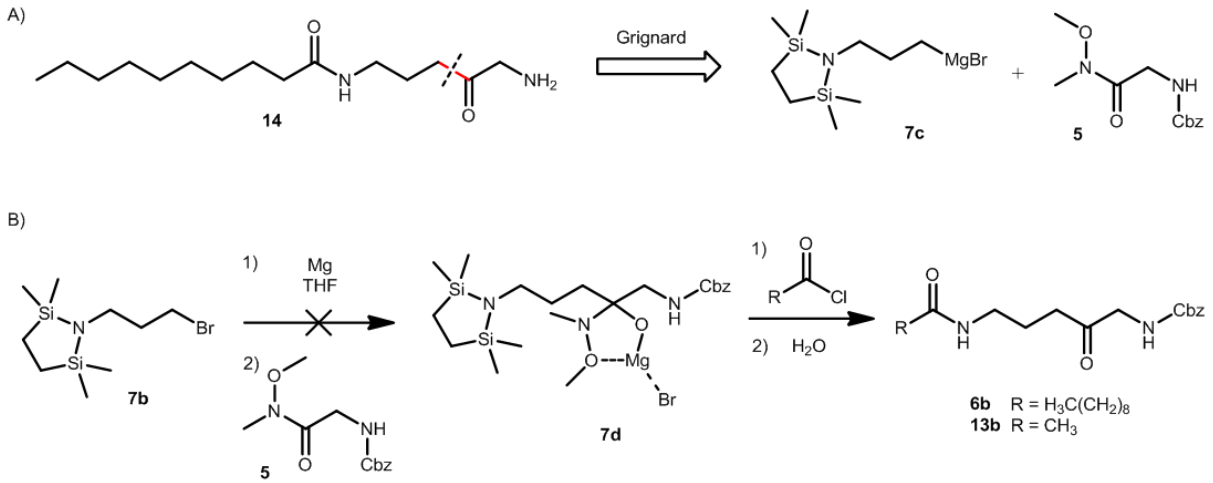
	R	R_1	X
2	H	CH_3	NH
8	H	C_9H_{19}	NH
14	H	C_9H_{19}	CH_2
16	CH_2OH	CH_3	NH
17	CH_3	CH_3	NH
21	CH_3	C_9H_{19}	CH_2

Figure 14: *Synthetic probes **2**, **8**, **14**, **16**, **17** and **21** whose synthesis was targeted during the laboratory work.*

5.2 Retrosynthetic Considerations

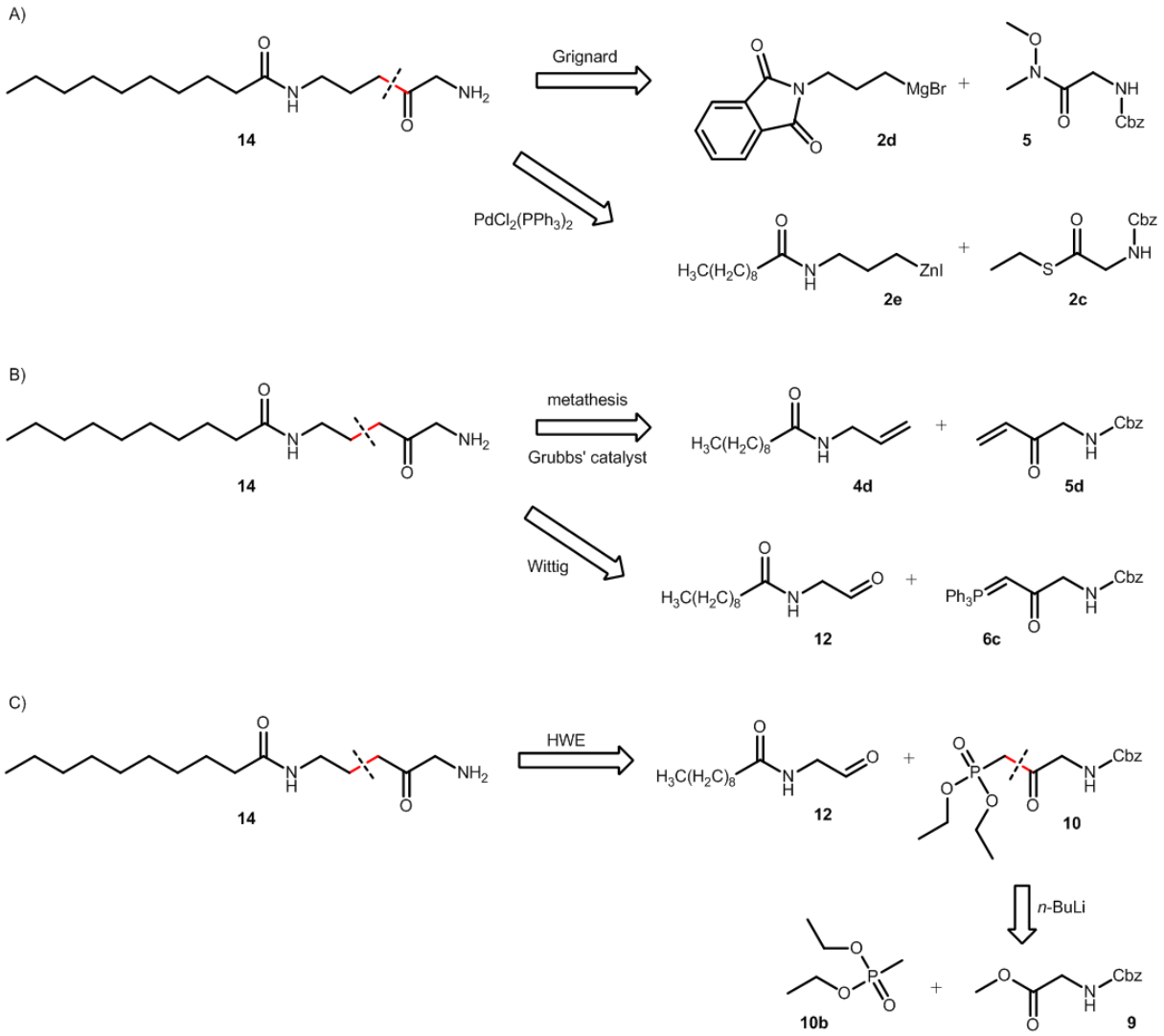
A strategy to synthesize aminoacyl amino(dethia)-*N*-acyl cysteamines was already developed in the Tosin group in previous projects, whereas previous attempts to synthesize aminoacyl carba(dethia)-*N*-acyl cysteamines were not successful. Hence, this section reports only retrosynthetic considerations regarding representatives of the latter group of which the probes **14** and **21** are examples.

As a general procedure to synthesize derivatives of various amino acids was desired, the most obvious bond to break in the retrosynthetic analysis is the one highlighted in red in Scheme 3 (A). One possibility to form a C-C bond next to a carbonyl group is the application of a Grignard reaction with a suitable Weinreb amide as substrate, together with appropriate protection of potentially disturbed functional groups. However, several attempts to follow the simple two-step reaction route shown in Scheme 3 (B) have been unsuccessful, even though the preparation of the Weinreb amide and the direct exchange of the silyl protecting group with an acyl group appeared to be working.



Scheme 3: *A) First retrosynthetic analysis of the desired product **14**. B) Route 1. The first attempt to synthesize **14** via Grignard reaction was unsuccessful.*

After this initial attempt turned out to be unsuccessful several other options have been devised as discussed below. As the silyl protecting group of route 1 (Scheme 3 (B)) is quite labile, the usage of another protecting group for the amino function of 3-Bromopropylamine, e.g. the *N*-phthalamide group, could potentially enable product formation. Also, the possibility to form a C-C bond *via* an organozinc reagent was considered (Scheme 4 (A)).

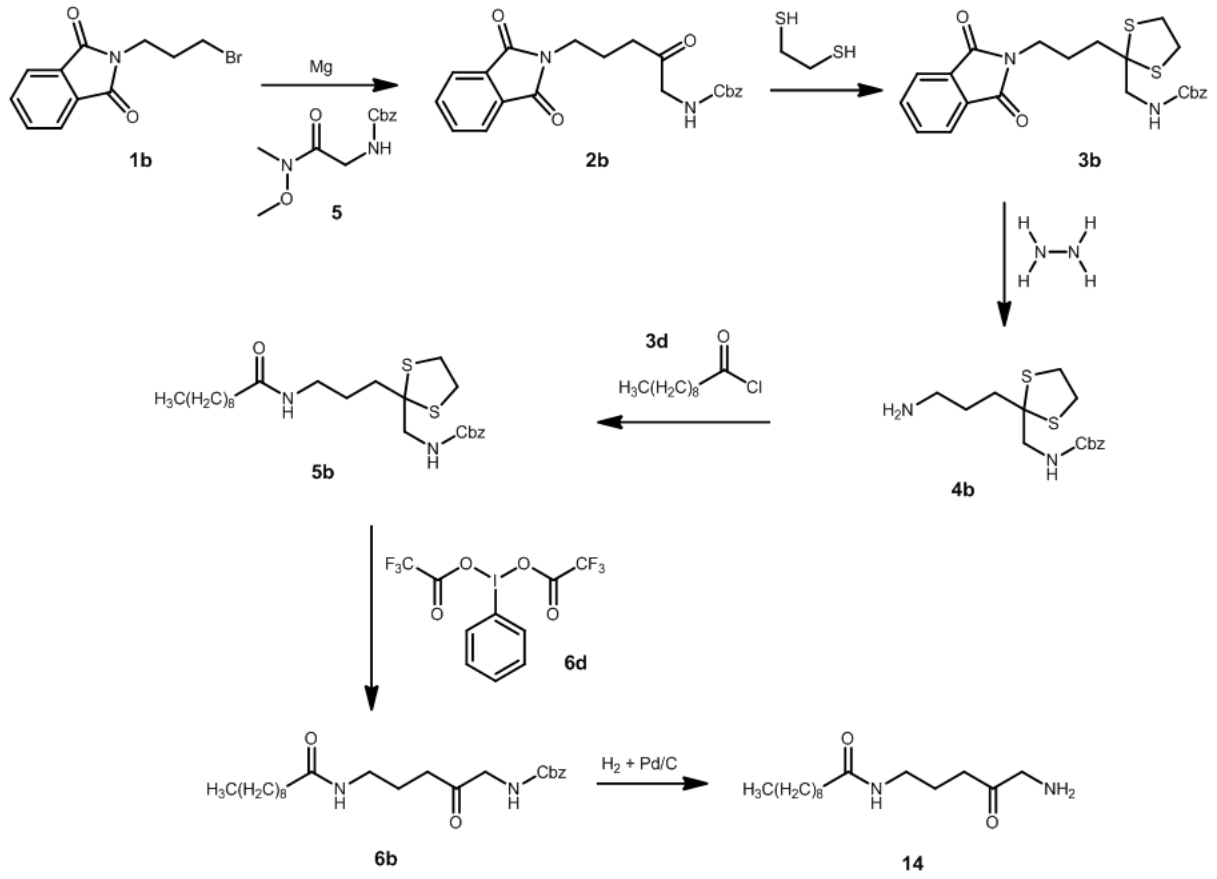


Scheme 4: *Further retrosynthetic analysis. A) Examples for C-C bond formation by nucleophilic carbonyl addition reactions. B) Proposed synthesis of compound* ***14*** *by using the double bond forming olefin metathesis or Wittig reaction. C) HWR reaction as alternative way to the Wittig reaction.*

Another possible bond to break in the retrosynthetic analysis would be the one shown in Scheme 4 (B). This bond could be formed by olefin metathesis using the Grubbs' catalyst, or by application of a Wittig reaction. In both cases, a double bond would be generated, which would have to be reduced by catalytic hydrogenation to form the desired product. The Cbz-group can be removed under the same conditions, therefore it represents a suitable protecting group for the amino functionality. However, even providing rather simple routes to synthesize the targeted product, the metathesis failed in previous studies while the Wittig approach was limited due to the restricted synthetic access of the required phosphoranes. In contrast to this, a relatively simple procedure was by me devised involving a Horner-Wadsworth-Emmons reaction (HWE), a variant of the Wittig reaction starting from phosphonate starting materials (Scheme 4 (C)).

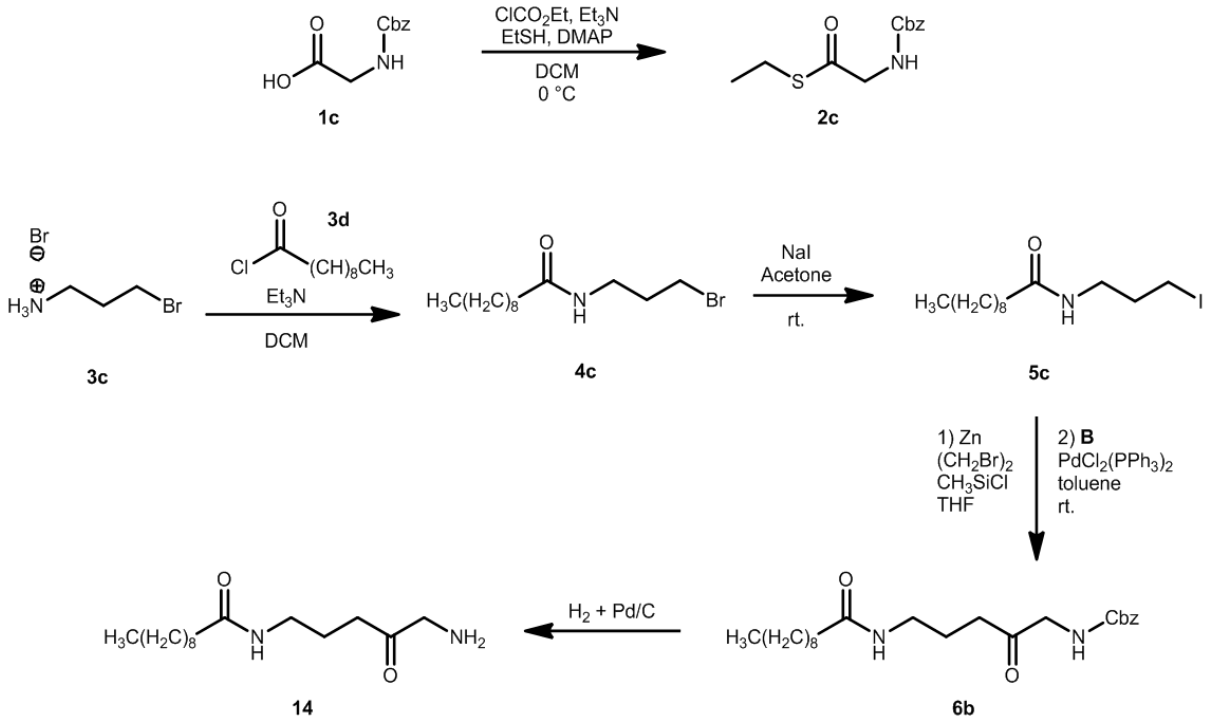
5.3 Potential Synthetic Routes to Probe 14

Based on the retrosynthetic reflections of the previous section, several synthetic routes were considered. Although the first attempt to use a Grignard reaction was not successful, the idea to use this kind of reaction to form the C-C bond next to the carbonyl group was not rejected immediately. Therefore the next devised route was also based on a Grignard reaction utilising an *N*-phthalamido protecting group in the starting material **1b**, which could possibly be compatible with Grignard conditions. This approach would involve the transformation of **1b** to the corresponding Grignard reagent **2d**, and its subsequent nucleophilic attack on the carbonyl group of the Weinreb amide. The resulting ketone group would have to be protected e.g. with 1,2-ethanedithiol, before deprotection of the amino group, in order to avoid cyclisation by intramolecular imine bond formation. After removal of the phthalamide group, the amino function could be acylated with the required acyl chloride and the final deprotection of the molecule would lead to the desired product **14** (Scheme 5).



Scheme 5: *Route 2. Proposed pathway to synthesize aminoacyl carba(dethia)-*N*-acyl cysteamines by application of a Grignard reaction as the key step forming the desired C-C bond and by using the* N*-phthalamide group to protect the amino group of 3-Bromopropylamine.*

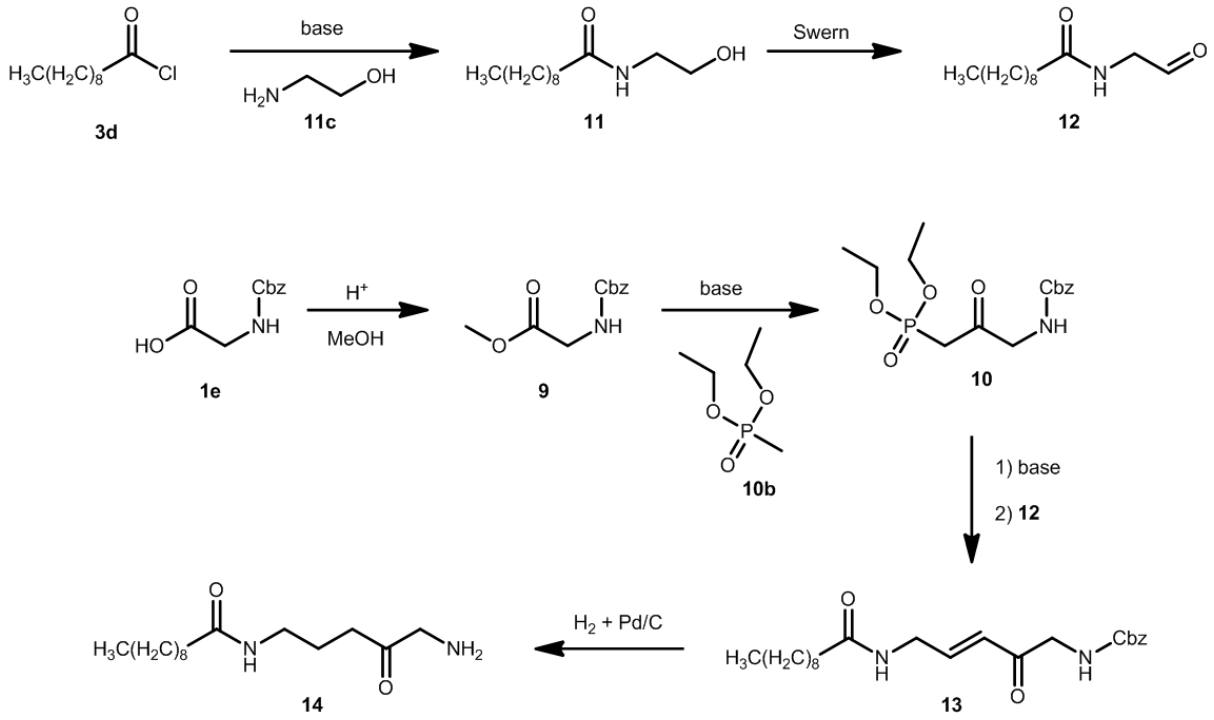
Another possibility would be to form the carbon-carbon bond by a palladium catalysed reaction between the ethanethiol ester of glycine and an appropriate organozinc reagent, which leads us to Route 3 represented in Scheme 6. The preparation of the required alkyliodide **5c** would start from 3-bromopropylamine hydrobromide **3c**. After *N*-acylation, the bromide could be converted into the corresponding iodide by reaction with NaI over several hours. The formation of ethanethiol esters from the corresponding carboxylic acids, as well as their palladium-mediated conversion to aldehydes and ketones by using organozinc reagents is well documented, (e.g. by H. Tokuyama *et. al.*).[41] The product **14** would be obtained after deprotection of **6c** by catalytic hydrogenation.



