Katholieke Universiteit Leuven
Group Biomedical Sciences
Faculty of Pharmaceutical Sciences
Rega Institute for Medical Research
Laboratory for Medicinal Chemistry

# Design, Synthesis and Evaluation of Aminoacyl-tRNA Synthetase Inhibitors Based on Microcin C and Albomycin

Gaston Vondenhoff

Doctoral Thesis in Pharmaceutical Sciences
Leuven, 2012

Katholieke Universiteit Leuven
Group Biomedical Sciences
Faculty of Pharmaceutical Sciences
Rega Institute for Medical Research
Laboratory for Medicinal Chemistry

# Design, Synthesis and Evaluation of Aminoacyl-tRNA Synthetase Inhibitors Based on Microcin C and Albomycin

Gaston Vondenhoff

**Jury:**
Promotor: Prof. Dr. Arthur Van Aerschot
Co-promotor: Prof. Dr. Konstantin Severinov
Chair: Prof. Dr. Ann Van Schepdael
Secretary: Prof. Dr. Lieve Vanmellaert
Members: Prof. Dr. Alfons Verbruggen, Prof. Dr. Jos Vanderleyden, Prof. Dr. Serge Van Calenbergh

Leuven, 02.04.2012

Doctoral thesis in Pharmaceutical Sciences

**Theses accompanying this dissertation**

- The world is like organic chemistry: practical, but far from perfect. An ideal world would be more like quantum mechanics: unfeasible.
- Most publishing is just another way of gathering credits for stuff that does not or only partially works.
- Too many ideas are a chemist's worst enemy.
- Serendipity is a chemist's best friend.
- Your compounds are like your babies: you make them, you clean them, you give them the best environment you can. They differ from real babies in that, if they are not active, they end up in the organic waste.
- According to the general relativity theory, for fast moving objects time moves slower, but when I work faster it definitely seems that time runs up faster as well.
- True devotion to chemistry shows in short overnight reactions.
- One day we will find a super-drug that will save millions of lives but after that human nature will kill billions.
- Cream always floats to the top! (unless it is dissolved in the organic layer)

# Table of Contents

# Preface

## Acknowledgements

Na onder andere 600 reacties, 350 silicakolommen, 60 prepTLC platen en talloze vlieguren aan de hplc, vindt U hier op deze bladzijden het resultaat van wat voor mij een bijna-obsessie-ervaring werd. De zoektocht naar het ultieme antibioticum was er een die zich wellicht het best laat karakteriseren met de termen frustratie, opluchting, euforie en spanning. Ik heb dit altijd een fantastisch doctoraatsproject gevonden omwille van het interdisciplinaire werk dat op het grensvlak van de farmacie, organische chemie, micro,- en moleculaire biologie balanceert. De vrijheid die ik heb mogen genieten om van alle disciplines te proeven heb ik bijzonder geapprecieerd en dit werd voornamelijk door twee personen mogelijk gemaakt die ik hiervoor heel erg dankbaar ben, namelijk mijn promotor Prof. Dr. Arthur Van Aerschot en mijn co-promotor Prof. Dr. Konstantin Severinov.

Arthur, U bedank ik hier in het bijzonder voor uw bereidwilligheid. Ondanks uw vaak overvolle agenda die gevuld was met allerhande taken, gaande van bestuur van het departement tot oefenzittingen en bijwonen van masterthesis verdedigingen uit alle regionen, was u er altijd om ons doctoraatsstudenten te helpen met een verbluffende snelheid en accuratesse. Dit niet in de laatste plaats met de organisatie van allerlei verplichtingen. Graag wil ik u ook bedanken voor het stimuleren van mijn onderzoek in de VS en Moskou. Daarnaast ben ik ervan overtuigd dat ons medicinaal chemie laboratorium zonder uw organisatie een stuk minder soepel zou functioneren.

Konstantin, what is there not to admire about someone able to run three laboratories on two continents with such enthusiasm and willingness to drink pivo and eat pizza every few days with his PhD's and postdocs. I thank you for all the opportunities you gave me to conduct research in your laboratories in the US and Russia that not only involved the synthesis but also the testing of my compounds. It's because of this boundary crossing that our project "flies" as you call it yourself. I guess the flexibility with which we did this collaboration was and is our strong point.

Voorts wil ik de leden van mijn de jury, te weten Prof. Dr. Van Calenberg, Prof. Dr. Van Schepdael, Prof. Dr. Vanderleyden, Prof. Dr. Vanmellaert en Prof. Dr. Verbruggen bedanken voor hun interesse in dit werk en hun kritische evaluatie van het finale manuscript.

PhD-projects are only fun if there are other people in the lab to share your frustrations with, fortunately I had the honor of meeting quite some: Alessandro Stella, Anastasia Parchina, Anna Ramadori, Bart Van den Brande, Bart Vanderhoydonck, Concetta Paolella, Dhuldeo Kachare, Elisabetta Groaz, Gregory Chattelain, Guy Schepers, Hassen Bel Abed, Johannes Vogel, Katrijn Bockstael, Kim Buts, Kishore Lingam Gangadhara, Luc Baudemprez, Martin Kögler, Michal Česnek, Michal Šála, Milind Saudi, Miyeon Jang, Shrinivas Dumbre, Sonja Kovackova, Sonja Mertens, Qiuya Huang, Xiaoping Song.

Special thanks to Bharat Gadakh, with whom I synthesized several of the compounds and who corrected this thesis so meticulously. Bharat, I'm honoured that you will carry on the torch and hopefully will ignite a stream of new antibiotics!

And my Russian colleagues at the Waksman Institute, IBG and IMG, that were über friendly in helping me find my way in New Jersey and Moscow and with whom it was a delight to hang out: Andrey Shadrin, Anna Lopatina, Ekatarina Semenova, Kate Bogdanova, Konstantin Kuznedelov, Leonid Minakin, Maria Novikova, Maxim Nagorkin, Svetlana Dubiley, Teymur Kazakov. Especially I'd like to thank Ksenia Pugach who is such a briljant animator, a dear friend and a great artist!

Prof. Dr. Roger Busson wil ik graag bedanken voor de vele uitleg en praktische hulp die ik heb gehad bij de synthese en de NMR karakterisatie. Ook Prof. Dr. Eveline Lescrinier bedank ik voor het opnemen van een heel aantal NMR spectra. Prof. Dr. Jef Rozenski bedank ik voor de vele honderden massa spectra die hij voor mij heeft opgenomen. Erkentelijkheid gaat uit naar Prof. Dr. Piet Herdewijn voor het gebruik mogen maken van alle infrastructuur en naar Chantal Biernaux voor alle administratie.

Ook mogen in hier "mijn" studenten niet ontbreken die zo moedig elke dag in alle vroegte stonden te trappelen om mij te helpen. Bart Blanchaert, Sophie Geboers, en Laurence Carlier, ontzettend bedankt voor al jullie inzet bij dit werk. Al heeft de een al wat meer tegenslag gehad dan de ander, jullie hebben alle drie ontzettend hard gewerkt om jullie deel tot een goed einde te brengen, chapeau!