Scheme 6: *Route 3. Proposed pathway to synthesize aminoacyl carba(dethia)-N-acyl cysteamines by palladium mediated reaction of ethanthiol esters and organozinc reagents as the key step forming the desired C-C bond.*

A further reaction route was elaborated by me on the basis of a HWE reaction and is represented in Scheme 7. As the preparation of phosphonate **10** from commercially available starting materials requires just two relatively simple steps, this pathway appears to be more feasible than the one based on the corresponding Wittig reaction. From the routes discussed in the present section, this one was the first to be tested and it ultimately led to the successful synthesis of the desired probe **14**. The proposed synthesis starts from ethanolamine, which has to be *N*-acylated with decanoylchloride and oxidized to the

corresponding aldehyde to form the carbonyl reagent **12**. The preparation of the phosphonate **10** required for the HWE reaction was planned by reacting diethyl methylphosphonate **10b** and the methyl ester of Cbz-protected glycine **9**. The latter would be prepared from **1e** in hot acidic methanol. According to the plan, the olefin **13** should be formed after deprotonation of compound **10** by addition of aldehyde **12** and subsequently be converted to the product **14** by a Pd-catalysed hydrogenation. More details about the development of this synthetic strategy are discussed in section 6.

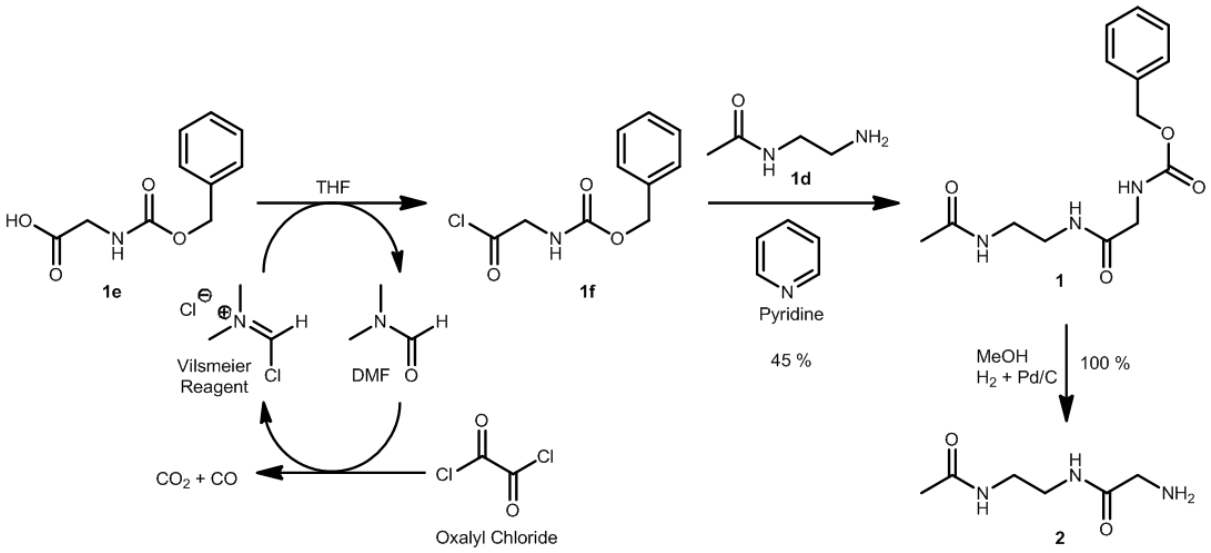


Scheme 7: *Route 4. Proposed pathway to synthesize aminoacyl carba(dethia)-*N*-acyl cysteamines by a HWE reaction as the key step.*

6 Results and Discussion

6.1 Preparation of Aminoacyl amino(dethia)-*N*-acetyl cysteamines

Probe **2**, which belongs to the group of aminoacyl amino(dethia)-*N*-acetyl cysteamines, was prepared by following procedures used by M. Tosin and co-workers in previous projects. These involve the preliminary conversion of the *N*-Cbz-protected amino acid **1e** to the corresponding *N*-Cbz-protected aminoacyl chloride using oxalyl chloride and a small amount of DMF, which catalyses the formation of the acid chloride group as shown in Scheme 8. In the course of the reaction, the DMF is intermediately transformed to the Vilsmeier reagent, which converts the carboxylic acid to the corresponding acid chloride, at the same time being converted back to DMF. The *in situ* formation of the acid chloride group was followed by the coupling with *N*-(2-aminoethyl)-acetamide **1d**, whereby the addition of pyridine is required to neutralize the newly generated hydrochloric acid. Subsequent workup and purification by column chromatography gave the product **1** in 63 % yield. The compound was finally converted to probe **2** by a Pd-mediated hydrogenation, which led to the cleavage of the Cbz-protecting group. For quantitative conversion at r.t., a reaction time of at least 30 min appears to be necessary.



Scheme 8: *Formation of an acid chloride from the corresponding carboxylic acid by using oxalyl chloride as chloride donor and DMF as catalyst. Probe* ***2*** *is prepared from* ***1*** *by Pd-catalysed hydrogenation which leads to cleavage of the Cbz-group.*

The final product **2** was characterised by ESI-HR-MS, ^{1}H-NMR and ^{13}C-NMR spectroscopy. Furthermore, HMBC and HMQC spectra helped to assign the signals. In Figure 15 (A) the calculated high-resolution mass spectrum (lower spectrum) is compared with the real measurement (upper spectrum) which shows a good accordance and proves the correct mass. The ^{1}H-NMR spectrum indicates a clean product since no unexpected signals are present (Figure 15 (B)). The signals of two CH_2 groups have very similar chemical shifts and unfortunately they overlap with the signal of the solvent (methanol) which results in a complex multiplet. However, the presence of all expected signals was proved with the aid of HMBC and HMQC experiments which allowed also the right assignment of the ^{13}C-signals (Figure 15 (C)).

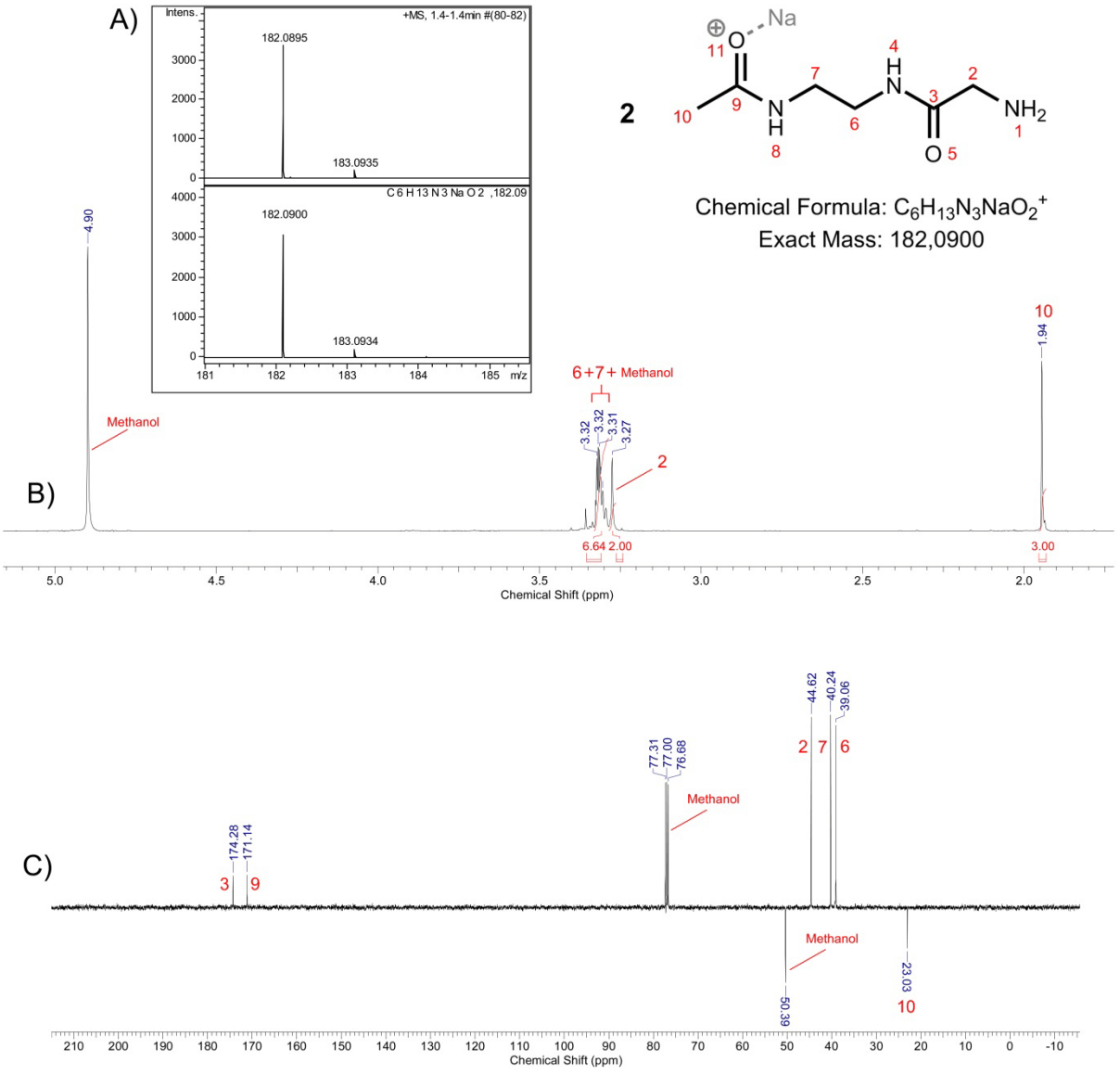
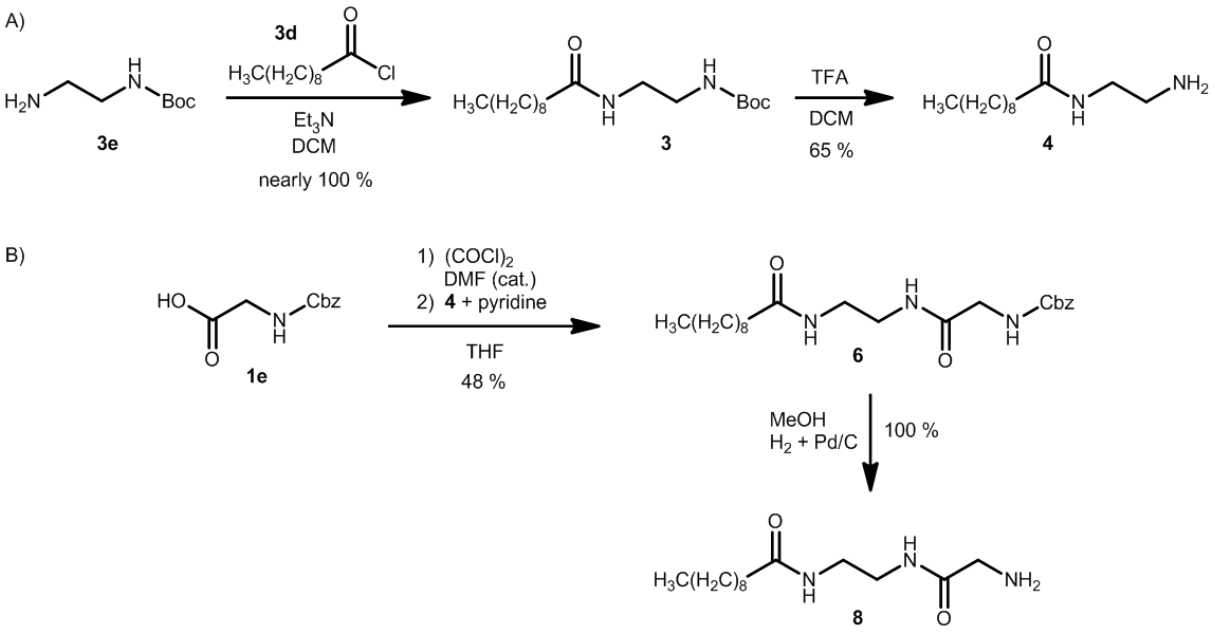


Figure 15: *Characterisation of substance* ***2****. A) ESI-HR-MS. B)* ^{1}*H-NMR. C)* ^{13}*C-NMR.*

6.2 Preparation of Aminoacyl amino(dethia)-*N*-decanoyl cysteamines

The preparation of probe **8**, which belongs to the group of aminoacyl amino(dethia)-*N*-decanoyl cysteamines, was carried out in a similar fashion as the synthesis of probe **2**. However, the reaction route is extended to the synthesis of *N*-(2-aminoethyl) decanamide **4**, as this compound was not found to be commercially available. This synthesis started from Boc-protected ethane-1,2-diamine **3e**, which was converted into **3** by acylation of the free amino group with decanoyl chloride **3d** as shown in Scheme 9 (A). The reaction was carried out in dry dichloromethane under argon atmosphere and addition of triethylamine enabled the neutralization of the newly generated hydrochloric acid. Compound **3** was dissolved in dichloromethane, mixed with trifluoroacetic acid and stirred at room temperature for 16 hours to give compound **4** in 65 % yield. After purification by column chromatography the conversion of amine **4** to compound **6** and subsequent deprotection to yield probe **8** (Scheme 9 (B)) were carried out, similarly to the synthesis of probe **2** (Scheme 8). Purification of compound **6** by column chromatography was not particularly satisfactory, as not all impurities could be removed. Instead, a high purity was achieved by a quick wash with cold methanol, a process which appeared to be as simple as it was crucial to reach also the desired high purity of probe **8** after the subsequent deprotection.



Scheme 9: *A) Reaction route for the synthesis of* N*-(2-aminoethyl) decanamide **4**. B) Subsequent coupling with Cbz-protected glycine yields compound **6** which can be converted to probe **8** by Pd-catalysed hydrogenation.*

Also here, the final product **8** was characterised by ESI-HR-MS, ^{1}H-NMR and ^{13}C-NMR spectroscopy. 2D-NMR experiments like COSY, HMQC and HMBC spectroscopy, helped to assign the signals. The comparison of the calculated mass spectrum with the measured one (Figure 16 (A)), shows good accordance and proves the correct mass. The ^{1}H-NMR spectrum indicates high purity of the product (Figure 16 (B)). Similar to the ^{1}H-NMR spectrum of probe **2**, there are two CH_2 signals with quite similar chemical shifts also in the spectrum of probe **8**. These signals appear again in the region around 3.30 and 3.36 ppm and thus they overlap with the signal of methanol. The result is a complex multiplet. The ^{13}C-signals were assigned with the aid of HMBC and HMQC experiments. However, the overlap of the signals 13-18 in the ^{1}H-NMR spectrum hindered the right assignment of the corresponding ^{13}C-signals which are labelled with the letter 'X' in Figure 16 (C).