I am indebted to the following organisations for their financial support: IWT (Agentschap voor Innovatie door Wetenschap en Technologie in Vlaanderen) voor het bekostigen van mij doctoraat. FWO (Fonds Wetenschappelijk Onderzoek – Vlaanderen) voor het ondersteunen van mijn onderzoek te Moskou. NIH (National Institutes of Health) for the financial support of my research in the USA.

Gelukkig zijn er toch af en toe momenten in een doctoraat waarin de overtollige kennis overboord gegooid mag worden: Chris, Mike, Paul, Paul en Peter, zonger al os gepils woar ut sowieso nooit get gewoare! Dank vuer alle vrungsjap durch de joare heen! En dat vur es god bleef nog mer veul pilskes moge numme!

Eline, jij bent in zekere zin de aanleiding geweest om dit doctoraat te beginnen, mijn voorbeeld om er hard voor te werken en mijn drive om het binnen korte tijd tot een goed einde te brengen. Dankjewel voor alle steun en toeverlaat over de jaren!

P & M en Michelleke bedank dat er d'r ummer vuer mich woart ondanks al mien sjpragietse, al ut geregel, ut verhoes.... Zonger uch vaak moedige besjlissinge zou ich dit neet hubbe bereikt!

Gaston Vondenhoff

Leuven, 2012

# List of abbreviations

| | |
|---|---|
| aa | amino acid |
| aaRS | aminoacyl-tRNA synthetase |
| aaRSi | aminoacyl-tRNA synthetase inhibitor |
| aaSA | 5'-*O*-(*N*-aminoacyl)-sulfamoyladenosine |
| ABC | ATP-binding cassette |
| AdT | aminoacyl-tRNA amidotransferase |
| AMP | adenosine-5'-monophosphate |
| ATP | adenosine-5'-triphosphate |
| Boc | *tert*-butoxycarbonyl |
| DBU | 1,8-diazabicyclo[5.4.0]undec-7-ene |
| DEAD | diisopropyl azodicarboxylate |
| DIC | diisopropylcarbodiimide |
| DIPEA | *N,N*-diisopropylethylamine |
| DMAc | *N,N*-dimethylacetamide |
| DMF | *N,N*-dimethylformamide |
| DMSO | dimethylsulfoxide |
| DTD | D-aminoacyl-tRNA deacylase |
| DTT | 1,4-dithiothreitol |
| EDCI·HCl | *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride |
| EF | elongation factor |
| FDA | food and drug administration |
| Fmoc | 9H-fluoren-9-ylmethoxycarbonyl |
| GluAdT | Glu-tRNA$^{Gln}$ amidotransferase |
| HOBt | *N*-hydroxybenzotriazole |
| HBTU | *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate |
| HPLC | high performance liquid chromatography |
| $IC_{50}$ | half maximal inhibitory concentration |
| LB | lysogeny broth |
| McC | microcin C |
| MIC | minimum inhibitory concentration |
| MS | mass spectrometry |
| NMR | nuclear magnetic resonance |
| NRPS | non-ribosomal peptide synthetase |
| -OSu | *N*-hydroxysuccinimide ester |
| PBP | penicillin-binding protein |
| PDF | peptide deformylase |
| PP | pyrophosphate |
| rt | room temperature |
| SDC | siderophore drug conjugate |
| *t*Bu | *tert*-butyl |
| TBDMS | *tert*-butyldimethylsilyl |
| TEAB | triethylammonium bicarbonate |

| | |
|---|---|
| TCA | trichloroacetic acid |
| TFA | trifluoroacetic acid |
| THF | tetrahydrofuran |
| tRNA | transfer ribonucleic acid |
| wt | wild type |
| Z | benzyloxycarbonyl |

IUPAC abbreviations for amino acids

| 3-letter code | 1-letter code | full name |
|---|---|---|
| Ala | A | Alanine |
| Arg | R | Arginine |
| Asn | N | Asparagine |
| Asp | D | Aspartic acid |
| Cys | C | Cysteine |
| Gln | Q | Glutamine |
| Glu | E | Glutamic acid |
| Gly | G | Glycine |
| His | H | Histidine |
| Ile | I | Isoleucine |
| Leu | L | Leucine |
| Lys | K | Lysine |
| Met | M | Methionine |
| Phe | F | Phenylalanine |
| Pro | P | Proline |
| Ser | S | Serine |
| Thr | T | Threonine |
| Trp | W | Tryptophan |
| Tyr | Y | Tyrosine |
| Val | V | Valine |

# Chapter 1

# General Introduction

**Abstract:** Increasing resistance to antibiotics is a major problem worldwide and provides the stimulus for development of new bacterial inhibitors with preferably different modes of action. In search for new leads, several new bacterial targets are being exploited beside the use of traditional screening methods. Hereto, inhibition of bacterial protein synthesis is a long-standing validated target. Aminoacyl-tRNA synthetases (aaRSs) play an indispensable role in protein synthesis and their structures proved quite conserved in prokaryotes and eukaryotes. However, some divergence has occurred allowing the development of selective aaRS inhibitors. Following an outline on the action mechanism of aaRSs, an overview will be given of already existing aaRS inhibitors, which are largely based on mimics of the aminoacyl-adenylates, the natural reaction intermediates. This is followed by a discussion on more recent developments in the field and the bioavailability problem and the so-called Trojan horse inhibitors, as based on microcin C and the sideromycins.

Parts of this chapter were earlier published in an adapted form as:

Microcin C: biosynthesis, mode of action, and potential as a lead in antibiotics development
G. H. Vondenhoff, A. Van Aerschot, *Nucleosides Nucleotides Nucleic Acids*, 2011, *30*, 465 [1].

and

Aminoacyl-tRNA synthetase inhibitors as potential antibiotics
G. H. Vondenhoff, A. Van Aerschot, *Eur J Med Chem*, 2011, *46*, 5227 [2].

## 1.1 Introduction

Antibiotics are molecules that either kill or stop bacteria or fungi from growing. Since the clinical introduction of antibiotics in the 1930s, bacteria inevitably respond by developing resistance. With bacterial resistance as an emerging problem, the need for new antibiotics is rising. Tremendous efforts dedicated worldwide in this field have led only to marginal results. By marketing daptomycin, linezolid and tigecycline, the range of treatment options was expanded against methicillin-resistant *S. aureus* (the highest profile pathogen). However, ground was lost on other pathogenic bacteria. The spread of extended spectrum β-lactamases, AmpC enzymes and quinolone resistance in Enterobacteriaceae forces the use of carbapenems to which resistance is increasing through acquisition of metallo-, KPC and OXA-48 β-lactamases. However, resistance against carbapenems is much more common in non-fermenters such as *Pseudomonas aerigunosa* and *Acinetobacter baumannii*. Also *Neisseria gonorrhoeae* is showing low-level resistance against the oxyimino cephalosporins [3].

Three mechanisms by which resistance to the already existing classes of antibiotics develops are: *i)* modification of the target, *ii)* functional bypassing of that target, or *iii)* the drug becoming ineffective due to bacterial impermeability, efflux or enzymatic inactivation [4, 5]. Strategies to overcome resistance involve further development of existing classes of antibiotics and the use of combinations of existing antibiotics, as well as searching for new classes of antibiotics. Indeed, the former strategy seems the most promising, since it can build on previous knowledge, and thus is relatively time-, labor- and cost-saving. However, there is a greater risk of rapid reoccurrence of resistance. Therefore new antibiotics with different modes of action need to be developed to prevent cross-resistance [6, 7].