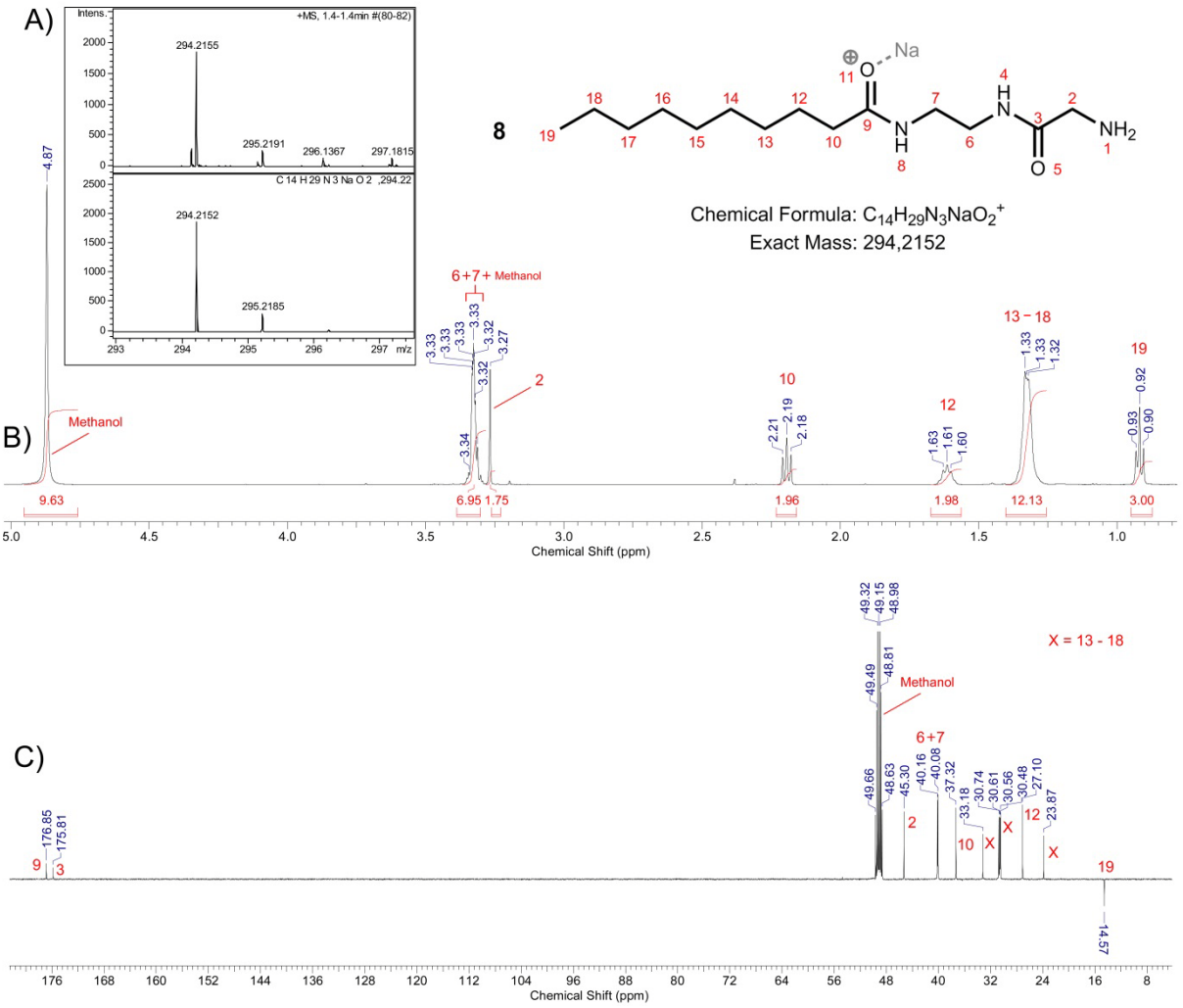
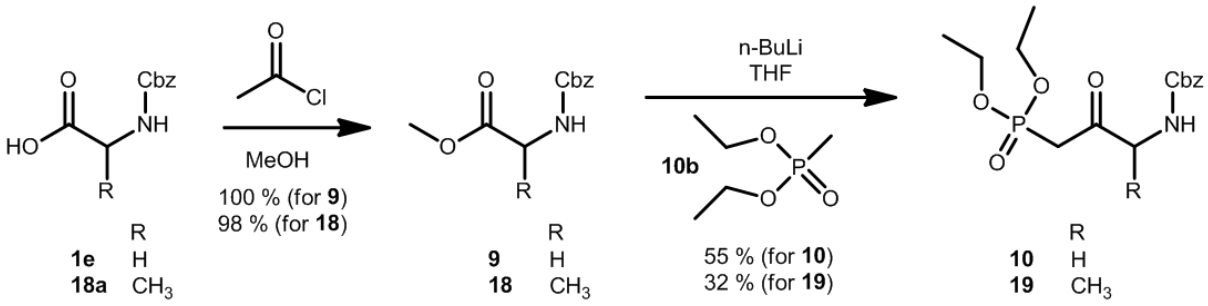


Figure 16: *Characterisation of substance **8**. A) ESI-HR-MS. B) ^{1}H-NMR. C) ^{13}C-NMR.*

6.3 Preparation of Aminoacyl carba(dethia)-*N*-decanoyl cysteamines

The preparation of amino carba(dethia)-*N*-decanoyl cystamines from amino acids proved more demanding than the synthesis of the corresponding amino(dethia)-analogues, in terms of the number and complexity of steps involved. In this section the first successful synthesis of the glycine-based carba(dethia) probe **14** will be presented and discussed. In parallel, the synthesis of the analogous alanine-based probe **21** will be presented and discussed. Based on the retrosynthetic reflections of section 5.2, several routes to prepare compound **14** have been elaborated and route 4 (Scheme 7), by me devised, ultimately proved successful. The pathway starts with the simple preparation of the methyl ester **9** from Cbz-protected glycine **1e**. The reaction was carried out with an oil bath temperature of 95 °C in boiling acidic methanol prepared from acetyl chloride and a large excess of dry methanol, leading to a reliable and quantitative conversion. Within a reaction time of 50 min, no formation of significant amounts of undesired by-products could be observed by TLC, MS and NMR. However, the following conversion of the methyl ester **9** to the phosphonate **10** was by far less efficient and reliable. For this conversion, the methyl group of diethyl methylphosphonate was deprotonated by *n*-BuLi in dry THF at -78 °C to allow the subsequent nucleophilic attack of the methylene group onto the methyl ester, affording the corresponding phosphonate in a maximum of 55 % yield. The reaction was tested several times whereby a slight variation in the reaction conditions led to a remarkable influence on the yield, as shown in Table 1.

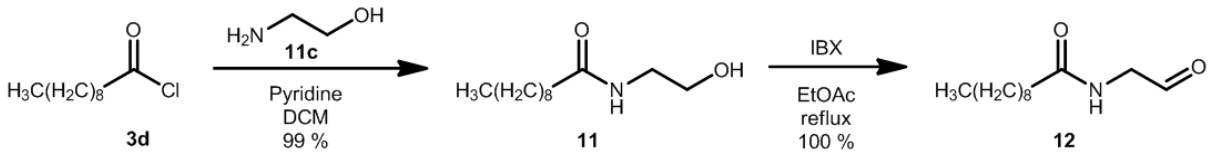


Scheme 10: *Conversion of the Cbz-protected amino acids glycine and alanine to the corresponding methyl esters and further conversion to the phosphonates **10** and **19**.*

Table 1: *Various reaction conditions for the conversion of the methyl esters **9** and **18** to the corresponding phosphonates **10** and **19** respectively.*

Reagent	Conditions	Reaction Time and Temperature	Yield
9	0.04 M; **10b** (2.5 eq.); *n*-BuLi (2.5 eq.);	30 min (-78 °C) then 60 min (-78 to 0 °C)	< 37 %
9	0.22 M; **10b** (2.5 eq.); *n*-BuLi (2.5 eq.);	45 min (-78 °C) then 60 min (-78 to 0 °C)	55 %
9	0.23 M; **10b** (2.0 eq.); *n*-BuLi (2.0 eq.);	80 min (-78 °C) then 80 min (-78 to 0 °C)	24 %
18	0.22 M; **10b** (2.5 eq.); *n*-BuLi (2.5 eq.);	45 min (-78 °C) then 45 min (-78 to 0 °C)	32 %

After the preparation of phosphonate **10**, the second reactant **12** was needed for the HWE reaction. Since this aldehyde was not found to be commercially available, it was synthesized from ethanolamine **11c** and decanoyl chloride **3d**. Although acid chlorides can react with both amino and hydroxyl groups, the coupling reaction was performed prior to oxidation of the hydroxyl group of ethanolamine. This is because the oxidation with IBX is relatively expensive and the amino compound is used in excess for the coupling reaction. Furthermore, the small molecule 2-aminoacetaldehyde is quite volatile and would also most likely be subject to dimerization, cyclisation and spontaneous pyrazine formation on air. In a first attempt to perform the coupling reaction, pure decanoyl chloride was added within one minute to a solution of ethanolamine and triethylamine in cold THF. The mixture was stirred for 30 min at 0 °C and overnight at room temperature. The result was a mix of three products, the *N*-acylated (**11**), the *O*-acylated (**11a**) and the *N,O*-diacylated ethanolamine (**11b**). However, by appropriate choice of the reaction conditions, it was possible to efficiently direct the reaction to the coupling of decanoyl chloride with the amino function, leaving the hydroxyl group unaffected as shown in Table 2. The next step was the oxidation of **11** to the corresponding aldehyde, a reaction which was initially planned to be performed by Swern oxidation. For this reaction, DMSO was added to a solution of oxalyl chloride in dichloromethane under argon atmosphere at low temperature. After addition of **11** and stirring of several minutes, followed by addition of base and workup, the aldehyde **12** was formed. However, in several attempts this method did not lead to a quantitative conversion, and efforts to separate the aldehyde from impurities and starting material by recrystallization and/or column chromatography were not quite satisfactory (43 % and 10 %). This led to the idea of using IBX as oxidative reagent. The use of this proved to be highly suitable for the desired conversion and ultimately led to the reliable formation of a pure product in quantitative yield over a period of 5 h in refluxing EtOAc (Table 3).



Scheme 11: *N-acylation of ethanolamine with decanoyl chloride in DCM using pyridine as base and subsequent oxidation of the hydroxyl group with IBX in refluxing EtOAc to afford pure aldehyde **12** in an overall yield of 99 %.*

Table 2: *Acylation of ethanolamine **11c** with decanoyl choride **3d**. Depending on the reaction conditions different amounts of product **11** (N-acylated) and by-products **11a** (O-acylated) and **11b** (N,O-diacylated) are formed.*

11c	Et_3N	Addition of 3d	Reaction Time and Temperature	11 : 11a : 11b	Yield*
0.42 M; 1.2 eq.	1.2 eq.	4.8 M; 1 min	30 min at 0 °C; 16 h at r.t	59 : 20 : 21	29 %
0.87 M; 2.5 eq.	1.2 eq.	0.3 M; 30 min	30 min at -78 °C; 2 h -78 to 0 °C	100 : 0 : 0	99 %
1.31 M; 3.1 eq.	1.5 eq.	0.3 M; 30 min	30 min at -78 °C; 2 h -78 to 0 °C	100 : 0 : 0	99 %

** Final yield of pure product **11**.*

Table 3: *Oxidation of alcohol **11** to aldehyde **12** using different methods and reaction conditions.*

Method	Conditions	Time	Yield
Swern	95 mM in DCM; DMSO (2.2 eq.); $(COCl)_2$ (1.1 eq.); Et_3N (5 eq.); -60 °C	20 min	74 %*
Swern	93 mM in DCM; DMSO (3.0 eq.); $(COCl)_2$ (1.5 eq.); Et_3N (6 eq.); -60 °C	30 min	43 %
Swern	175 mM in DCM; DMSO (3.6 eq.); $(COCl)_2$ (2.0 eq.); Et_3N (8 eq.); -78 °C	70 min	10 %
IBX	186 mM in EtOAc; IBX (1.8 eq.); reflux 95 °C	5 h	100 %
IBX	198 mM in EtOAc; IBX (1.8 eq.); reflux 95 °C	5 h	100 %

** Calculated on the basis of the integrals of the NMR signals, purification was not successful.*

After compounds **10** and **12** were successfully prepared in good to excellent yields, the HWE reaction, the actual key step of the presently discussed reaction route, was tested. The theory prescribes the usage of a strong base to deprotonate the methylene group adjacent to the phosphorus of phosphonate **10** to enable its nucleophilic attack on the carbonyl group of aldehyde **12**. A variety of different bases and solvents has been tested by Abrunhosa-Thomas *et. al.* to deprotonate representatives of very similar phosphonates. Reportedly the best results could be achieved by using 1.3 eq. of $Ba(OH)_2$ as base with a mixture of THF/H_2O (40:1) as the solvent.[42]

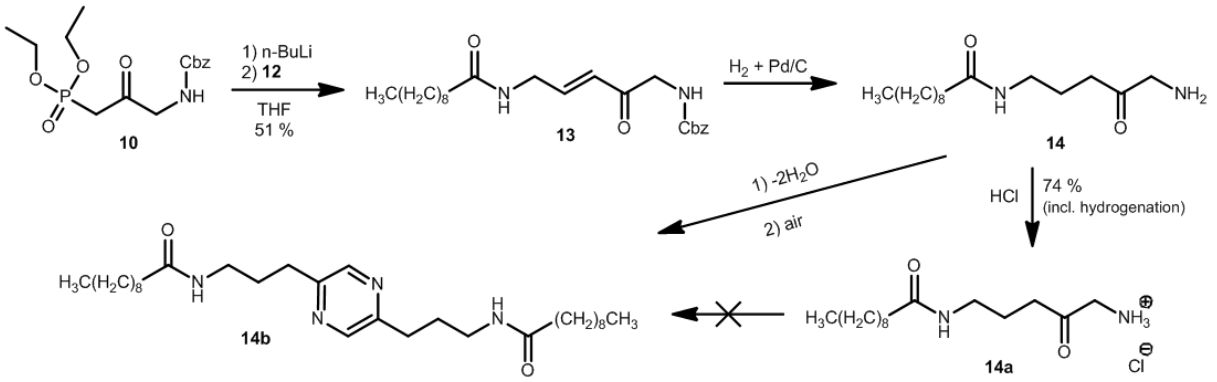
However, these conditions have been proved unsuitable for a successful reaction of **10** with aldehyde **12**. Another base was required and the application of *n*-BuLi led finally to the successful formation of olefin **13**. The phosphonate was dissolved in dry tetrahydrofuran under argon atmosphere and cooled to -78 °C before *n*-BuLi was added. A solution of aldehyde **12** in THF was added dropwise and the mixture was stirred for some time at this temperature, before allowing to reach room temperature. Subsequent workup and purification by recrystallization or column chromatography gave pure product **13** as a white solid. Nevertheless this reaction was very difficult to control and several attempts to perform the reactions under similar conditions led to unreliable yields of purified product in a range between 0 and 51 % (Table 4).

Table 4: *Various conditions for the HWR reaction between phosphonates **10** or **19** and aldehyde **12** have been tested. Yields of pure product appeared to be variable and ranged from 0 to 51 %.*

Reagent	Base (eq.)	Aldehyde	Solvent	Time and Temperature	Yield
10; 47 mM	$Ba(OH)_2$ (1.25 eq.)	**12** (1.05 eq.)	THF/H_2O (40:1)	50 min r.t.	traces*
10; 35 mM	*n*-BuLi (1.10 eq.)	**12** (1.00 eq.)	THF	45 min -78 °C; 70 min r.t.	0 %
10; 26 mM	*n*-BuLi (1.00 eq.)	**12** (0.95 eq.)	THF	30 min -78 °C; 5 h r.t.	traces*[1]
10; 33 mM	*n*-BuLi (1.00 eq.)	**12** (1.05 eq.)	THF	80 min -78 °C; 1 h to 0 °C	88 %*[1]
10; 29 mM	*n*-BuLi (1.00 eq.)	**12** (1.05 eq.)	THF	1 h -78 °C; 1 h to 0 °C	44 %
10; 36 mM	*n*-BuLi (1.00 eq.)	**12** (1.05 eq.)	THF	1 h -78 °C; 1 h to 0 °C	5 %
10; 37 mM	*n*-BuLi (1.00 eq.)	**12** (1.05 eq.)	THF	1 h -78 °C; 75 min to 0 °C	14 %
10; 38 mM	*n*-BuLi (1.00 eq.)	**12** (1.05 eq.)	THF	1 h -78 °C; 1 h to 0 °C	51 %
19; 38 mM	*n*-BuLi (1.00 eq.)	**12** (1.05 eq.)	THF	1 h -78 °C; 1 h to 0 °C	16 %

** According to mass spectra, not purified. *[1] Calculated from integrals of ^{1}H-NMR signals, not purified.*

The final step of the pathway to probe **14** was the cleavage of the Cbz-protecting group and the reduction of the double bond. This could be achieved under the same reaction conditions, namely by hydrogenation mediated by palladium dust on activated carbon. The reaction was carried out in cold methanol under hydrogen atmosphere in the presence of the catalyst. Whereas the reduction of the double bond was ended within approximately five minutes, complete removal of the protecting group took at least 20 times as long, as determined by TLC and MS. Even if this final step was expected to be simple, it ultimately proved quite challenging. After the catalyst was filtered off and the solvent was evaporated, no product was detectable by NMR spectroscopy or mass spectrometry in the first instance. Instead, an unexpected signal appeared in the ^{1}H-NMR spectrum in the aromatic region. This led to the supposition of spontaneous dimerization and cyclisation of substance **14** followed by oxidation on air to the corresponding pyrazine **14b**, an assumption that could be confirmed by ^{13}C-NMR and mass spectrometry. A way to bypass this problem was identified as the conversion of **14** to the corresponding hydrochloride **14a** was proved to enhance its stability towards dimerization (Scheme 12). The addition of 10 equivalents $HCl_{(aq.)}$ 35 % immediately after filtration of the reaction mixture was found to be the most convenient way to achieve this conversion and thus to prevent dimerization. Evaporation of the solvent and further purification led to the pure hydrochloride product **14a** (Table 5).



Scheme 12: *HWR reaction between phosphonate **10** and aldehyde **12** in THF using* n-*BuLi as base. Subsequent deprotection and reduction of the double bond by catalytic hydrogenation followed by treatment with HCl leads to hydrochloride **14a** which seems to be stable toward dimerization and aromatization.*

Table 5: *Various reaction conditions for the simultaneous cleavage of the Cbz-protecting group and the reduction of the double bond of compound **13** and conversion of amine **14** to the corresponding hydrochloride **14a**.*

Reagent	Pd/C	Time and Temperature	HCl	Yield
13; 9 mM	10 mol%	16 h; r.t.	-	0 %
13; 12 mM	10 mol%	80 min; 50 °C to r.t.	-	33 %*
13; 5 mM	10 mol%	100 min; -13 °C to -4 °C	$HCl_{(aq.)}$ 10 %; extraction of crude product	26 %
13; 4 mM	10 mol%	100 min; 0 °C	$HCl_{(aq.)}$ 10 % (excess); addition after filtration	70 %*
13; 7 mM	10 mol%	4 h; 0 °C	$HCl_{(aq.)}$ 35 % (10 eq.); addition after filtration	74 %

** Calculated from integrals of ^{1}H-NMR signals; purification not successful.*

Figure 17 illustrates the characterisation of the final product **14a** by ESI-HR-MS, ^{1}H-NMR and ^{13}C-NMR spectroscopy. HMBC and HMQC spectra were used to assign the signals. By comparison of the calculated mass spectrum with the measured one, the correct mass was proved (Figure 17 (A)). Figure 17 (B) shows a clean ^{1}H-NMR spectrum without the presence of unexpected signals. This proves the first successful synthesis and purification of substance **14a**, the key compound of this project. The ^{13}C-signals were assigned with the aid of HMBC and HMQC experiments. Also here, the right assignment of the ^{13}C-signals of the decanoyl chain, which are labelled with the letter 'X' in Figure 17 (C), was hindered due to their very similar chemical shifts and the overlap of the corresponding ^{1}H-signals. The insignificant signal at 203.72 ppm, labelled with the number 3 in the ^{13}C-spectrum, was clearly present in the HMBC spectrum at this chemical shift.

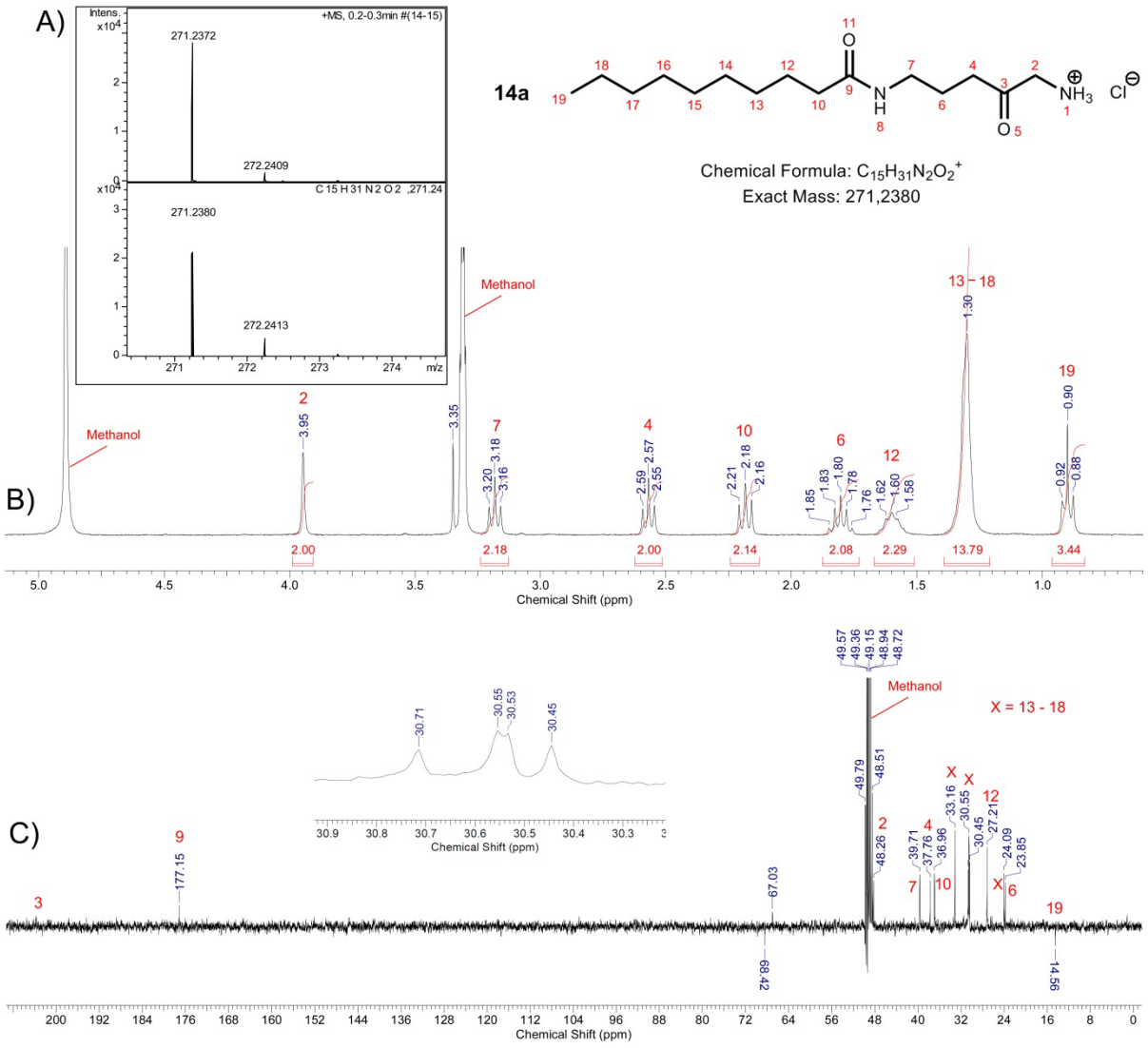


Figure 17: *Characterisation of substance **14a**. A) ESI-HR-MS. B) ^{1}H-NMR. C) ^{13}C-NMR.*

6.4 Culturing of *Streptomyces Lasaliensis* and Analysis

After their preparation and purification, probes **2**, **8**, **17** and **14a** were preliminarily tested for their ability to off-load intermediates from the echinomycin NRPS in an engineered strain of *Streptomyces lasaliensis* (*S. lasaliensis* ACP12 (S970A) mutant). This strain was previously constructed from *S. lasaliensis* NRRL 3382. A point mutation at position 970 in ACP12, caused a replacement of its active serine by alanine. As a result the strain doesn't produce the polyketide lasalocid A, but only echinomycin.[40]

A frozen sample (100 µL) of *S. lasaliensis* ACP12 (S970A) was thawed and added to 10 mL of M79 liquid medium. After two to three days of incubation at 30 °C, this preculture was ready to be transferred into MYM liquid medium to generate a production culture, which was grown for 5 days at 30 °C. The feeding of the probes was carried out by daily addition of a methanolic solution of the probe of interest. The increase in the final concentration was in the range of 0.1 to 1 mM per day. Subsequent extraction of the cultures with EtOAc and BuOH, followed by evaporation of the solvents, resolution of the residue in MeOH/H_2O and filtration, afforded suitable samples for HR-LC-MS analyses (Figure 18).

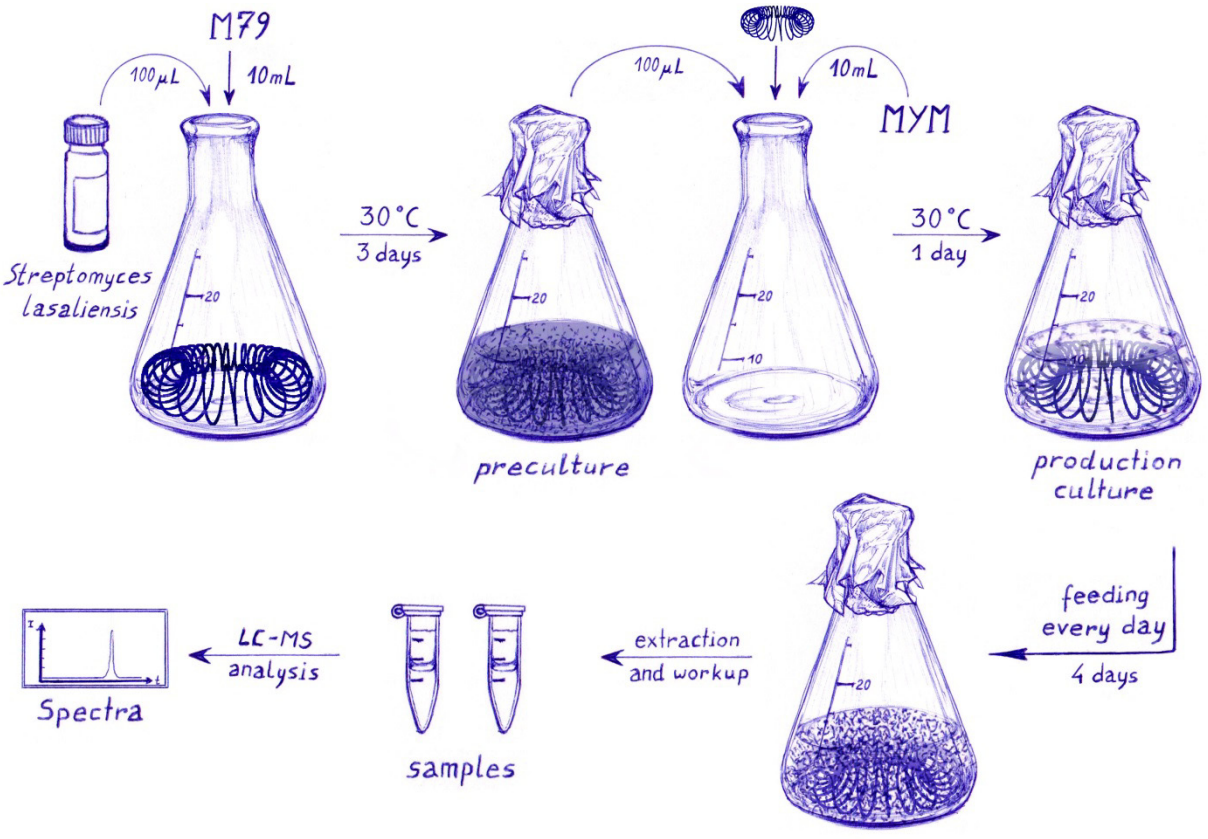


Figure 18: *Culturing, feeding and extraction of* S. lasaliensis *ACP12 (S970A) and subsequent LC-MS analyses.*

The HR-LC-MS analyses provided spectra which gave the signal intensity in dependency of the retention time and the *m/z* ratio of the ions. This allows the automated filtering of the signal/time spectra of individual ions, which strongly facilitates the detection of eventually off-loaded intermediates. The high resolution of the analyses is crucial to assign a detected mass to the correct compound. Moreover, the high number of different substances in the extracts of biological systems results in a huge number of the corresponding signals in the MS-spectra and an overlap of signals could falsify the result.

In order to enable an automated filtering of the signal/time spectra of specific *m/z* values, the corresponding software was provided with a list of all the masses of the potentially off-loaded intermediate species as H^+, Na^+ or NH_4^+ adducts. A list of the intermediates which can possibly be off-loaded by probe **2** is illustrated in Figure 19.

So far neither one of these masses nor a mass corresponding to an intermediate expected to be off-loaded by probe **8** could be detected. This may possibly be due to the observed toxic effects of the probes, which resulted in the death of the bacteria at high concentrations (~1 mM per day) while nontoxic concentrations (~0.5 mM per day) might be too low to allow the successful trapping of detectable amounts of intermediates. Accurate adjustment of the right dose will be crucial to handle this problem in further projects. On the other hand even the selectivity of the C domains could likely be responsible for the so far unsuccessful off-loading of intermediates. To verify this, probes derived from alanine were fed to *S. lasaliensis* ACP12 (S970A) with the aim to off-load at least the corresponding tripeptide, the intermediate formed by incorporation of alanine in the echinomycin biosynthesis. However, the alanine probe **17** (Figure 14) was not synthesized by me and the preparation of the alanine probe **21** could not be finished in time. Moreover, due to time constrains, extracts of feeding experiments with the probes **17** and **14a** could not be analysed in the course of the laboratory work. Later analyses have not delivered the expected results so far.

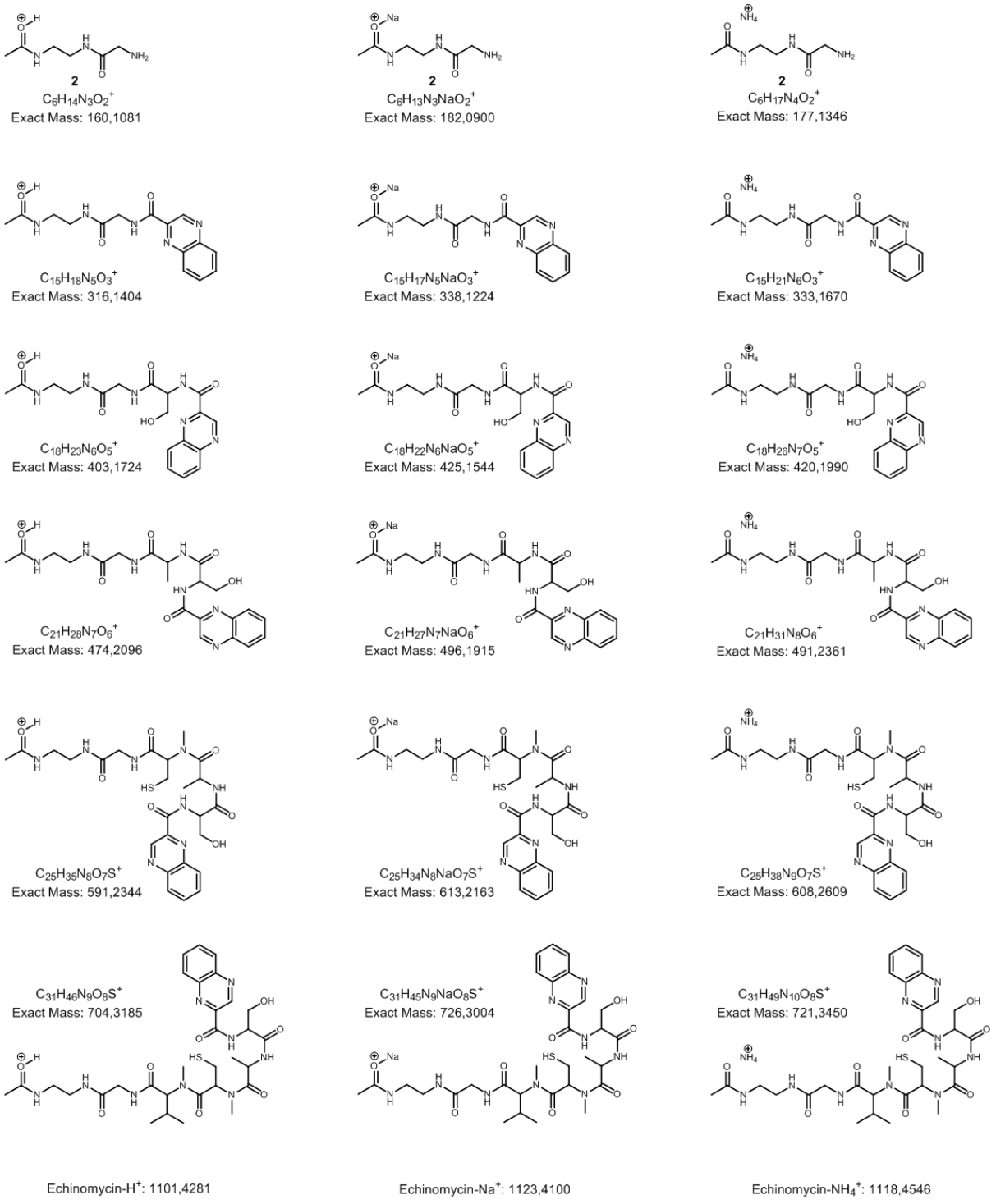


Figure 19: *List of expected intermediates to be identified in the extracts from* S. lasaliensis *ACP12 (S970A) fed with probe* ***2****. Only the masses corresponding to the probe itself and to echinomycin were found so far.*

7 Conclusions and Future Work

The task of this master project can be subdivided into three parts. The main part was the synthesis and validation of novel chemical probes, which are expected to be suitable for the *in vivo* off-loading of intermediates from NRPSs. This was followed by the second part, the culturing of the echinomycin producing strain *S. lasaliensis* ACP12 (S970A), the feeding of the purified probes and the extraction of the fed cultures. The third and last step was the HR-LC-MS analysis of the extracts and the evaluation of the generated spectra.

To the best of my knowledge, this work presents the first successful synthesis, purification and characterisation of the key compound **14a** as well as of probe **8** and various precursors, as illustrated in Figure 20. In the course of the laboratory work, the amino(dethia) probes **2**, **8** and **17** and the carba(dethia) probe **14a** were fed to cultures of *S. lasaliensis* ACP12 (S970A) in different feeding experiments. The adjustment of the right concentration of the probes was proved to be crucial for the survival of the bacteria. Due to time constrains, not all the extracts of the cultures could be analysed by HR-LC-MS during the laboratory work, but they could recently be analysed by other group members (see section 9.19). Unfortunately, so far no off-loaded intermediates could be detected in any of the analysed extracts. Instead, the mass spectra of the extracts showed always a good amount of intact probe and echinomycin. An example of this is illustrated in Figure 21. This observation indicates that nontoxic concentrations of the tested probes do possibly not affect the biosynthesis of echinomycin in *S. lasaliensis* ACP12 (S970A).