New antibiotics have to fulfill three criteria: the target should be vital for the cell function of the pathogen, it should be very selective for the bacterial target, and it should be difficult for the bacteria to develop resistance by mutations [8]. One of the more recent targets for intervention is the translation of mRNA and thus bacterial protein synthesis. One way of intervention is by inhibiting the charging of transfer ribonucleic acid (tRNA) with its cognate amino acid by aminoacyl-tRNA synthetases (aaRSs). These enzymes are found in all living organisms and most organisms contain at least 20 different aaRSs, one for each amino acid. Aside from the 20 standard amino acids, quite a few non-standard amino acids are known, including selenocysteine and pyrrolysine [9]. In addition, aaRSs are already clinically validated as valuable target for development of antibiotics, *e.g.* Bactroban® (also known as mupirocin), which is responsible for the inhibition of isoleucin-tRNA synthetase (IleRS).

Inhibition at this level is interesting for a number of reasons. First, aaRSs have a pivotal role in translation of messenger RNA (mRNA), and thus are of vital importance. Second, strong

structural conservation in the catalytic domains of the synthetase exists throughout evolution, implying that one type of drug directed against a typical active site may inhibit a range of synthetases. Little structural variation may also imply that it could be difficult to develop resistance by mutations in genes coding for the synthetases. This also means that if great homology exists between eukaryotic and prokaryotic aaRS, selectivity of a potential inhibitor for the latter may be hard to achieve. Third, depending on which synthetase is considered, a full canonical pattern exists, meaning that there are great sequence differences between prokaryotes and eukaryotes. This is, almost without exception, true for AspRS, GluRS, PheRS, LeuRS, IleRS, HisRS, ProRS, and MetRS. However, structurally, great differences are not always observed. The architecture of the active site is in a structural sense quite conserved amongst different species [10-12]. Both *in vitro* and *in vivo,* most eukaryal aaRSs can complement bacterial enzymes [13]. Further combined biochemical, bioinformatics, and structural studies are needed to reveal the exact variations in active sites of aaRSs, which are the key to rational drug design.

Although several other compounds are found in nature as well, most inhibitors of aaRSs developed to date are non-cleavable mimics of the natural reaction intermediates (*i.e.* aminoacyl-adenylates (aa-AMP)), that act through competitive binding to the aaRSs. Most of these aa-AMP analogues are aminoacyl-sulfamoyl-adenosines (aaSAs), *vide infra*. Although these compounds are potent inhibitors of aaRSs, with MIC values in the nanomolar range [14-17], their *in vivo* activity is considerably lower and poor uptake has been suggested to be the main reason [18]. The lack of selectivity and poor bioavailability are the most prominent problems for this new potential class of antibiotics. One potential solution is, once more, provided by nature. Several natural compounds have been identified that cunningly make use of an active uptake mechanism inside the bacterial membranes, after which the respective compound is being taken up and metabolized inside the sensitive cell, releasing the active moiety of the prodrug.

In this thesis several solutions to these problems are reviewed, with special attention to microcin C (McC) and albomycin, which have been the lead compounds for the here described aaRS inhibitors.

## 1.2 Aminoacyl-tRNA-synthetases: basic mechanisms and actions

### 1.2.1 Coupling of amino acids to a cognate tRNA

To create an aminoacyl-tRNA unit, a tRNA-subunit must be covalently attached to a specific amino acid. This reaction is catalyzed by aaRSs, which are specific for each amino acid and a corresponding group of tRNAs (isoacceptors). These enzymes have to recognize two

substrates: first, a set of tRNAs which share a collection of 'identity elements' and second, an amino acid that may be distinguished by small differences in side-chain properties.

The actual coupling of an amino acid to the corresponding tRNA comprises two steps. First, the amino acid (aa) is activated by nucleophilic attack on the α-phosphate of adenosine triphosphate (ATP) giving aminoacyl-adenosine-monophosphate (aaAMP) and pyrophosphate. The second step constitutes the esterification by a nucleophilic attack of the 2'- or 3' ribose hydroxyl group at the 3'-end (A76) of the cognate tRNA to the activated carboxyl group of the aaAMP generating the activated aa-tRNA species (Fig. 1.1). The correct aa-tRNAs interact with elongation factors (EF-1α in eukaryotes and Archaea, EF-Tu in prokaryotes) to translate the mRNA within the A site of the ribosome [8]. This process has been adequately documented many times (see *e.g.* Ataide *et al.* [19] and Mirande [20]).

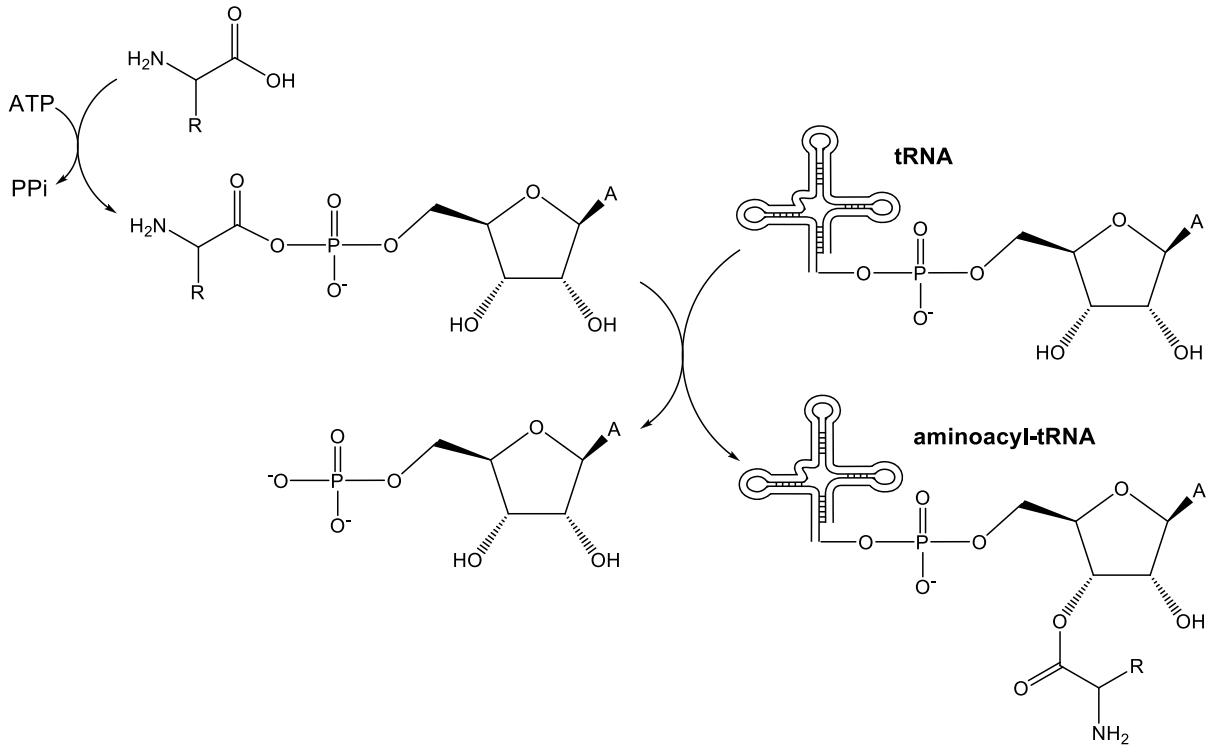



**Figure 1.1.** Aminoacylation occurs in two steps, both catalyzed by aaRSs. The aminoacylation reaction depicted here takes place at the 3'-hydroxyl moiety and thus is catalyzed by a class II enzyme. The 2'-hydroxyl is acylated by class I aaRSs.