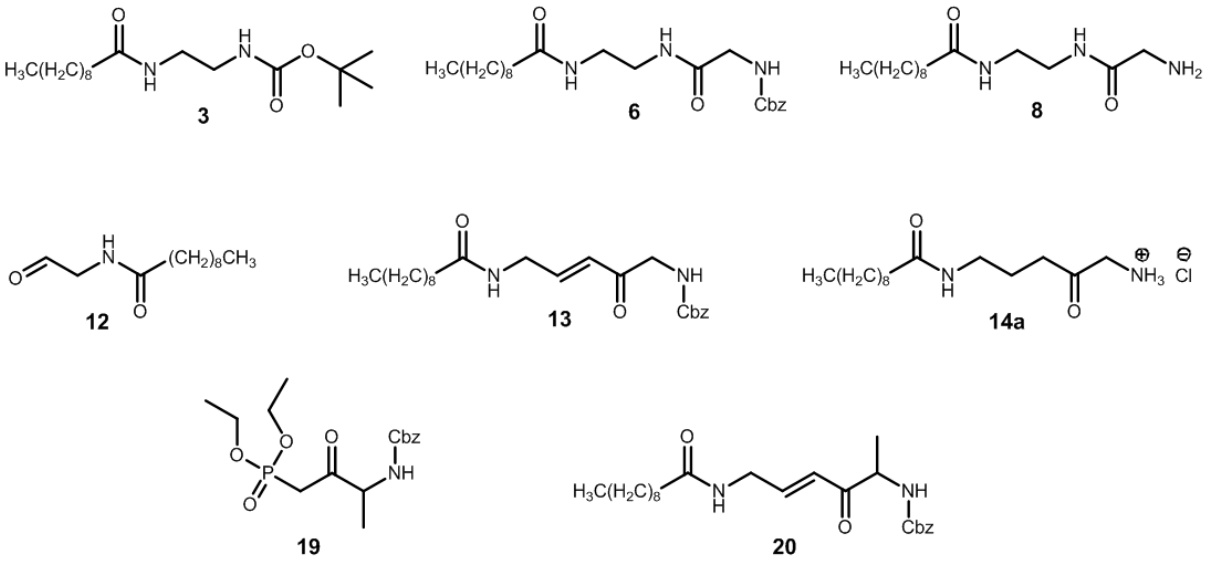


Figure 20: *In the course of the laboratory work, these compounds were prepared, purified and characterised for the first time.*

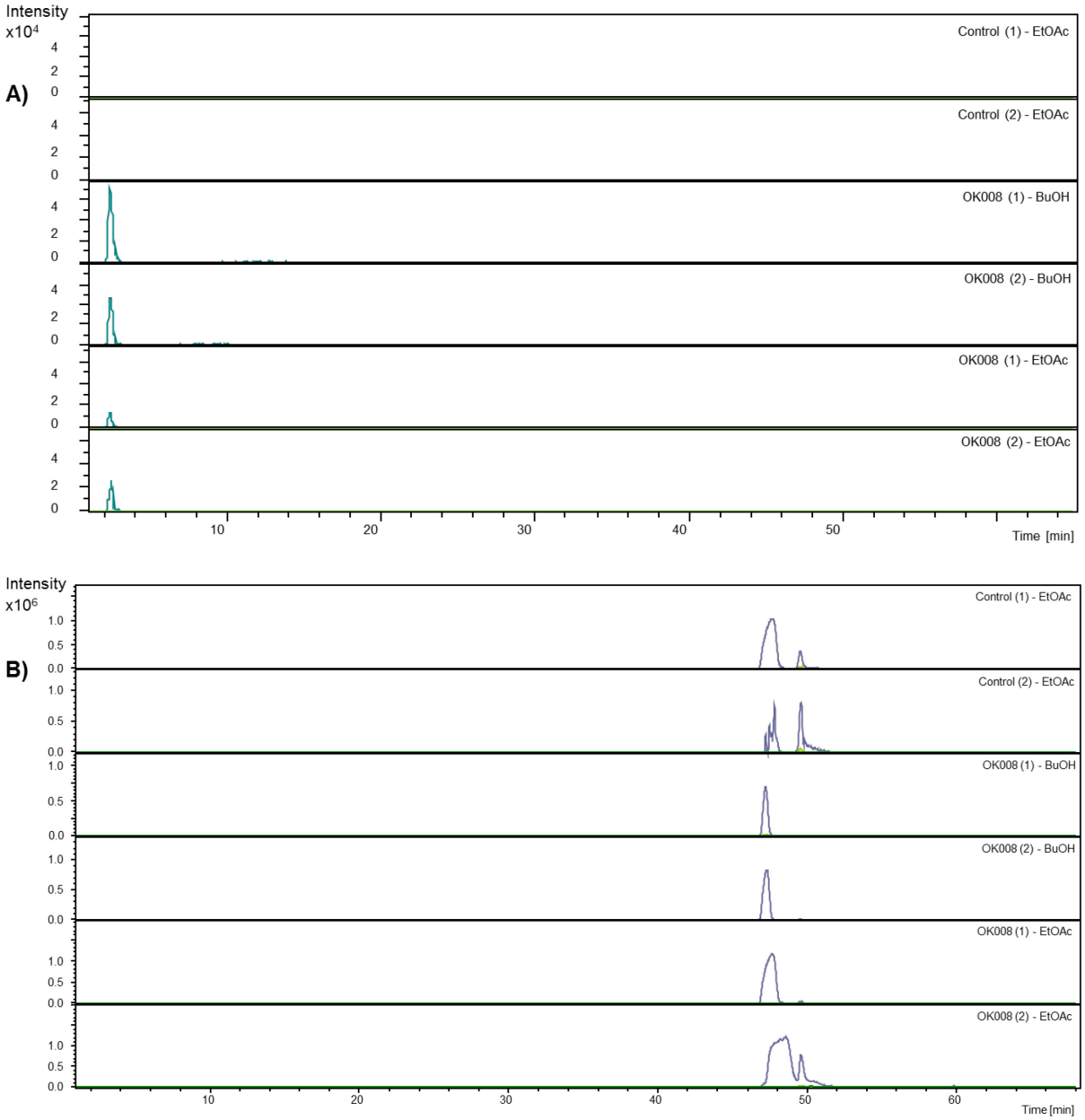


Figure 21: *HR-LC-MS spactra of extracts of* S. lasaliensis *ACP12 (S970A) fed with probe* ***2****. A) The spectra show the signals of the* Na^+ *adduct of probe* ***2*** *(mass: 182.0900) as a function of the retention time. The signals of the corresponding* H^+ *and* NH_4^+ *adducts are present with much lower intensity at the same retention time. The peaks prove the presence of probe* ***2*** *in the EtOAc and BuOH extracts. B) The spectra show the signals of the* H^+ *adduct of echinomycin (mass: 1101.4281) as a function of the retention time. The signals of the corresponding* Na^+ *and* NH_4^+ *adducts are present with much lower intensity at the same retention time. The peaks prove the presence of echinomycin in the EtOAc and BuOH extracts.*

Future work needs to be done which will involve the synthesis of other amino(dethia) and carba(dethia) compounds (such as e.g. probe **16**, or deuterated probes), the retesting of this probes in further feeding experiments and different conditions, the usage of different NRP producing strains and the adjustment of the optimal dose of the tested probes, for the feeding to the corresponding bacteria.

8 General Experimental Procedures

8.1 Materials and Methods for Synthetic Chemistry

- In general the progress of a reaction was determined by TLC and suitable mixtures of different solvents were used as mobile phase. TLC staining was carried out by dipping the TLC plate into a solution of $KMnO_4$, and subsequent heating with a heat gun, as long as the substance of interest was not absorbing ultraviolet light. In certain cases the staining occurred by immersing the TLC plates into suitable dips containing ninhydrin or vanillin.
- For water free reactions, the glassware was dried in the drying oven over night. The apparatus was set up while the glassware was still hot and the system was immediately flushed with argon and closed with glass stoppers or septa. Substrates, reagents and solvents were transferred into the flasks by the use of syringes or cannulas.
- Cooling baths in a temperature range between -78 and -20 °C were prepared by appropriate mixtures of acetone and dry ice, cooling baths between -20 and 10 °C were prepared by mixing ice with sodium chloride or water.
- The storage of sensitive products or chemicals occurred in the refrigerator or in the freezer and if necessary under argon atmosphere.
- The separation of the products from by-products or other impurities was usually carried out by using column chromatography with silica gel as stationary phase and suitable mixtures of different solvents as eluent. Other common methods of purification were recrystallization, washing and filtration.
- Magnetic stirring was the common method used for complete mixing of the reactants.
- Normally solvents were removed on the rotary evaporator, the complete removal of the last traces of solvent occurred under vacuum (<1 mbar) for several hours and if necessary at elevated temperatures.
- The storage of anhydrous solvents occurred over suitable desiccants and stabilizers in dark glass bottles and usually under argon atmosphere.
- ^{1}H- and ^{13}C-NMR spectra as well as COSY, HMQC and HMBC spectra were measured by using various spectrometers from Bruker. The chemical shifts are indicated in parts per million [ppm] and the coupling constants (*J*) are indicated in hertz [Hz]. Usually the measurements were carried out at room temperature in deuterated chloroform or methanol and the spactra were referenced on the signal of the respective solvent.
- Low resolution MS data were recorded on Agilent 6130B single Quad (ESI) and high resolution MS data were acquired using ESI on a MaXis UHR-TOF (Bruker Daltonics).

8.2 Materials and Methods for Microbiology

- All glass-ware and media were sterilized by autoclaving before being used and media were transferred with the aid of sterile disposable pipets in a horizontal laminar flow hood.
- Cultures were grown in Erlenmeyer flasks equipped with a steel coil by shaking at 30 °C in a *New Brunswick Innova 44 Incubator Shaker*.
- Precultures were grown in M79 medium over a period of two to three days; production cultures were grown in MYM medium over a period of 5 days.
- Feeding was carried out by daily addition of a methanolic solution of 1 to 10 µmol of the probe of interest over a period of 4 days. This corresponds to an increase in the final concentration of 0.1 to 1 mM per day.
- Bacteria (*S. lasaliensis* ACP12 (S970A)) were stored in a low temperature freezer at -78 °C.
- The recipes for the liquid media used for the cultivation of *S. lasaliensis* ACP12 (S970A) are as follows:

  M79 (250mL): 2.5 g Glucose, 2.5 g Peptone, 0.5 g Yeast extract, 1.5 g NaCl, 2.5 g Casamino Acids, Tab water, pH = 7.1 (adjusted with NaOH), autoclaved.

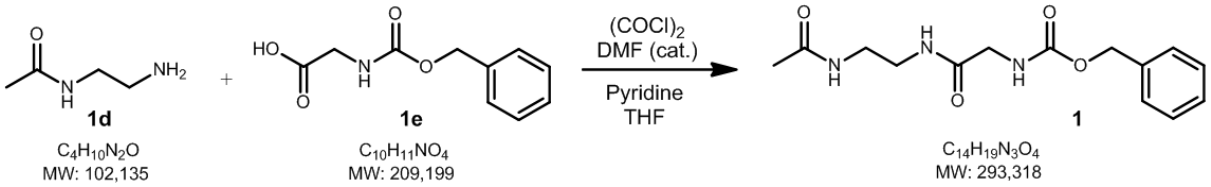
  MYM (500mL): 2 g Maltose, 2 g Yeast extract, 5 g Malt extract, Tab water, pH = 7.0 (adjusted with NaOH), autoclaved.

8.3 Materials and Methods LC-MS analyses

UPLC-HR-ESI-MS analyses of the extracts of *S. lasaliensis* ACP12 (S970A) were recorded on a MaXis Impact UHR-TOF (Bruker Daltonics) instrument. 5 µL of the samples were injected onto an Acquity UPLC HSS T3 column (150 mm x 1.0 mm, 1.8 µm). As mobile phase a gradient of water and acetonitrile was used while both solvents were mixed with 0.1 % TFA. For the analyses of the extracts the following method was applied (A = 0.1 % TFA in H_2O, B = 0.1 % TFA in MeCN): 10% B 0-2.7 min; 10-100% B 2.7-42.7 min; 100% B 42.7-62.7 min; 100-10% B 62.7-65.7 min; 10% B 65.7-77.7 min. The flow rate was set to 0.05 mL/min. All spectra were recorded in positive ionisation mode and scanned from *m/z* 100 to 3000.

9 Experimental Section

9.1 Benzyl (2-((2-acetamidoethyl)amino)-2-oxyethyl)carbamate (1)



Scheme 13: *Synthesis of Benzyl (2-((2-acetamidoethyl)amino)-2-oxyethyl)carbamate*

Table 6: *Substances used for the synthesis of benzyl (2-((2-acetamidoethyl)amino)-2-oxyethyl)carbamate:*

Substance	m[g]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
Z-glycine amide	1.00	209.20	4.78	-	-	1.00
N-(2-aminoethyl)-acetamide	0.60	102.135	5.87	-	-	1.23
Oxalyl chloride	0.61	126.93	4.78	1.48	0.41	1.00
DMF	0.10	73.10	1.30	0.95	0.10	0.27
Pyridine	0.80	79.10	10.16	0.98	0.82	2.13
THF	-	-	-	-	50 + 10	solvent

Procedure:

Z-glycine amide was dissolved in dry THF (50 mL) and cooled to 0 °C. The flask was filled with argon before oxalyl chloride and DMF were added to the solution. The mixture was stirred for 2 h at 0 °C and 2 h at room temperature. In a separate flask *N*-(2-aminoethyl)-acetamide and pyridine were dissolved in dry THF (10 mL). This solution was completely dried in the presence of 3 Å molecular sieves. Following, the dried solution was added to the solution of the Z-glycine amide. The solution was cooled to 0 °C for 15 min and stirred over night at room temperature. The solvent was removed under reduced pressure providing a yellowish product (2 phases, solid and oil). The crude product was purified by flash chromatography (silica gel (400 mesh); EtOAc/MeOH, 10:1 to 7:3) to give compound **1** as a white solid.

Result:

Yield	NMR	Name	Solvent	Notes
625 mg (45 %)	^{1}H	*OK 001 F 27-40*	CD_3OD	pure product
254 mg (18 %)	^{1}H	*OK 001 F 41-49*	CD_3OD	product (slightly impure)

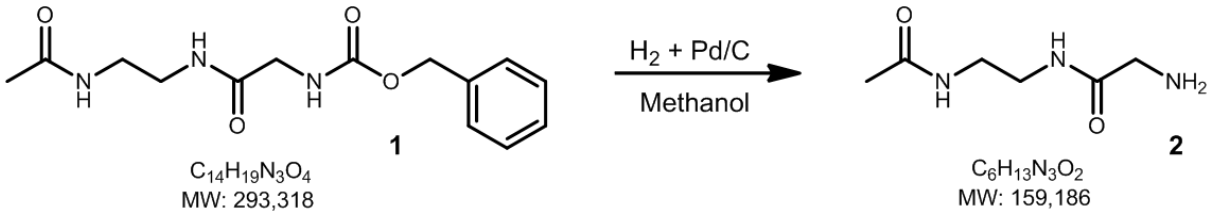
***R_f*:**
0.42 (EtOAc:MeOH, 4:1)

^{1}H-NMR (CD_3OD; 400 MHz) δ [ppm]
δ = 1.93 (s, 3 H), 3.25-3.32 (m, 4 H), 3.37 (s, 2 H), 5.11 (s, 2 H), 7.27-7.40 (m, 5 H)

^{13}C-NMR (CD_3OD; 151 MHz) δ [ppm]
δ = 22.8, 40.1, 40.2, 45.1, 68.1, 129.1, 129.2, 129.6, 138.2, 159.3, 172.8, 173.9

ESI-HR-MS:
m/z calculated for $C_{14}H_{20}N_3O_4^+$ $[M+H]^+$: 294.1448, found: 294.1452

9.2 *N*-(2-acetamidoethyl)-2-aminoacetamide (2)



$C_{14}H_{19}N_3O_4$
MW: 293,318

$C_6H_{13}N_3O_2$
MW: 159,186

Scheme 14: *Synthesis of* N-*(2-acetamidoethyl)-2-aminoacetamide*

Table 7: *Substances used for the synthesis of* N-*(2-acetamidoethyl)-2-aminoacetamide:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[w%]	V[mL]	eq.
1	100	293.318	0.341	-	-	1
Pd/C	36	106.420	0.034	10	-	0.1
dry methanol	-	-	-	-	5 + 5	solvent
H_2	-	-	-	-	-	excess

Procedure:

A 25-mL round bottom flask was equipped with a magnetic stirrer and filled with argon. Pd/C and 5 mL dry MeOH were added and the argon was replaced by H_2. Substance **1** was dissolved in 5 mL dry MeOH under argon atmosphere and added all at once to the suspension of Pd/C. The reaction mixture was stirred for 2 h at room temperature and filtered over Celite. The solvent was removed under reduced pressure providing a white solid product.

Result:

Yield - crude	NMR	Name	Solvent	Notes
54 mg (100 %)	^{1}H	*OK 055 crude*	$CDCl_3$	product (traces of solvents)

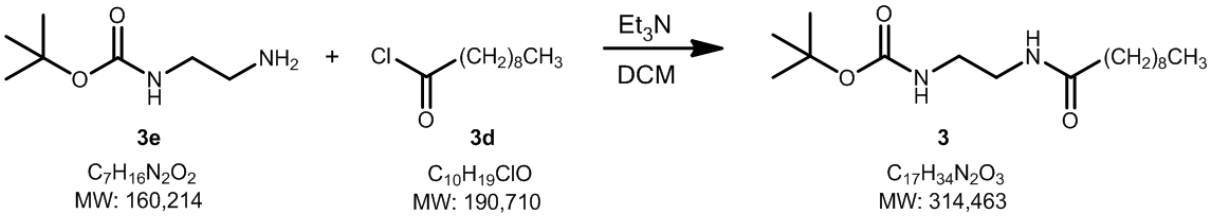
***R_f*:**
0.44 (Acetone:MeOH, 3:1 + 1% NH_3)

1H-NMR (CD_3OD; 400 MHz) δ [ppm]
δ = 1.94 (s, 3 H), 3.27 (s, 2 H), 3.28-3.35 (m, 4 H + MeOD-signal)

13C-NMR (CD_3OD; 101 MHz) δ [ppm]
δ = 23.0, 39.1, 40.2, 44.6, 171.1, 174.3

ESI-HR-MS:
m/z calculated for $C_6H_{13}N_3NaO_2^+$ $[M+Na]^+$: 182.0900, found: 182.0895

9.3 *tert*-Butyl (2-decanamidoethyl)carbamate (3)



Scheme 15: *Synthesis of* tert-*butyl (2-decanamidoethyl)carbamate*

Table 8: *Substances used for the synthesis of* tert-*butyl (2-decanamidoethyl)carbamate*

Substance	m[mg]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
tert-butyl (2-aminoethyl)carbamate **3e**	500	160.21	3.12	1.016	0.492	1
Decanoyl chloride	773	190.71	4.06	0.919	0.842	1.3
Et_3N	632	101.19	6.24	0.728	0.868	2
DCM	-	-	-	-	15	solvent

Procedure:

tert-Butyl (2-aminoethyl)carbamate was dissolved in dry DCM under argon atmosphere. Et_3N was added and the mixture was cooled to 0 °C. After 15 min of stirring the decanoyl chloride was added. The mixture was stirred for further 30 min at 0 °C and overnight at room temperature. The solution was washed with 10 mL of 1 M HCl, 10 mL of sat. $NaHCO_{3\,(aq.)}$ and 10 mL of brine. The organic phase was separated, dried with Na_2SO_4 and filtered. The solvent was removed under reduced pressure to yield compound **3** as a white porous solid.