### 1.2.2 Two distinct classes comprise twenty one aminoacyl-tRNA-synthetases

The 21 aaRSs are classified into two distinct classes: 11 in class I and 10 in class II, with LysRS found in each class. This partition is based on consensus motifs of the catalytic domains. Class I aaRSs contain two dinucleotide-binding Rossman folds, which is a structural motif that can bind nucleotides. It is composed of three or more β-strands linked by two α-helices. Since each Rossman fold can only bind one nucleotide, class I aaRSs contain two paired Rossman folds [21]. The active site of a class II aaRS is a barrel-like structure of

antiparallel β-sheets surrounded by loops and α-helices [22]. This structure forms a template that binds the respective amino acid and ATP. Furthermore, class I proteins differ from class II proteins in the position of esterification at the ribose moiety of the 3'-adenosine of the tRNA with the amino acid. Class I synthetases esterify at the 2'-hydroxyl group, whereas class II synthetases esterify at the 3'-hydroxyl group of the ribose [8, 23, 24]. This can be explained by the fact that class I proteins approach the tRNA acceptor stem from the minor groove side, whereas class II enzymes approach the tRNA from the major groove [8]. Further subdivisions in each class are made, based on sequence homology and domain architecture [22]. Table 1.1. shows the classification of aaRSs in the six subclasses.

**Table 1.1. Classification of the different aaRSs**

| **Class I** | | | **Class II** | | |
|---|---|---|---|---|---|
| **Ia** | **Ib** | **Ic** | **IIa** | **IIb** | **IIc** |
| LeuRS | | | HisRS | | |
| IleRS | TyrRS | ArgRS | ProRS | AspRS | |
| ValRS | TrpRS | GlnRS | SerRS | AsnRS | PheRS |
| CysRS | LysRSI | GluRS | ThrRS | LysRSII | |
| MetRS | | | GlyRS | | |
| | | | AlaRS | | |

The recognition of the cognate tRNA is for all aaRSs dependent on the discriminator base N73, the acceptor stem and the anti-codon of the tRNA. To maintain high fidelity in the catalytic coupling of tRNA to amino acids, all aaRSs contain a distinct structural domain for anticodon recognition. In addition, some aaRSs contain a zinc-binding domain that is involved in the recognition of the acceptor stem [22].

Interestingly, LysRS is found in both class I and class II. Most organisms contain a class II LysRS, but some bacteria and Archaea only possess a structurally distinct class I LysRS instead. As class I LysRS is not found in Eukarya and differs in substrate specificity from its class II analogue, this class I LysRS may be an interesting species-specific target for antibacterial drug development [25].

### 1.2.3 An editing site hydrolyses the misactivated amino acids

Correct aminoacylation depends on the selection of two appropriate substrates, the tRNA and the amino acid, by the corresponding aaRS. Since the tRNA is a relatively large unit and

therefore has a large number of 'identity elements', the selection of tRNA is much easier than the selection of the smaller amino acids. Amino acids have to be selected by the nature of their side chains [25]. Although each amino acid has a different structure, some have similar chemical and/or structural properties.

Two different editing mechanisms exist to decrease the number of incorrectly aminoacylated tRNAs. In pre-transfer editing, the misactivated amino acid is hydrolyzed into the amino acid and AMP, whereas in the post-transfer editing, the incorrectly aminoacylated tRNA is hydrolyzed into the amino acid and tRNA [26].

The synthetic site is mostly specific enough so that only the correct amino acid can be activated and transferred, due to the recognition of specific properties of each amino acid and the steric exclusion of amino acids with larger side-chains. Also, it has been reported that the difference in sugar puckering and the orientation of the C(4')-C(5') bond of the adenosine plays an important role on how aaRSs discriminate cognate from non-cognate aminoacyl-AMP [15].

Apart from the synthetic site, an editing site exists to hydrolyze misactivated amino acids. The presence of two catalytic sites with different activities led to the proposal of a double-sieve model. In this model the synthetic site of the enzyme acts as the first sieve, excluding amino acids that are too large or do not establish the right interactions with the active site. The smaller amino acids that can establish sufficient interactions however, may slip through this first sieve and may be incorrectly activated. The editing site, which is too small to fit the cognate amino acids, is capable of hydrolyzing misactivated amino acids. This double sieving mechanism raises the accuracy to about one mistake in 40,000 aminoacylation reactions [27]. The interplay between pre- and post-transfer editing in tRNA synthetases has been reviewed recently by Martinis and Boniecki [28].

### 1.2.4 Indirect biosynthesis of $tRNA^{Asn}$ and $tRNA^{Gln}$ by transamidation

Beside the sieving mechanism in the aaRSs, Archaea and bacteria have an additional system involved in the editing of non-cognate aminoacylated tRNA. The aminoacyl-tRNA amidotransferase (AdT) can modify the coupled amino acid to obtain the cognate aminoacyl-tRNA. This is not only an error reducing mechanism, but also an indirect pathway for the biosynthesis of Asn-$tRNA^{Asn}$ and Gln-$tRNA^{Gln}$, which is for some bacteria the only source to obtain these charged tRNAs, *e.g.* for *Helicobacter pylori* [29]. The misacylated tRNAs are synthesized by non-discriminating GluRS and AspRS, which also aminoacylate Glu onto $tRNA^{Gln}$ and Asp onto $tRNA^{Asn}$ [30]. Since this mechanism is not present in eukaryotic cells, aminoacyl-tRNA amidotransferases are interesting targets for drug development. Hereto, some analogues bearing resemblance to the 3'-end of aminoacylated tRNA like aspartycin and glutamycin (**1a,b;** Fig. 1.2) or to the reaction intermediates of the transamidation reaction

like **3**, have been synthesized and tested for antibacterial activity [30]. More recently a series of chloramphenicol analogues was synthesized, uncovering compound **4** within their series as the most active inhibitor of the transamidase activity with respect to Asp-tRNA$^{Asn}$ with a $K_i$ value of 27 μM [31].

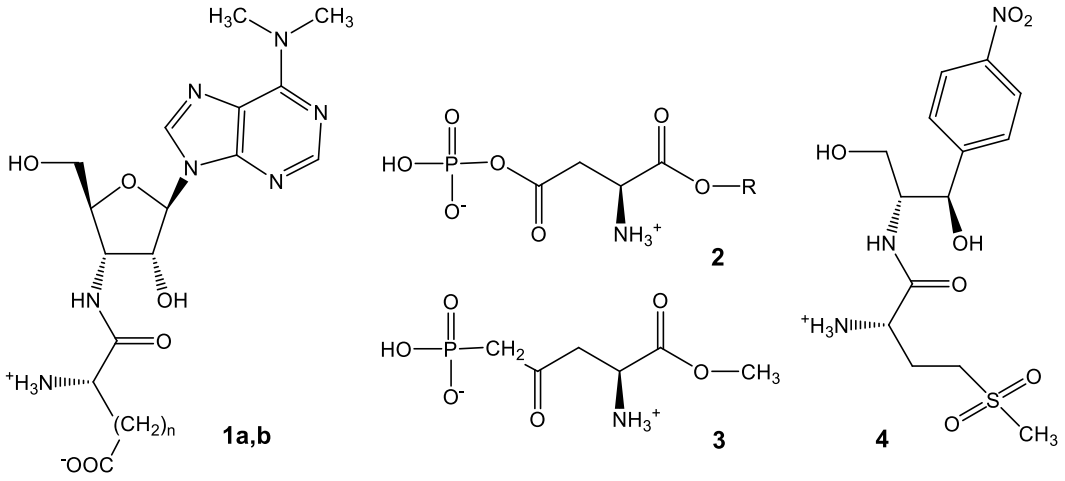



**Figure 1.2.** Structures for the amidotransferase inhibitors aspartycin (**1a**, n=1) and glutamycin (**1b**, n=2), and for the chloramphenicol analogue **4**. While structure **2** depicts α-phosphoryl-aspartyl-tRNA$^{Asn}$, the reaction intermediate in the transamidation reaction, with R indicating the remainder of the tRNA, compound **3** is a stable phosphonate mimic as small molecule inhibitor of the reaction.

### 1.2.5 Non-ribosomal peptide synthetases fulfill a similar role as aaRSs

Many important peptides, produced by bacteria and fungi, are synthesized by non-ribosomal peptide synthetases (NRPSs) with cyclosporine A, gramicidin S and bleomycin A2 being examples of such peptides. NRPSs can be seen as a series of modules, in which each module is responsible for the incorporation of an amino acid into the growing polypeptide chain. In contrast to aaRSs, NRPSs are both template and peptide producing enzymes. Each module can be subdivided into three domains, which are similar to the ribosomal peptide machinery. Hence, both systems use aminoacyl-adenylates as their building blocks. As a consequence, also the NRPSs can be inhibited by 5'-*O*-(*N*-aminoacyl)-sulfamoyladenosines (aaSAs, *vide infra)* at concentrations in the low nanomolar range [32]. However, both systems bind these inhibitors in different conformations.

Peptide synthesis by the ribosomal machinery is extraordinarily efficient and occurs with high fidelity, but is restrained to a set of 20-22 amino acids. In contrast, peptide synthesis by NRPS lacks proofreading activity, but can use a variety of substrates, including D-amino acids, fatty acids, and aryl acids [33]. This makes NRPS ideal targets for potential antibiotics.

## 1.3 Existing aminoacyl-tRNA-synthetase inhibitors

Most inhibitors of aaRSs act by competitive binding at the active site where normally the cognate amino acid would bind. Many inhibitors known to date are natural products or derivatives of them. Only few compounds have reached the stage of clinical development. A selection of the most important compounds (all depicted in Fig. 1.3) will be discussed in the next section.

### 1.3.1 Mupirocin is the only approved aaRS inhibitor

Of the numerous aaRS inhibiting compounds mupirocin (**5**; Fig. 1.3), marketed as Bactroban® by GSK, is the only aaRS inhibitor approved by the FDA [19, 34]. Originally it was isolated from *Pseudomonas fluorescens*. It is targeted against IleRS, and functions as a competitive inhibitor at the synthetic active site ($K_i$: 2.5 nM for *Escherichia coli* IleRS). The tetrahydropyran ring binds at the place where ribose would normally bind, the epoxy-group binds in the amino acid pocket and the fatty acid binds inside the adenine pocket. Mupirocin is primarily active against Gram-positive bacteria, *e.g.* methicillin resistant *S. aureus* (MRSA) [23] (MIC: 0.25-0.5 µg/mL) showing an 8000-fold selectivity for pathogenic aaRS over human aaRS [24]. It is less active against Gram-negative bacteria (MIC: 128 µg/mL for *(E. coli)*). Because it is used as topical ointment, high local concentrations can be achieved, making it sometimes suitable for Gram-negative bacteria as well.

Unfortunately, it appears that resistance is developing against this antibiotic as well. Low-level resistance has been reported by mutation of its target, IleRS, whereas high-level resistance is found due to the presence of a second IleRS with many similarities to eukaryotic enzymes, due to acquisition of the *mupA* gene [23, 35]. Another drawback of mupirocin is its poor bioavailability; since its ester function is highly unstable, it is rapidly hydrolyzed in blood and tissue. Hence, its use is limited to topical treatment. Therefore, many analogues of mupirocin have been created, although none of these have reached the clinic. The most successful analogue was SB-234764 (**6**), which combined structural features of IleSA and mupirocin. However, with the aim of gaining selectivity, further modifications especially to the base moiety have been envisaged, culminating in substitution of a phenyltetrazole moiety for the adenine (**7**, CB-432, *vide infra*).

### 1.3.2 Clinical development candidates

Indolmycin (**8**) is an inhibitor of tryptophanyl-tRNA synthetase (TrpRS) [36]. Indolmycin is a biosynthetic derivative of Trp, which has a number of other intracellular functions that affect viability [19]. Probably due to its hydrophobicity, which may impair cellular uptake, the overall *in vivo* inhibition of TrpRS is limited. Pfizer filed a patent already in 1965, although further

development was discontinued, since it appeared that indolmycin was not sufficiently active against the majority of commonly occurring pathogenic bacteria like streptococci, enterococci and Enterobacteriaceae [22]. More recently, indolmycin has been shown to exert a bacteriostatic activity against *S. aureus*. However, certain strains have been isolated that have adopted resistance *via* a point mutation (H43N) in TrpRS [37]. Also *Streptomyces griseus* was shown to adopt resistance to indolmycin. *Helicobacter pylori* on the other hand, showed to be unable to develop resistance [38].