Result:

Yield - crude	NMR	Name	Solvent	Notes
1.006 g (105 %)	^1H	*OK 009 crude*	$CDCl_3$	product + water

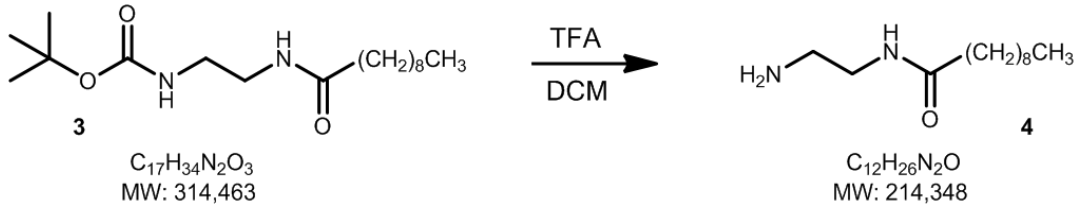
***R_f*:**
0.50 (EtOAc)

1H-NMR ($CDCl_3$; 400 MHz) δ [ppm]
δ = 0.88 (t, *J* = 7.0 Hz, 3 H), 1.20-1.35 (m, 12 H), 1.45 (s, 3 H), 1.57-1.70 (m, 2 H), 2.17 (t, *J* = 8.0 Hz, 2 H), 3.25-3.32 (m, 2 H), 3.34-3.40 (m, 2 H), 4.92 (br s, 1 H), 6.15 (br s, 1 H)

13C-NMR (CD_3OD; 151 MHz) δ [ppm]
δ = 14.6, l23.9, 27.1, 28.9, 30.5, 30.6, 30.6, 30.7, 33.2, 37.3, 40.6, 41.1, 80.3, 158.7, 176.8

ESI-HR-MS:
m/z calculated for $C_{17}H_{35}N_2O_3^+$ $[M+H]^+$: 315.2642, found: 315.2641

9.4 *N*-(2-Aminoethyl)decanamide (4)



Scheme 16: *Synthesis of* N-*(2-aminoethyl)decanamide*

Table 9: *Substances used for the synthesis of* N-*(2-aminoethyl)decanamide:*

Substance	m[mg]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
3	1000	314.46	3.18	-	-	1
TFA	3626	114.02	31.80	1.489	2.435	10
DCM	-	-	-	-	40	solvent

Procedure:

Substance **3** was dissolved in DCM, TFA was added and the mixture was stirred at room temperature for 16 hours. The sovent was removed under reduced pressure and residual TFA was quenched with Et_3N. The yellow oil was purified by column chromatography (silica gel; Acetone/MeOH 2:1) to give compound **4** as a white solid (446 mg, 65 % yield).

Result:

Yield	NMR	Name	Solvent	Notes
446 mg (65 %)	^{1}H	*OK 011 F12-25*	CD_3OD	product

***R_f*:**
0.45 (Acetone/MeOH, 2:1)

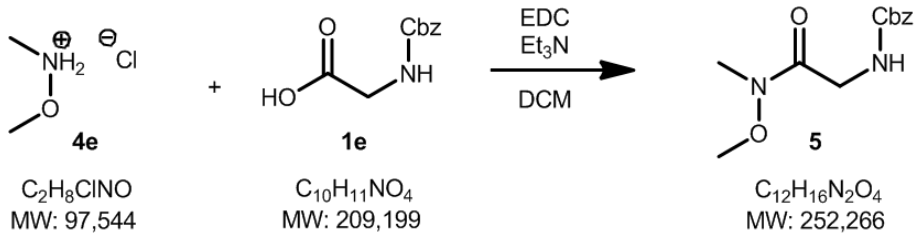
^{1}H-NMR (CD_3OD; 400 MHz) δ [ppm]
δ = 0.90 (t, *J* = 7.0 Hz, 3 H), 1.22-1.38 (m, 12 H), 1.55-1.65 (m, 2 H), 2.19 (t, *J* = 7.5 Hz, 2 H), 2.72 (t, *J* = 6.4 Hz, 2 H), 3.24 (t, *J* = 6.3 Hz, 2 H)

^{13}C-NMR (CD_3OD; 151 MHz) δ [ppm]
δ = 14.6, 23.9, 27.1, 30.5, 30.5, 30.6, 30.7, 33.2, 37.3, 42.1, 42.9, 176.6

ESI-HR-MS:
m/z calculated for $C_{12}H_{27}N_2O^+$ $[M+H]^+$: 215.2118, found: 215.2109

Notes:
The reaction time of 16 h is probably too much.

9.5 Benzyl (2-(methoxy(methyl)amino)-2-oxoethyl)carbamate (5)



Scheme 17: *Synthesis of benzyl (2-(methoxy(methyl)amino)-2-oxoethyl)carbamate*

Table 10: *Substances used for the synthesis of benzyl (2-(methoxy(methyl)amino)-2-oxoethyl)carbamate:*

Substance	m[mg]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
N-Carbobenzyloxy glycine	500	209.199	2.39	-	-	1
N,O-Dimethyl-hydroxylamine * HCl	256	97.544	2.63	-	-	1.1
EDC hydrochloride	504	191.702	2.63	-	-	1.1
Et_3N	484	101.19	4.78	0.728	0.664	2
DCM	-	-	-	-	15	solvent

Procedure:

N-Carbobenzyloxy glycine was dissolved in dry DCM under argon atmosphere. Et_3N, *N,O*-dimethyl-hydroxylamine * HCl and EDC were added and stirred for ~65 h at room temperature. The mixture was washed with 10 mL of 1 M HCl, 10 mL of sat. $NaHCO_{3\ (aq.)}$ and 10 mL of brine. The organic phase was dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure leaving a colourless milky oil which crystallized after some days.

Result:

Yield - crude	NMR	Name	Solvent	Notes
396 mg (66 %)	^1H	*OK 005 crude*	$CDCl_3$	product + water

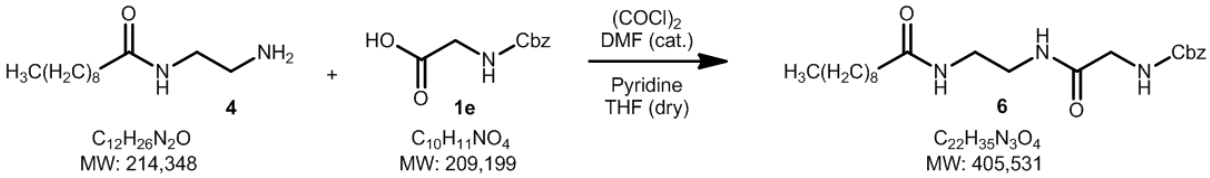
***R_f*:**
0.50 (EtOAc)

1H-NMR ($CDCl_3$; 400 MHz) δ [ppm]
δ = 3.22 (s, 3 H), 3.73 (s, 3 H), 4.16 (d, *J* = 4.3 Hz, 2 H), 5.14 (s, 2 H), 5.54 (br s, 1 H), 7.30-7.41 (m, 5 H)

13C-NMR (CD_3OD; 151 MHz) δ [ppm]
δ = 32.7, 42.8, 62.0, 67.9, 129.0, 129.1, 129.6, 138.4, 159.3, 172.3

ESI-HR-MS:
m/z calculated for $C_{12}H_{17}N_2O_4^+$ $[M+H]^+$: 253.1183, found: 253.1183

9.6 Benzyl (2-((2-decanamidoethyl)amino)-2-oxoethyl)carbamate (6)



Scheme 18: *Synthesis of benzyl (2-((2-decanamidoethyl)amino)-2-oxoethyl)carbamate*

Table 11: *Substances used for the synthesis of benzyl (2-((2-decanamidoethyl)amino)-2-oxoethyl)carbamate:*

Substance	m[mg]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
4	319	214.348	1.488	-	-	1
N-carbobenzyloxy glycine	342	209.199	1.637			1.1
Oxalyl chloride	208	126.930	1.637	1.48	0.140	1.1
DMF	29	73.100	0.402	0.95	0.031	0.27
Pyridine	251	79.100	3.170	0.98	0.256	2.13
THF	-	-	-	-	20 + 10	solvent

Procedure:

Substance **4** and pyridine were dissolved in 20 mL dry THF under argon atmosphere and stirred for 2 h at room temperature in the presence of 3 Å molecular sieves. In a separate round bottom flask the *N*-carbobenzyloxy glycine was dissolved in 10 mL dry THF under argon atmosphere and stirred for a few minutes at 0° C before oxalyl chloride and DMF were added. This mixture was stirred for 1 h at 0° C and for 10 min at room temperature. The solution of substance **4** was added and the reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure providing a brown solid residue which was suspended in ~ 2 mL cold MeOH and filtered. The clean and white solid was washed with Et_2O and dried under vacuum.

Result:

Yield	NMR	Name	Solvent	Notes
289 mg (48 %)	^{1}H	*OK 014 washed*	$CDCl_3$	clean product

R_f:
0.44 (EtOAc/MeOH, 9:1)

^{1}H-NMR ($CDCl_3$; 400 MHz) δ [ppm]

δ = 0.88 (t, *J* = 6.8 Hz, 3 H), 1.20-1.32 (m, 12 H), 1.57-1.64 (m, 2 H), 2.17 (t, *J* = 7.7 Hz, 2 H), 3.41 (s, 4 H), 3.85 (d, *J* = 5.8 Hz, 2 H), 5.36 (br s, 1 H), 5.99 (br s, 1 H), 6.70 (br s, 1 H), 7.33-7.38 (m, 5 H)

^{13}C-NMR (CD_3OD; 126 MHz) δ [ppm]

δ = 14.4, 23.7, 27.0, 30.4, 30.5, 30.5, 30.6, 33.1, 37.4, 40.1, 40.5, 45.4, 68.1, 129.0, 129.2, 129.6, 138.3, 159.5, 172.7, 176.9

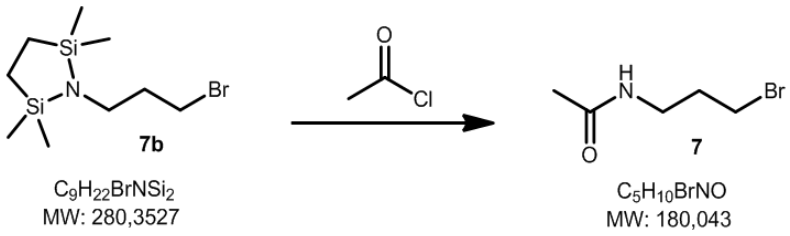
ESI-HR-MS:

m/z calculated for $C_{22}H_{35}N_3NaO_4^+$ $[M+Na]^+$: 428.2520, found: 428.2523

Note:

The ^{13}C-signal on position 159.50 could only be detected in HMBC experiments.

9.7 *N*-(3-Bromopropyl)acetamide (7)



Scheme 19: *Synthesis of* N-*(3-bromopropyl)acetamide*

Table 12: *Substances used for the synthesis of* N-*(3-bromopropyl)acetamide:*

Substance	m[mg]	M[g/mol]	n[mmol]	V[mL]	eq.
7b	70	280,35	0,25	-	1
Acetyl chloride	-	-	-	0,5	solvent

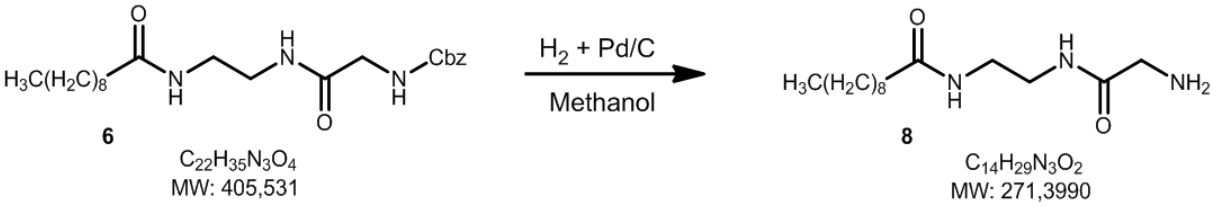
Procedure:

The starting material was dissolved in acetyl chloride and stirred for 24 h at room temperature. The mixture was quenched with water and sat. $NaHCO_3$ and subsequently extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure leaving a colorless oil.

Result:

Yield	NMR	Name	Solvent	Notes
Unknown	^1H	*OK 007*	$CDCl_3$	product + impurity

9.8 *N*-(2-(2-Aminoacetamido)ethyl)decanamide (8)



Scheme 20: *Synthesis of* N-*(2-(2-aminoacetamido)ethyl)decanamide*

Table 13: *Substances used for the synthesis of* N-*(2-(2-aminoacetamido)ethyl)decanamide:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[w%]	V[mL]	eq.
6	200	405.531	0.493	-	-	1
Pd/C	52	106.420	0.049	10	-	0.1
dry methanol	-	-	-	-	40	solvent
H_2	-	-	-	-	-	excess

Procedure:

Substance **6** was dissolved in 30 mL dry MeOH under argon atmosphere by heating up the suspension until a clear solution was formed. A 50-mL round bottom flask was equipped with a magnetic stirrer and filled with argon. Pd/C and 10 mL dry methanol were added, the flask was warmed up to approx. 60 °C by a water bath and the argon was replaced by H_2. The hot solution of **6** was added all at once and stirred overnight while cooling down to room temperature within approx. 30 min. The mixture was filtered over Celite and the solvent was removed under reduced pressure to give a clean and white solid product.

Result:

Yield - crude	NMR	Name	Solvent	Notes
133 mg (100 %)	^{1}H	*OK 018 crude*	CD_3OD	clean product

***R_f*:**

0.60 (Acetone/MeOH, 3:2 + 1% NH_3)

^{1}H-NMR (CD_3OD; 500 MHz) δ [ppm]

δ = 0.92 (t, *J* = 5.4 Hz, 3 H), 1.26-1.38 (m, 12 H), 1.58-1.66 (m, 2 H), 2.19 (t, *J* = 5.0 Hz, 2 H), 3.27 (s, 2 H), 3.32-3-34 (m, 4 H + MeOD-signal)

^{13}C-NMR (CD_3OD; 126 MHz) δ [ppm]

δ = 14.6, 23.9, 27.1, 30.5, 30.6, 30.6, 30.7, 33.2, 37.3, 40.1, 40.2, 45.3, 175.8, 176.9

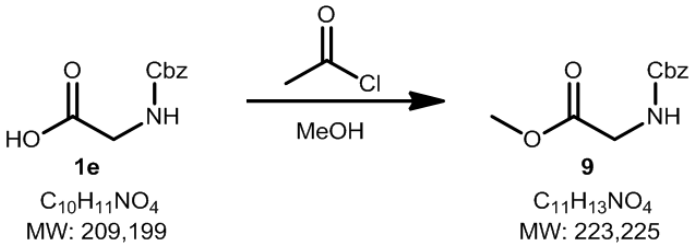
ESI-HR-MS:

m/z calculated for $C_{14}H_{29}N_3NaO_2^+$ $[M+Na]^+$: 294.2152, found: 294.2155

Notes:

The purification of substance **8** was associated with many problems. To avoid the necessity of purification it is strongly recommended to use a very clean starting material **6** whose purification can be done easily by washing it with cold MeOH or by recrystallization from hot MeOH if necessary.

9.9 Methyl 2-(((benzyloxy)carbonyl)amino)acetate (9)



Scheme 21: *Synthesis of methyl 2-(((benzyloxy)carbonyl)amino)acetate*

Table 14: *Substances used for the synthesis of methyl 2-(((benzyloxy)carbonyl)amino)acetate:*

Substance	m[g]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
Z-Glycine amide	0.100	209.199	0.478	-	-	1
Acetyl chloride	0.113	78.498	1.43	1.104	0.102	3
MeOH (dry)	-	-	-	-	5	solvent

Procedure:

Dry MeOH was cooled to 0 °C under argon atmosphere and acetyl chloride was added. The solution was stirred for 5 min before Z-Gly-OH was added. The mixture was refluxed for 50 min at 95 °C and subsequently cooled again to 0 °C. The acidic solution was quenched with sat. $NaHCO_{3\ (aq.)}$, concentrated under reduced pressure until all the methanol was evaporated and extracted three times with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure leaving a colourless oil.