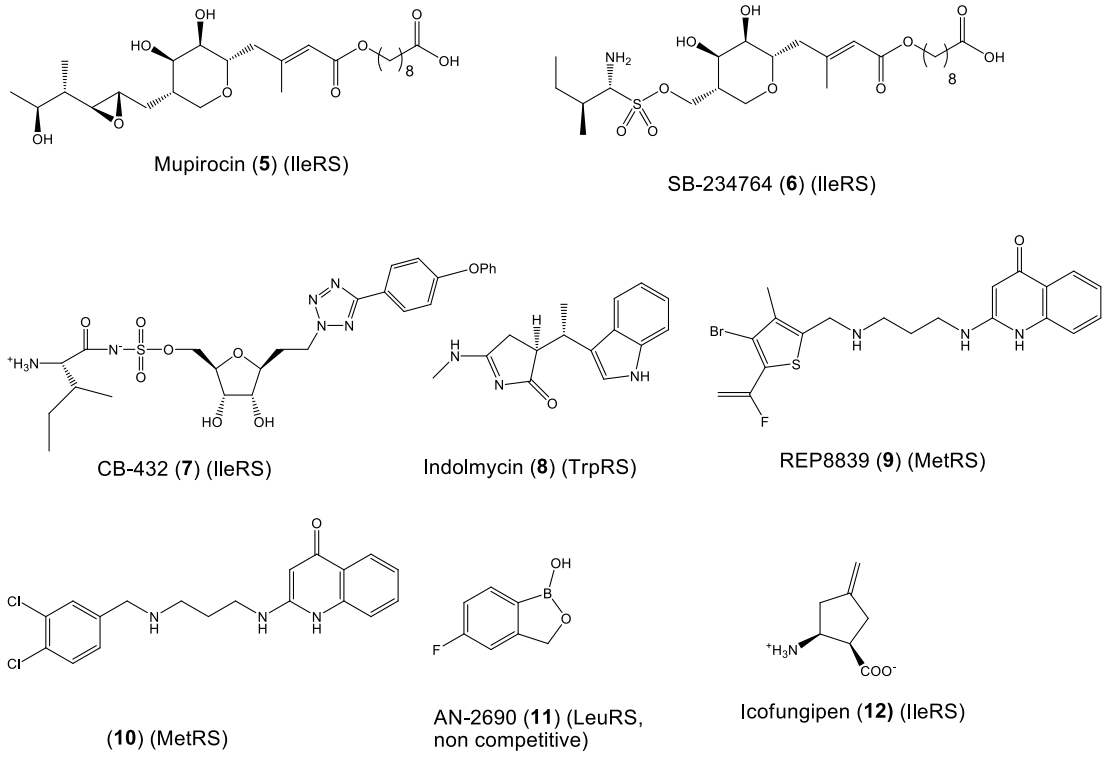



**Figure 1.3.** Structures of some well-known synthetic aaRS inhibitors.

REP8839 (**9**) is a fluorvinylthiophene linked *via* a 1,3-diaminopropane with a quinolone. It is a very potent analogue of the original quinoline derivative **10** found *via* a high throughput screening effort by Jarvest *et al* [39]. REP8839 is a fully synthetic inhibitor of MetRS, currently in phase I clinical trials for the treatment of skin and wound infections of *S. aureus.* Aside from mupirocin- or oxacillin-resistant *S. aureus* strains ($MIC_{90}$: 0.5 µg/mL), this compound also showed good activity against *Streptococcus pyogenes* (MIC: 0.03-0.5 µg/mL) as well as against a number of other staphylococci and enterococci. Interestingly, the $K_i$ of REP8839 for *S. aureus* MetRS was found to be ~10 pM, while this compound showed significantly weaker inhibition for *E. coli* MetRS with a $K_i$ value of 300 nM. Moreover, no inhibition of mamalian rat liver MetRS was found, while against human mitochondrial and cytoplasmic MetRS this compound showed $K_i$ values three- to six-fold higher than *S. aureus* MetRS. Thus REP8839

is a relatively selective compound with high potential [40]. The compound also proved to be well tolerated when applied for intranasal ointments [41].

AN-2690 (**11**) is a fluorinated benzoxaborole with activity against dermatophytes, yeasts, and molds. It is perfectly capable to penetrate nail tissue. For this feature it is being pursued to treat onychomycosis (infection of the nail). In contrast to most other aaRS inhibitors, it is a non-competitive inhibitor of LeuRS, and binds in the editing site of the enzyme [14]. Here it traps $tRNA^{Leu}$ through the formation of two covalent bonds of boron with the 2',3'-hydroxyl groups of the 3'-terminal adenosine of $tRNA^{Leu}$ with formation of acyclic borate structure. Phase I and II clinical trials have shown efficacy and safety [22], with phase III trials ongoing, carried out by its developer Anacor. Very recently analogues of AN2690 were found to strongly inhibit LeuRS of *Trypanosoma brucei*, paving the way likewise for anti-parasitic drug development [42].

Icofungipen (**12**) is an antifungal that inhibits IleRS. Through active transport by permeases this compound accumulates in yeast cells up to 200-fold of the extracellular concentration [43]. It was discovered through a program directed towards a more potent derivative of cispentacin (**13**). The 1*R*, 2*S*-configuration was found to be essential, and, aside from the methylene addition at the 4-position, no other additions or substitutions were allowed, in order to retain high activity [44]. Good clinical efficacy and safety were observed in phase I and II clinical trials, although low mycologic eradication rates were observed in HIV-positive patients. To this end, higher dosage may be desirable [22].

### 1.3.3 Other natural aaRS inhibiting compounds

Cispentacin (**13**) (PLD-118; Fig. 1.4) is a cyclic β-amino acid that has been isolated from two species: *Bacillus cereus* and *Streptomyces setonii.* It is effective against *Candida albicans* infection in mice. Although icofungipen is a follow-up of this compound and inhibits IleRS [22, 23], cispentacin itself is a millimolar inhibitor of ProRS.

Chuangxinmycin (**14**) bears some resemblance to indolmycin, and as a consequence it also inhibits TrpRS. Initially it was reported for its activity against a range of Gram-positive and Gram-negative bacteria. It showed good efficacy against *Shigella dysenteria* and *E. coli* in infected mice. Despite its apparent potency, there are no reports on further development of this compound [23].

In contrast to most other aaRS inhibitors, borrelidin (**15**) is an allosteric inhibitor. By binding to a hydrophobic patch of ThrRS, it impairs catalytic conformational changes necessary for Thr and ATP binding [45]. Apart from its ThrRS inhibition, borrelidin also activates caspases 3 and 8, hence inducing apoptosis. As of these effects, borrelidin is evaluated in further studies for its potency for angiogenesis inhibition. The compound showed good absorption and

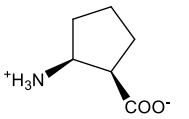
membrane permeability, and proved to be non-mutagenic in the Ames test, but an inhibitor of CYP3A [46, 47].

Non-hydrolyzable analogues of the aminoacyl-AMP form the largest class of potentially active compounds against aaRSs [34]. Agrocin 84 (**16**, Fig. 4) is such a well-known aaRS inhibitor and is used to inhibit the formation of plant tumors caused by *Agrobacterium tumefaciens*. Agrocin 84 is a derivative of leucyl-adenylate, but contains a D-glucofuranosyloxyphosphoryl moiety which is important for uptake by the pathogen. This moiety is cleaved off intracellularly following uptake [19]. A similar mode of action is presented by microcin C (McC, **21a**), which will be further discussed in the next sections. The toxic moiety of agrocin 84 inhibits cellular leucyl-tRNA synthetases, but the agrocin 84 producing *Agrobacterium radiobacter* K84 strain carries a second, self-protective copy of the synthetase, termed AgnB2 and providing immunity to the antibiotic [48]. The genetic basis for the production and self-protection to agrocin 84 has been discussed by Kim *et al.* [49]. The full synthesis of agrocin 84 was already described by Moriguchi *et al*. [50].

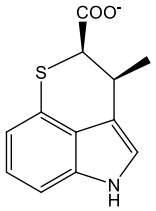
Another interesting natural antibiotic, which is also an aa-AMP analogue, is ascamycin (**17**), produced by a *Streptomyces* strain and carrying a 2-chloroadenine moiety. Ascamycin inhibits incorporation of phenylalanine in *Xanthomonas citri* and *X. oryzae.* No activity was observed against *E. coli*., which was blamed on the compound being charged, hampering permeation of the bacterial membrane [51]. *X. citri* and *X. oryzae* possess an Xc-aminopeptidase at their cell surfaces that metabolizes ascamycin into its dealanyl derivative. The dealanyl analogue showed activity against a range of Gram-negative and Gram-positive bacteria [52].

Phosmidosine (**18**) is a proline-AMP analogue and according to its structure, most likely also targets the corresponding ProRS, although this has never been reported. The natural compound was first described in 1991 as an antifungal nucleotide antibiotic inhibiting spore formation of *Botrytis cinerea* at the concentration of 0.25 µg/mL [53]. In view of its rare *O*-methylated phosphoramidate structure, the compound is rather base unstable. Sekine *et al*. [54] tried to circumvent this instability in synthesizing different analogues and studied the structure-activity relationship of this potent antitumoral compound with its unique property of arresting cell growth at the G1 phase in the cell cycle. While both phosphoramidate isomers proved equally active, presence of the L-proline part was mandatory for the activity.

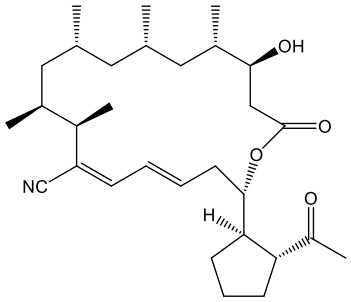
Several natural compounds were identified as sideromycins by their ability to chelate iron and subsequently being recognized as siderophore. Albomycin (**19**, **20**) also exhibits a Trojan horse mode of action like McC. Albomycin will be further discussed in section 1.9.1.



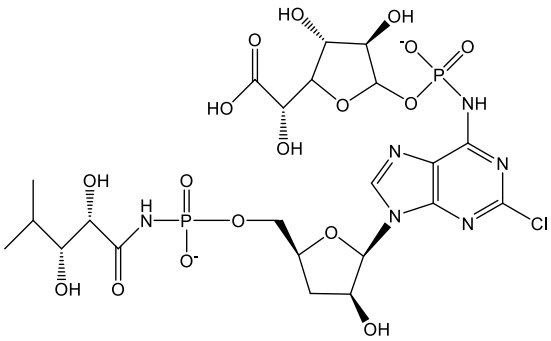
Cispentacin (**13**)
(ProRS)



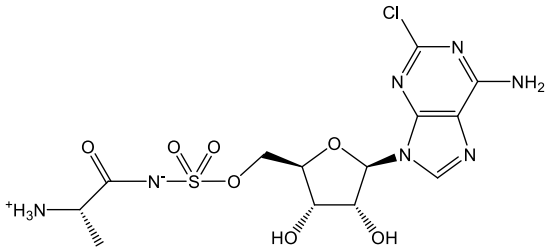
Chuangxinmycin (**14**
(TrpRS)



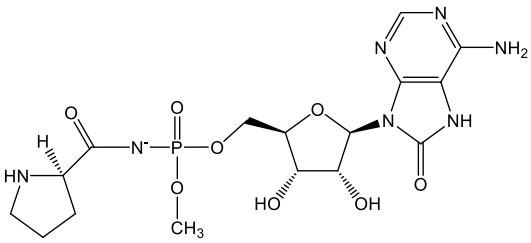
Borrelidin (**15**)
(ThrRS, allosteric)



Agrocin 84 (**16**) (LeuRS)



Ascamycin (**17**) (PheRS)



Phosmidosin (**18**) (ProRS?)

R = H: SB-217452 (**19**) SerRS Y = N-CO-$NH_2$

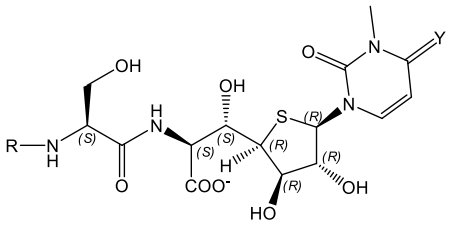
Albomycin (**20**)

Y = N-CO-$NH_2$ delta2

Y = NH epsilon

Y = O delta 1

R=



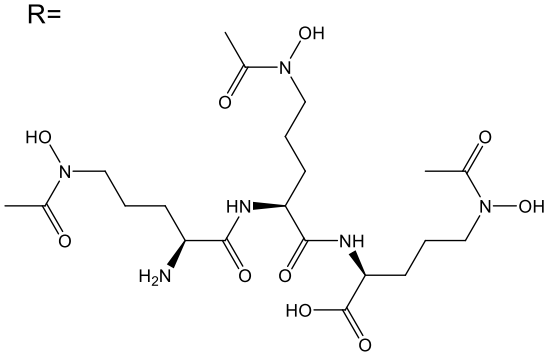


**Figure 1.4**. Chemical structures for some well-known natural aaRS inhibitors.

## 1.4 Microcin C

### 1.4.1 Biochemical synthesis

Microcin C (McC, **21a**) (Fig. 1.5) is another natural compound that forms, together with albomycin, the lead compound for this thesis. The compound is produced by Enterobacteriaceae, and belongs to the bacteriocins which are peptidic antibiotics encoded on gene clusters, with the colicins being produced by *E. coli* (formerly called 'colicines,' meaning 'coli killers'). Microcins are related to colicins, which have the same mode of action and are produced by the same family of bacteria. Colicins differ from microcins in their size: 25 to 80 kDa versus less than 10 kDa, respectively. Also the production and secretion of microcins are different. While colicin production is triggered by the SOS system, microcin production starts when the prokaryotic cell approaches the stationary growth phase (G1-phase). Usually the G1-phase is set when the cell senses a depletion of nutrients. Furthermore, colicins are selectively leaked into the extracellular environment upon activation of a cellular phospholipase by a lysis-protein which is encoded on the colicin operon. This leaking usually results in cell death of colicin-producing organisms. Microcin-producing organisms however, are capable of protecting themselves against the self-made microcin antibiotic.

In *E. coli*, six structural genes are found in the *mccC7* cluster, which determine the production, maturation and secretion of McC, and provide self-immunity. McC51, previously believed to be a different microcin, is identical to McC7, and both are referred to as McC, as both microcin products are identical [55-57]. The first gene in the cluster, *mccA*, with only 21 base pairs, is the smallest bacterial gene known, it encodes the heptapeptide precursor of McC (f-MRTGNAN) [58]. This precursor is extensively modified during a maturation process, in which it is linked to adenosine *via* a phosphoramidate linkage. Hereto, the *mccB* product catalyzes the condensation of AMP to the *C*-terminus of the heptapeptide, according to a two-stage reaction mechanism (Fig. 1.5) which was finally unraveled by Roush *et al*.[59] In the first step the α-carboxylate of the Asn7 moiety attacks the Pα, and hereby an unstable acyl adenylate is formed. Although a weak nucleophile, the nitrogen at the amide of the Asn7-side chain then attacks the *C*-terminal carbonyl, expelling AMP and forming a *C*-terminal succinimide. Subsequently, the nitrogen of the succinimidyl moiety attacks the Pα of the second ATP molecule, releasing pyrophosphate. By nucleophilic attack of water at the γ-carbonyl of the cyclized Asn7, ring-opening takes place. Hence, the heptapeptide is linked to adenosine via a P-N bond, while the *C*-terminal Asn7 is modified to an aspartyl-residue.