Result:

Yield - crude	NMR	Name	Solvent	Notes
107 mg (100 %)	^{1}H	*OK 020 crude*	$CDCl_3$	Product (traces of solvents)

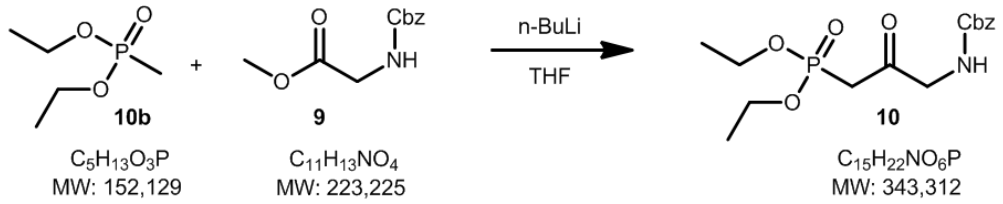
***R_f*:**
0.55 (EtOAc/PE, 3:2)

^{1}H-NMR (CD_3OD; 400 MHz) δ [ppm]
δ = 3.77 (s, 3 H), 4.01 (d, *J* = 5.5 Hz, 2 H), 5.14 (s, 2 H), 5.25 (br s, 1 H), 7.33-7.38 (m, 5 H)

^{13}C-NMR (CD_3OD; 101 MHz) δ [ppm]
δ = 42.6, 52.3, 67.1, 128.1, 128.2, 128.5, 136.2, 156.2, 170.4

ESI-HR-MS:
m/z calculated for $C_{11}H_{13}NNaO_4^+$ $[M+Na]^+$: 246.0737, found: 246.0738

9.10 Benzyl (3-(diethoxyphosphoryl)-2-oxopropyl)carbamate (10)



$C_5H_{13}O_3P$ MW: 152,129 | $C_{11}H_{13}NO_4$ MW: 223,225 | $C_{15}H_{22}NO_6P$ MW: 343,312

Scheme 22: *Synthesis of benzyl (3-(diethoxyphosphoryl)-2-oxopropyl)carbamate*

Table 15: *Substances used for the synthesis of benzyl (3-(diethoxyphosphoryl)-2-oxopropyl)carbamate:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[mol/L]	p[g/mL]	V[mL]	eq.
Z-Gly-OMe **9**	1015	223.225	4.55	-	-	-	1
Diethyl methylphosphonate	1729	152.129	11.37	-	1.041	1.661	2.5
n-BuLi	728	64.055	11.37	1.6	-	7.105	2.5
THF	-	-	-	-	-	10 + 10	solvent

Procedure:

Diethyl methylphosphonate was dissolved in 10 mL dry THF under argon atmosphere and cooled to -78 °C. *n*-BuLi was added dropwise and the milky mixture was stirred for 45 min. A solution of Z-Gly-OMe **9** in 10 mL dry THF was stirred under argon atmosphere for 1 h in the presence of 3 Å molecular sieves and added dropwise to the reaction mixture at -78 °C. The clear but slightly yellow solution was stirred for 45 min at -78 °C and allowed to reach 0 °C within 1 h. The mixture was quenched with saturated $NH_4Cl_{(aq.)}$ and extracted twice with EtOAc. The solvent was removed under reduced pressure leaving 2.296 g of a slightly yellow oil. In order to remove residual diethyl methylphosphonate by distillation the crude product was stirred at 95 °C and <1 mbar in a 100-mL round bottom flask. After 2.5 h the mass spectra showed no more traces of diethyl methylphsphonate. The higher boiling residue in the flask was further purified by column chromatography (silica gel 400 mesh; cyclohexane/EtOAc 3:1 to EtOAc to EtOAc/MeOH 8:2) to give compound **10** as a slightly yellow oil (853 mg, 55 % yield).

Result:

Yield	NMR	Name	Solvent	Notes
131 mg (8 %)	^{1}H	*OK 023 F45*	$CDCl_3$	Product (traces of solvents)
407 mg (26 %)	^{1}H	*OK 023 F46*	$CDCl_3$	Product (traces of solvents)
315 mg (20 %)	^{1}H	*OK 023 F47-49*	$CDCl_3$	Product (traces of solvents)

R_f:

0.15 (EtOAc)

^{1}H-NMR ($CDCl_3$; 400 MHz) δ [ppm]

δ = 1.35 (t, *J* = 7.0 Hz, 6 H), 3.13 (d, *J* = 22.8 Hz, 2 H), 4.12-4.19 (m, 4 H), 4.24 (d, *J* = 5.0 Hz, 2 H), 5.14 (s, 2 H), 5.55 (br s, 1 H), 7.33-7-38 (m, 5 H).

^{13}C-NMR ($CDCl_3$; 151 MHz) δ [ppm]

δ = 16.3 (d, *J* = 5.8 Hz, 2 C), 39.9 (d, *J* = 127,2 Hz, 1 C), 51.5, 62.90 (d, *J* = 7.0 Hz, 1 C), 67.0, 128.1, 128.2, 128.5, 136.2, 156.1, 197.5 (d, *J* = 6.9 Hz, 1 C)

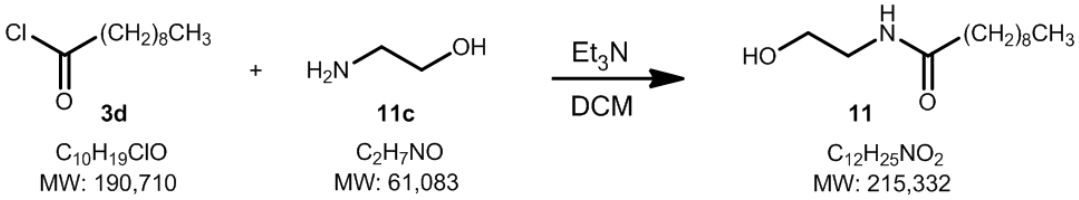
^{31}P-NMR ($CDCl_3$; 121 MHz) δ [ppm]

δ = 17.5-18.3 (m, 1 P)

ESI-HR-MS:

m/z calculated for $C_{15}H_{22}NNaO_6P^+$ $[M+Na]^+$: 366.1077, found: 366.1070

9.11 *N*-(2-Hydroxyethyl)decanamide (11)



Scheme 23: *Synthesis of* N-*(2-hydroxyethyl)decanamide*

Table 16: *Substances used for the synthesis of* N-*(2-hydroxyethyl)decanamide:*

Substance	m[mg]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
Decanoyl chloride	2000	190.710	10.49	0.919	2.176	1
Ethanolamine	1601	61.083	26.22	1.012	1.582	2.5
Et3N	1273	101.190	12.585	0.728	1.749	1.2
DCM	-	-	-	-	30+40	solvent

Procedure:

A 250-mL round bottom flask was equipped with a magnetic stirrer and filled with argon. Ethanolamine, 30 mL dry DCM and Et_3N were added and cooled to -78 °C while the solution was stirred vigorously. A white suspension was formed. Decanoyl chloide was dissolved in 40 mL dry DCM and added dropwise to the suspension within 30 min. The reaction mixture was stirred for a further 30 min at -78 °C and allowed to reach room temperature within 2 h. The mixture was washed twice with 50 mL 1 M HCl, once with 100 mL sat. $NaHCO_{3\,(aq.)}$ and once with brine. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure providing compound 11 as a porous white solid.

Result:

Yield - crude	Notes
2.24 g (99 %)	Very clean according mass spectra and TLC.

***R_f*:**

0.14 (EtOAc)

^{1}H-NMR (CD_3OD; 300 MHz) δ [ppm]

δ = 0.87 (t, *J* = 6.7 Hz, 3 H), 1.18-1.37 (m, 12 H), 1.55-1.68 (m, 2 H), 2.20 (t, *J* = 7.6 Hz, 2 H), 3.26 (br s, 1 H), 3.41 (dd, *J* = 5.3, 10.17 Hz, 2 H), 3.66-3.75 (m, 2 H), 6.19 (br s, 1 H)

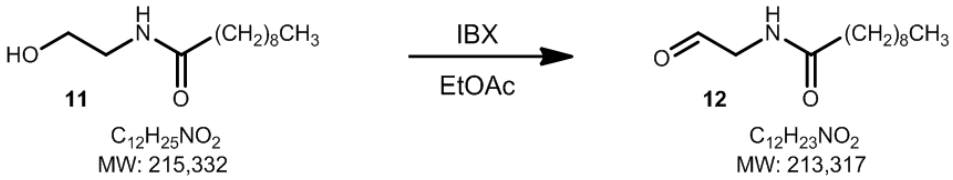
^{13}C-NMR (CD_3OD; 75 MHz) δ [ppm]

δ = 14.1, 22.6, 25.7, 29.2, 29.3, 29.3, 29.4, 31.8, 36.7, 42.4, 62.3, 174.6

ESI-HR-MS:

m/z calculated for $C_{12}H_{25}NNaO_2^+$ $[M+Na]^+$: 238.1778, found: 238.1778

9.12 *N*-(2-Oxoethyl)decanamide (12)



Scheme 24: *Synthesis of* N-*(2-oxoethyl)decanamide*

Table 17: *Substances used for the synthesis of* N-*(2-oxoethyl)decanamide:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[mol/L]	V[mL]	eq.
N-(2-hydroxyethyl)decanamide	200	215.332	0.93	0.2	-	1
IBX	468	280.020	1.67	-	-	1.8
EtOAc	-	-	-	-	5	solvent

Procedure:

N-(2-hydroxyethyl)decanamide **11**, IBX and EtOAc were mixed in a 25-mL round bottom flask and stirred under reflux in a 95 °C hot oil bath. After 5 h the reaction was completed and the mixture was allowed to cool down for 2 min. The warm solution was filtered over Celite and the solvent was removed under reduced pressure to afford compound **12** as a white solid.

Result:

Yield - crude	NMR	Name	Solvent	Notes
199 mg (100 %)	^{1}H	*OK 032 crude*	$CDCl_3$	Clean product

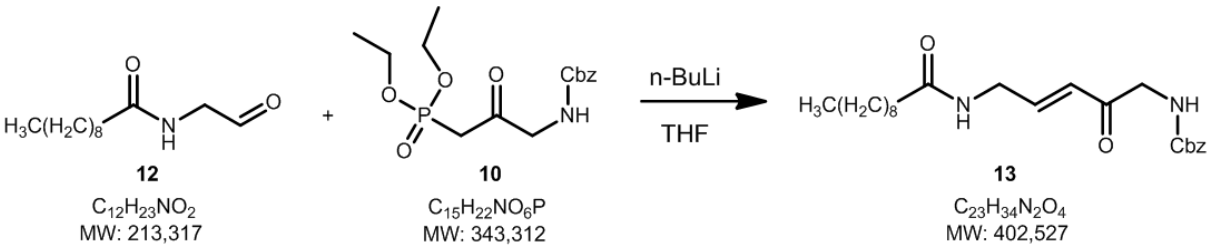
***R_f*:**
0.42 (EtOAc)

^{1}H-NMR ($CDCl_3$; 300 MHz) δ [ppm]
δ = 0.88 (t, *J* = 6.7 Hz, 3 H), 1.17-1.40 (m, 12 H), 1.57-1.73 (m, 2 H), 2.27 (t, *J* = 7.6 Hz, 2 H), 4.24 (d, *J* = 4.7 Hz, 2 H), 6.15 (br s, 1 H), 9.69 (s, 1 H)

^{13}C-NMR ($CDCl_3$; 75 MHz) δ [ppm]
δ = 14.1, 22.6, 25.5, 29.2 (2 C), 29.3, 29.4, 31.8, 36.3, 50.3, 173.5, 196.5

ESI-HR-MS:
m/z calculated for $C_{12}H_{23}NNaO_2^+$ $[M+Na]^+$: 236.1621, found: 236.1621

9.13 (*E*)-Benzyl (5-decanamido-2-oxopent-3-en-1-yl)carbamate (13)



Scheme 25: *Synthesis of (E)-benzyl (5-decanamido-2-oxopent-3-en-1-yl)carbamate*

Table 18: *Substances used for the synthesis of (E)-benzyl (5-decanamido-2-oxopent-3-en-1-yl)carbamate:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[mol/L]	V[mL]	eq.
10	309	343.312	0.900	-	-	1
n-BuLi	58	64.055	0.900	1.6	0.563	1
N-(2-oxoethyl)decanamide **12**	202	213.317	0.945	-	-	1.05
THF	-	-	-	-	12 + 12	solvent

Procedure:

Benzyl (3-(diethoxyphosphoryl)-2-oxopropyl)carbamate **10** was dissolved in 12 mL dry THF under argon atmosphere and cooled to -78 °C. n-BuLi was added and the mixture was stirred for 15 min. The aldehyde **12** was dissolved in 12 mL dry THF under argon atmosphere and added dropwise to the reaction mixture within 10 min. The mixture was stirred for 60 min at -78 °C and allowed to reach 0 °C within another 60 min. The reaction was quenched with 5 mL sat. $NH_4Cl_{(aq.)}$ and 20 mL of sat. $NaHCO_{3\ (aq.)}$ were added. The organic phase was separated and the aqueous layer was extracted three times with 20 mL DCM. The organic phases were combined, washed with brine, dried over $MgSO_4$ and the solvent was removed under reduced pressure leaving a white crystalline solid. The crude product was suspended in 3 mL Et_2O, sonicated, filtered, washed three times with 1 mL of cold Et_2O and dried to yield compound **13** as a white powder.

Result:

Yield	NMR	Name	Solvent	Notes
186 mg (51 %)	^{1}H	*OK 053 ether wash*	$CDCl_3$	Pure product

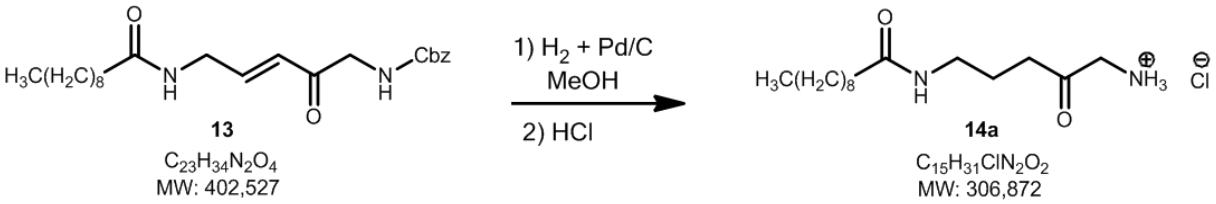
***R_f*:**
0.49 (EtOAc)

^{1}H-NMR ($CDCl_3$; 400 MHz) δ [ppm]
δ = 0.89 (t, *J* = 6.9 Hz, 3 H), 1.25-1.35 (m, 12 H), 1.61-1.70 (m, 2 H), 2.24 (t, *J* = 7.7 Hz, 2 H), 4.10 (t, *J* = 4.7 Hz, 2 H), 4.27 (d, *J* = 4.8 Hz, 2 H), 5.14 (s, 2 H), 5.57 (br s, 1 H), 5.60 (br s, 1 H), 6.20 (d, *J* = 16.1 Hz, 1 H), 6.90 (dt, *J* = 15.8, 4.9 Hz, 1 H), 7.30-7.40 (m, 5 H)

^{13}C-NMR ($CDCl_3$; 176 MHz) δ [ppm]
δ = 14.1, 22.6, 25.6, 29.2, 29.3 (2 C), 29.4, 31.8, 36.6, 40.3, 49.0, 67.0, 126.6, 128.1, 128.2, 128.5, 136.2, 144.2, 156.1, 173.2, 193.4

ESI-HR-MS:
m/z calculated for $C_{23}H_{34}N_2NaO_4^+$ $[M+Na]^+$: 425.2411, found: 425.2418

9.14 5-Decanamido-2-oxopentan-1-aminium chloride (14a)



13
$C_{23}H_{34}N_2O_4$
MW: 402,527

14a
$C_{15}H_{31}ClN_2O_2$
MW: 306,872

Scheme 26: *Synthesis of 5-decanamido-2-oxopentan-1-aminium chloride*

Table 19: *Substances used for the synthesis of 5-decanamido-2-oxopentan-1-aminium chloride:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[w%]	p[g/mL]	V[mL]	eq.
13	60	402.530	0.149	-	-	-	1
Pd/C	32	106.420	0.030	10	-	-	0.2
dry methanol	-	-	-	-	-	10 + 10	solvent
$HCl_{(aq.)}$ 35%	155	36.460	1.491	35	1.2	0.129	10
H_2	-	-	-	-	-	-	excess

Procedure:

A 25-mL round bottom flask was equipped with a magnetic stirrer and filled with argon. Pd/C and 10 mL dry MeOH were added, the argon was replaced by H_2 and the flask was cooled to 0 °C. (*E*)-benzyl (5-decanamido-2-oxopent-3-en-1-yl)carbamate **13** was dissolved in 10 mL dry MeOH under argon atmosphere and added all at once to the suspension of Pd/C. After 4 h of stirring at 0 °C the reaction was completed and the mixture was filtered quickly over Celite which was prewashed with methanol. Immediately after filtration 130 µL of 35 % $HCl_{(aq.)}$ were added and the solvent was removed *in vacuo*. Subsequently 4 mL of 10 % $HCl_{(aq.)}$ and 4 mL Et_2O were added to the residue and the mixture was sonicated for 1 min. The two phases were separated and the HCl phase was filtered over sand and concentrated under reduced pressure. The residue was dissolved in 1 mL of methanol and concentrated again to provide hydrochloride **14a** as a white solid crystalline product.

Result:

Yield - crude	NMR	Name	Solvent	Notes
34 mg (74 %)	^1H	*OK 060 HCl fraction*	CD_3OD	Product (traces of arom. dimer)

***R_f*:**
0.61 (Acetone:MeOH, 3:1 + 1 % NH_3 + 2 % H_2O)

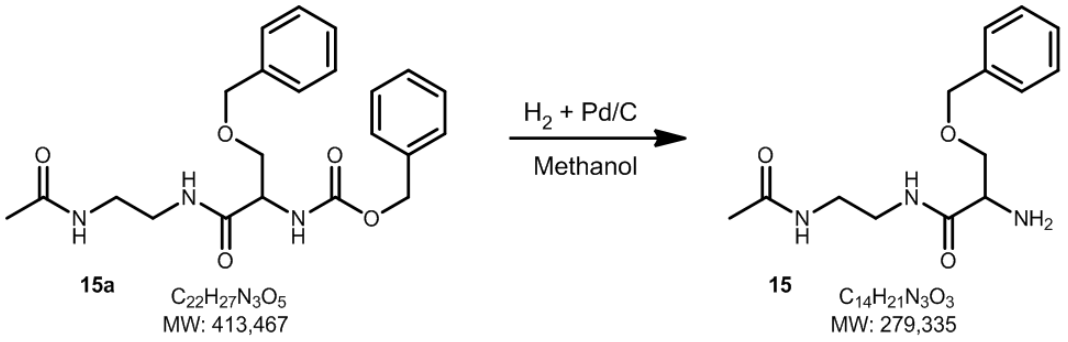
^{1}H-NMR (CD_3OD; 300 MHz) δ [ppm]
δ = 0.90 (t, *J* = 6.7 Hz, 3 H), 1.26-1.36 (m, 12 H), 1.55-1.65 (m, 2 H), 1.80 (tt, *J* = 6.8, 6.8 Hz, 2 H), 2.18 (t, *J* = 7.4 Hz, 2 H), 2.57 (t, *J* = 7.0 Hz, 2 H), 3.18 (t, *J* = 6.9 Hz, 2 H), 3.95 (s, 2 H)

^{13}C-NMR (CD_3OD; 101 MHz) δ [ppm]
δ = 14.6, 23.9, 24.1, 27.2, 30.5, 30.5, 30.6, 30.7, 33.2, 37.0, 37.8, 39.7, 48.3, 177.2, 203.7

ESI-HR-MS:
m/z calculated for $C_{15}H_{31}N_2O_2^+$ $[M+H]^+$: 271.2380, found: 271.2372

Notes:
The ^{13}C signal on position 103.72 could only be detected by using the HMBC spectrum.

9.15 *N*-(2-Acetamidoethyl)-2-amino-3-(benzyloxy)propanamide (15)



Scheme 27: *Synthesis of* N-*(2-acetamidoethyl)-2-amino-3-(benzyloxy)propanamide*

Table 20: *Substances used for the synthesis of* N-*(2-acetamidoethyl)-2-amino-3-(benzyloxy)propanamide:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[w%]	V[mL]	eq.
15a	200	413.47	0.484	-	-	1
Pd/C	51	106.42	0.048	10	-	0.1
dry methanol	-	-	-	-	35	solvent
H_2	-	-	-	-	-	excess

Procedure:

A 100-mL round bottom flask was equipped with a magnetic stirrer and filled with argon. Pd/C and 25 mL dry MeOH were added, the argon was replaced by H_2 and the flask was warmed up to 30 °C. Substance **15a** was dissolved in 10 mL dry MeOH under argon atmosphere and added all at once to the suspension of Pd/C. The mixture was stirred at room temperature for 75 min, filtered over Celite and the solvent was removed under reduced pressure to afford compound **15** as a white solid.

Result:

Yield - crude	NMR	Name	Solvent	Notes
128 mg (95 %)	^{1}H	*OK037 crude*	$CDCl_3$	Product (traces of Et_2O)

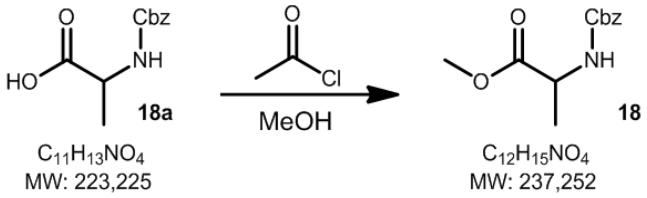
***R_f*:**

0.59 (Acetone:MeOH, 3:1 + 1 % NH_3 + 2 % H_2O)

^{1}H-NMR ($CDCl_3$; 300 MHz) δ [ppm]

δ = 1.66 (br s, 2 H), 1.92 (s, 3 H), 3.35-3.45 (m, 4 H), 3.58 (t, *J* = 5.9 Hz, 1 H), 3.72 (d, *J* = 5.1 Hz, 2 H), 4.55 (s, 2 H), 6.26 (br s, 1 H), 7.28-7.40 (m, 5 H), 7.84 (br s, 1 H)

9.16 Methyl 2-(((benzyloxy)carbonyl)amino)propanoate (18)



Scheme 28: *Synthesis of methyl 2-(((benzyloxy)carbonyl)amino)propanoate*

Table 21: *Substances used for the synthesis of methyl 2-(((benzyloxy)carbonyl)amino)propanoate:*

Substance	m[g]	M[g/mol]	n[mmol]	p[g/mL]	V[mL]	eq.
Z-alanin amide	1.000	223.225	4.480	-	-	1
Acetyl chloride	1.055	78.498	13.44	1.104	0.956	3
MeOH	-	-	-	-	20	solvent

Procedure:

Dry MeOH was cooled to 0 °C under Argon atmosphere and Acetyl chloride was added. The solution was stirred for 5 min before Z-Ala-OH was added. The mixture was refluxed for 50 min at 95 °C and subsequently cooled again to 0 °C. The acidic solution was quenched with sat. $NaHCO_{3\ (aq.)}$, concentrated under reduced pressure until all the methanol was evaporated and extracted three times with EtOAc. The organic phase was washed with brine, dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure leaving a colourless oil.

Result:

Yield - crude	NMR	Name	Solvent	Notes
1.040 g (98 %)	^{1}H	*OK 054*	$CDCl_3$	Product (+additional peak)

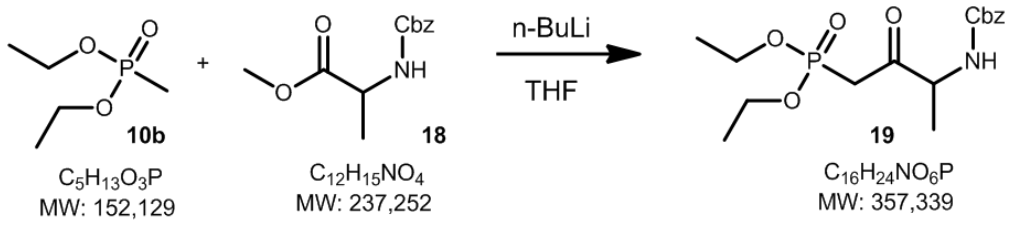
R_f:
0.75 (EtOAc)

^{1}H-NMR ($CDCl_3$; 400 MHz) δ [ppm]
δ = 1.42 (d, *J* = 7.0 Hz, 3 H), 3.76 (s, 3 H), 4.36-4.47 (m, 1 H), 5.12 (s, 2 H), 5.34 (br s, 1 H), 7.29-7.40 (m, 5 H)

^{13}C-NMR (CD_3OD; 176 MHz) δ [ppm]
δ = 18.6, 49.5, 52.4, 66.9, 128.1, 128.1, 128.5, 136.2, 155.6, 173.4

ESI-HR-MS:
m/z calculated for $C_{12}H_{15}NNaO_4^+$ $[M+Na]^+$: 260.0893, found: 260.0884

9.17 Benzyl (4-(diethoxyphosphoryl)-3-oxobutan-2-yl)carbamate (19)



Scheme 29: *Synthesis of benzyl (4-(diethoxyphosphoryl)-3-oxobutan-2-yl)carbamate*

Table 22: *Substances used for the synthesis of benzyl (4-(diethoxyphosphoryl)-3-oxobutan-2-yl)carbamate:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[mol/L]	p[g/mL]	V[mL]	eq.
18	326	237.252	1.37	-	-	-	1
diethyl methylphosphonate	523	152.129	3.44	-	1.041	0.502	2.5
n-BuLi	220	64.055	3.44	1.6	-	2.147	2.5
THF	-	-	-	-	-	3 + 3	solvent

Procedure:

Diethyl methylphosphonate was dissolved in 3 mL dry THF under argon atmosphere and cooled to -78 °C. *n*-BuLi was added dropwise and the milky mixture was stirred for 45 min. A solution of Z-Ala-OMe **18** in 3 mL dry THF was added to the reaction mixture and stirring was continued for 45 min at -78 °C. The reaction mixture was allowed to reach 0 °C within 45 min and stirred for other 10 min at this temperature before it was quenched with 10 mL $NH_4Cl_{(aq.)}$. The mixture was extracted three times with EtOAc, the organic phases where combined, dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure leaving 690 mg of a colourless oil. In order to remove residual diethyl methylphosphonate by distillation the crude product was stirred at 95 °C and <1 mbar in a 100-mL round bottom flask. After 4 h the mass spectra showed no more traces of diethyl methylphsphonate. The higher boiling residue in the flask was further purified by column chromatography (silica gel 400 mesh; cyclohexane/EtOAc 1:1 to EtOA) to give compound **19** as a colourless oil.

Result:

Yield	NMR	Name	Solvent	Notes
156 mg (32 %)	^{1}H	*OK 056 F14-21*	$CDCl_3$	Product

***R_f*:**
0.33 (EtOAc)

^{1}H-NMR ($CDCl_3$; 700 MHz) δ [ppm]

δ = 1.26-1.36 (m, 6 H), 1.40 (d, *J* = 7.3 Hz, 3 H), 3.08 (dd, *J* = 13.8, 22.3 Hz, 1 H), 3.33 (dd, *J* = 13.8, 23.0 Hz, 1 H), 4.10-4.20 (m, 4 H), 4.47-4.52 (m, 1 H), 5.12 (s, 2 H), 5.86 (d, *J* = 7.0 Hz, 1 H), 7.30-7.33 (m, 1 H), 7.33-7.38 (m, 4 H)

^{13}C-NMR ($CDCl_3$; 176 MHz) δ [ppm]

δ = 16.2, 16.3, 17.3, 38.9 (d, *J* = 129.0 Hz, 1 C), 56.4, 62.8 (d, *J* = 6.9 Hz, 1 C), 62.8 (d, *J* = 6.9 Hz, 1 C), 66.9, 128.0 (2 C), 128.2, 128.5 (2 C), 136.3, 155.7, 201.4 (d, *J* = 6.9 Hz, 1 C)

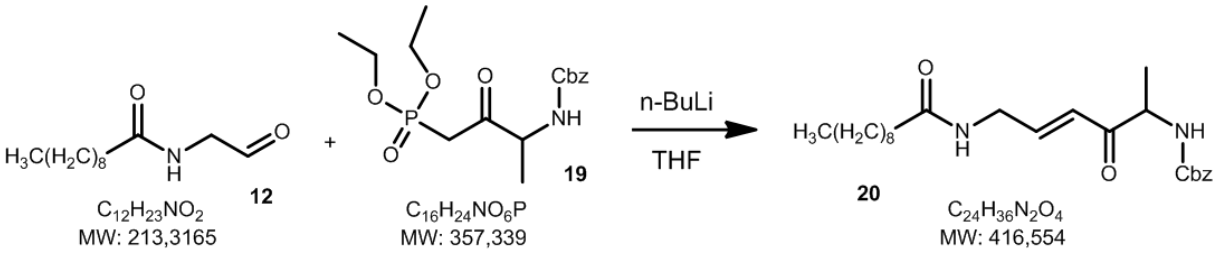
^{31}P-NMR ($CDCl_3$; 121 MHz) δ [ppm]

δ = 18.3-19.0 (m, 1 P)

ESI-HR-MS:

m/z calculated for $C_{16}H_{24}NNaO_6P^+$ $[M+Na]^+$: 380.1233, found: 380.1236

9.18 (*E*)-Benzyl (6-decanamido-3-oxohex-4-en-2-yl)carbamate (20)



Scheme 30: *Synthesis of (E)-benzyl (6-decanamido-3-oxohex-4-en-2-yl)carbamate*

Table 23: *Substances used for the synthesis of (E)-benzyl (6-decanamido-3-oxohex-4-en-2-yl)carbamate:*

Substance	m[mg]	M[g/mol]	n[mmol]	c[mol/L]	V[mL]	eq.
19	137	357.339	0.383	-	-	1
n-BuLi	25	64.055	0.383	1.6	0.240	1
N-(2-oxoethyl)decanamide **12**	86	213.317	0.403	-	-	1.05
THF	-	-	-	-	5 + 5	solvent

Procedure:

Benzyl (4-(diethoxyphosphoryl)-3-oxobutan-2-yl)carbamate **19** was dissolved in 5 mL dry THF under argon atmosphere and cooled to -78 °C. n-BuLi was added and the mixture was stirred for 15 min. The aldehyde **12** was dissolved in 5 mL dry THF under argon atmosphere and added dropwise to the reaction mixture within 10 min. The mixture was stirred for 60 min at -78 °C and allowed to reach 0 °C within another 60 min. The reaction was quenched with sat. $NH_4Cl_{(aq.)}$ and 10 mL of sat. $NaHCO_{3\ (aq.)}$ were added. The organic phase was separated and the aqueous layer was extracted three times with 10 mL DCM. The organic phases were combined, washed with brine, dried over $MgSO_4$ and the solvent was removed under reduced pressure leaving white crystals suspended in a colourless oil. The crude product was suspended in Et_2O, sonicated, filtered, washed twice with 2 mL of cold Et_2O and dried on air to yield compound **20** as a white powder.

Result:

Yield	NMR	Name	Solvent	Notes
26 mg (16 %)	^{1}H	*OK 059 ether wash*	$CDCl_3$	Pure product

***R_f*:**
0.57 (EtOAc)

^{1}H-NMR ($CDCl_3$; 400 MHz) δ [ppm]

δ = 0.88 (t, *J* = 6.9 Hz, 3 H), 1.24-1.34 (m, 12 H), 1.36 (d, *J* = 7.3 Hz, 3 H), 1.60-1.70 (m, 2 H), 2.23 (t, *J* = 7.7 Hz, 2 H), 4.05-4.15 (m, 2 H), 4.56-4.66 (m, 1 H), 5.10 (s, 2 H), 5.64 (d, *J* = 6.8 Hz, 1 H), 5.75 (brt, 1 H), 6.26 (d, *J* = 15.8 Hz, 1 H), 6.93 (dt, *J* = 15.8, 5.0 Hz, 1 H), 7.29-7.40 (m, 5 H)

^{13}C-NMR ($CDCl_3$; 101 MHz) δ [ppm]

δ = 14.1, 18.4, 22.6, 25.6, 29.2, 29.3 (2 C), 29.4, 31.8, 36.6, 40.3, 53.9, 66.8, 125.8, 128.0 (2 C), 128.1, 128.5 (2 C), 136.3, 144.7, 155.6, 173.1, 197.3

ESI-HR-MS:

m/z calculated for $C_{24}H_{36}N_2NaO_4^+$ $[M+H]^+$: 439.2567, found: 439.2556

Specific rotation:

$[\alpha]_{589}^{25}$ = +0.222 ± 0.004 (c = 0,91 g/100 mL; $CHCl_3$).

9.19 Feeding of *Streptomyces Lasaliensis*

Preculture:

To set up the precultures, 10 mL of M79 medium and 100 µL of *S. lasaliensis* were transferred into a 100 mL Erlenmeyer flask under sterile conditions and incubated for three days at 30 °C.

Production Culture:

For each production culture, 100 µL of the corresponding preculture were transferred into another 100 mL Erlenmeyer flask filled with 10 mL of MYM medium. The samples were incubated for one day at 30 °C.

Feeding:

In general, two flasks containing a production culture were used as blank samples in each feeding experiment. Further production cultures were used for the feeding with the substance of interest. For this purpose the required amount of the substance was dissolved in 100 µL MeOH and added to the production culture which was incubated at 30 °C. The feeding was repeated every day for a period of four days in total.

Work-up:

Unless otherwise stated, the cultures were extracted by shaking them twice for 1 h with 10 mL EtOAc and twice for 1 h with 10 mL n-BuOH. The extractions of the same solvent were combined and the solvent was removed under reduced pressure. The residue was dissolved in 1 mL of MeOH, diluted 1:1 with water, filtered and analysed by UPLC-HR-ESI-MS.

Data of Individual Experiments:

Table 24: *Feeding experiment 1.*

Probe	Substance N°	Concentration/day	Result
Control A	-	-	control
Control B	-	-	control
Probe 1	**2**	1 mM/day	No off-loaded intermediate detected
Probe 2	**2**	1 mM/day	No off-loaded intermediate detected

Note: It was not clear if the fed bacteria survived the entire feeding period.

Table 25: *Feeding experiment 2.*

Probe	Substance N°	Concentration/day	Result
Control A	-	-	control
Probe 1	**8**	1 mM/day	No off-loaded intermediate detected
Probe 2	**8**	1 mM/day	No off-loaded intermediate detected

Note: For some unknown reason the production culture of "control B" didn't start to grow. The amount of cells in probe 1 and 2 was very small after the end of the feeding period.

Table 26: *Feeding experiment 3.*

Probe	Substance N°	Concentration/day	Result
Control A	-	-	control
Control B	-	-	control
Probe 1 A	**8**	0.1 mM/day	No off-loaded intermediate detected*
Probe 1 B	**8**	0.1 mM/day	No off-loaded intermediate detected*
Probe 2 A	**8**	1 mM/day	Bacteria died after the second day
Probe 2 B	**8**	1 mM/day	Bacteria died after the second day

*Note: The bacteria fed with a concentration of 1 mM/day of substance **8** died after two days of feeding. Each culture was extracted by using a Vortex Mixing System. The extraction was done twice for 2 min with 10 mL EtOAc and twice for 2 min with 10 mL n-BuOH. *Analyses were carried out by other group members after the end of my laboratory works.*

Table 27: *Feeding experiment 4; repetition of feeding experiment 3.*

Probe	Substance N°	Concentration/day	Result
Control A	-	-	control
Probe 1 A	**8**	0.1 mM/day	No off-loaded intermediate detected*
Probe 1 B	**8**	0.1 mM/day	No off-loaded intermediate detected*
Probe 2 A	**8**	1 mM/day	Bacteria died after the second day
Probe 2 B	**8**	1 mM/day	Bacteria died after the second day

*Note: For some unknown reason the production culture of "control B" died after three days; bacteria fed with a concentration of 1 mM/day of substance **8** died after two days of feeding. The cultures were extracted by using a Vortex Mixing System. The extraction was done twice for 2 min with 10 mL EtOAc and twice for 2 min with 10 mL n-BuOH. *Analyses were carried out by other group members after the end of my laboratory works.*

Table 28: *Feeding experiment 5.*

Probe	Substance N°	Concentration/day	Result
Control A	-	-	control
Control B	-	-	control
Probe 3	**2**	0.5 mM/day	No off-loaded intermediate detected*
Probe 4	**2**	0.5 mM/day	No off-loaded intermediate detected*
Probe 5	**17**	0.5 mM/day	No off-loaded intermediate detected*
Probe 6	**17**	0.5 mM/day	No off-loaded intermediate detected*
Probe 7	**14a**	0.33 mM/day	No off-loaded intermediate detected*
Probe 8	**14a**	0.33 mM/day	No off-loaded intermediate detected*

*Note: Each culture was extracted by using a Vortex Mixing System. The extraction was done twice for 2 min with 10 mL EtOAc and twice for 2 min with 10 mL n-BuOH. *Analyses were carried out by other group members after the end of my laboratory works.*

10 Curriculum Vitae

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Italian	Fluent
English	Fluent
Dolomitic Ladin	Basic knowledge
driving license	**B**

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