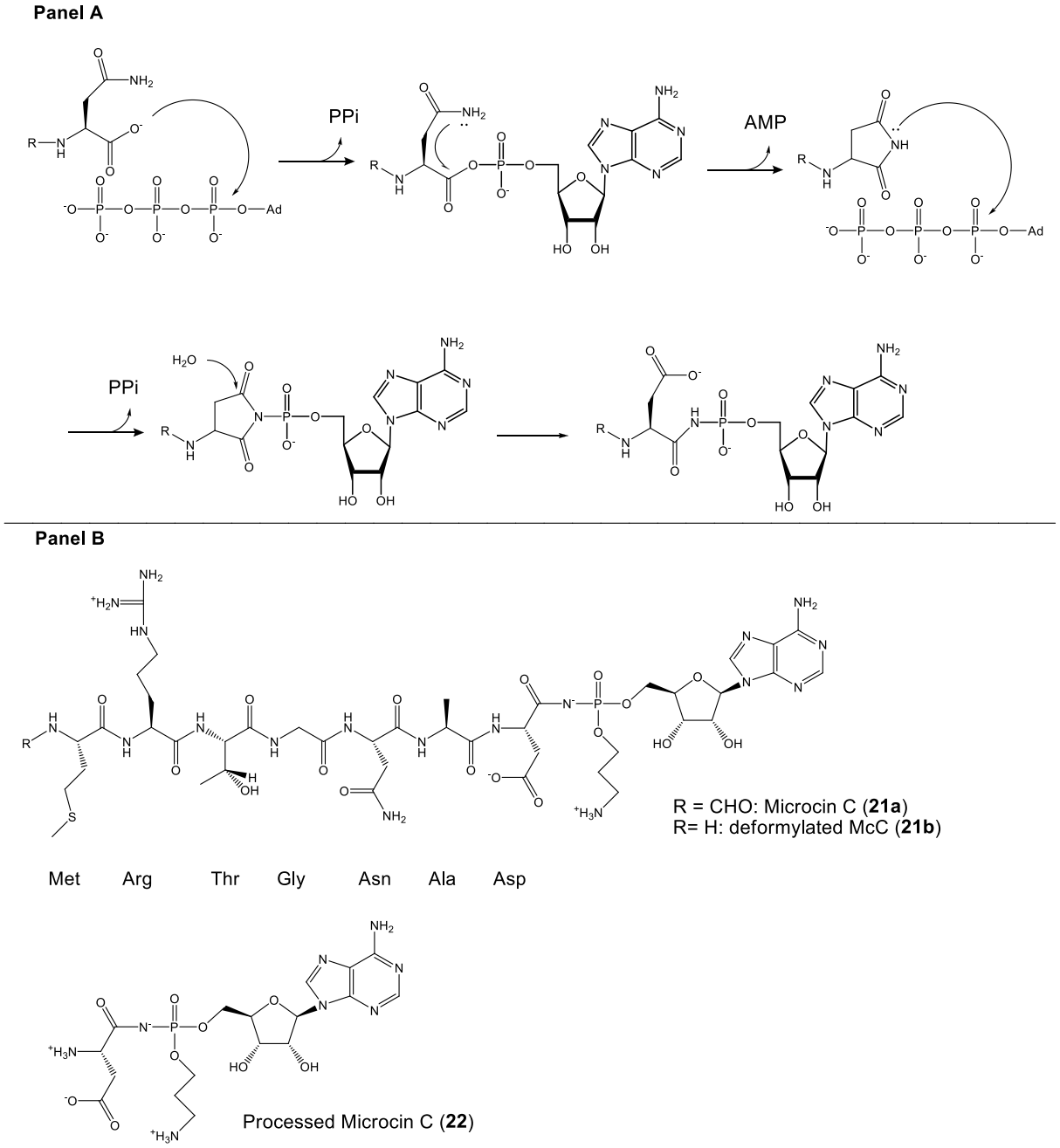



**Figure 1.5.** Panel A. Coupling of the heptapeptide to ATP as catalyzed by MccB. Panel B. Full structure of McC (**21a**) and the deformylated analogue (**21b**) and processed McC (**22**) as found after metabolization.

Following correct formation of the phosphoramidate, the *mccD* and *mccE* genes are required for attachment of the 3-aminopropyl group to the phosphate of McC and it has been demonstrated that the latter group increases the potency for inhibition of the McC target, the aspartyl-tRNA synthetase [60]. Since MccC displays 11 transmembrane domains and also shows great similarity with known transport proteins, it has been suggested that this protein may be involved in the export of McC to the extracellular environment [56]. Disruption of the

*mccC* gene was shown to be highly toxic to the McC-producing cell. Moreover, these cells were highly susceptible to exogenously added McC [61].

Once McC is assembled, it becomes already prone to internal processing by different oligopeptidases with the release of a non-hydrolyzable aspartyl-adenylate. Processed McC (**22**) therefore would inevitably accumulate in the producing cell, and this would inhibit AspRS, leading to the cessation of translation. If in addition, the *mccE* gene is disrupted, these cells become very sick [61]. Hence, there is a serious risk of self-poisoning by McC, which – apart from export by MccC – seems to be counteracted *via* two additional mechanisms. Following conversion to the active aspartyl-adenylate analogue, MccE, in addition to its involvement in attachment of the aminopropyl moiety, acetylates the processed McC and converts it into a non-toxic compound.[61] The authors speculate that MccE which is a Rim family member may detoxify various aminoacyl-nucleotides, either exogenous or those generated inside the cell.

Second, next to the *mccABCDE* operon, the additional *mccF* gene which is read in opposite direction, codes for a serine protease-like enzyme, providing resistance to exogenous McC. It has been shown recently that MccF detoxifies both intact and processed McC by cleaving the amide bond of the acyl phosphoramidate linkage [62]. This is a rather unexpected finding and in addition it was shown that some acyl sulfamoyladenylate analogues likewise were cleaved, releasing sulfamoyl adenylate, which was earlier shown to be a broad-spectrum antibacterial agent by itself with unknown mode of action [63].

### 1.4.2 Mechanism of action

Recently, Novikova *et al*.[64] found a single locus, *yejABEF*, encoding an inner membrane ABC transporter responsible for uptake of McC. Deletion of any one of the *yej* genes leads to McC resistance. Once inside the cell, McC is processed to liberate the active compound (Fig. 1.6). As do most ribosomally synthesized bacterial peptides, the *N*-terminus of the peptide moiety of McC bears a formyl cap. McC derivatives lacking the formyl group (**21b**) display a much lower activity than the native McC (**21a**), and a specific deformylase is necessary to start the processing of McC. *In vitro*, the rate-limiting step of McC processing has been shown to be deformylation of the first methionine residue [65].

Subsequently, the peptide tail of the deformylated compound McC is sequentially removed by one or more of the three broad-specificity peptidases - PepA, B, and N [65]. Hence, the resulting product set free is a relatively stable analogue of aspartyl adenylate, with the propylamine still attached to the phosphoramidate linkage. This compound is able to selectively inhibit AspRS [66], and according to its mechanism, McC is referred to as a Trojan horse inhibitor.

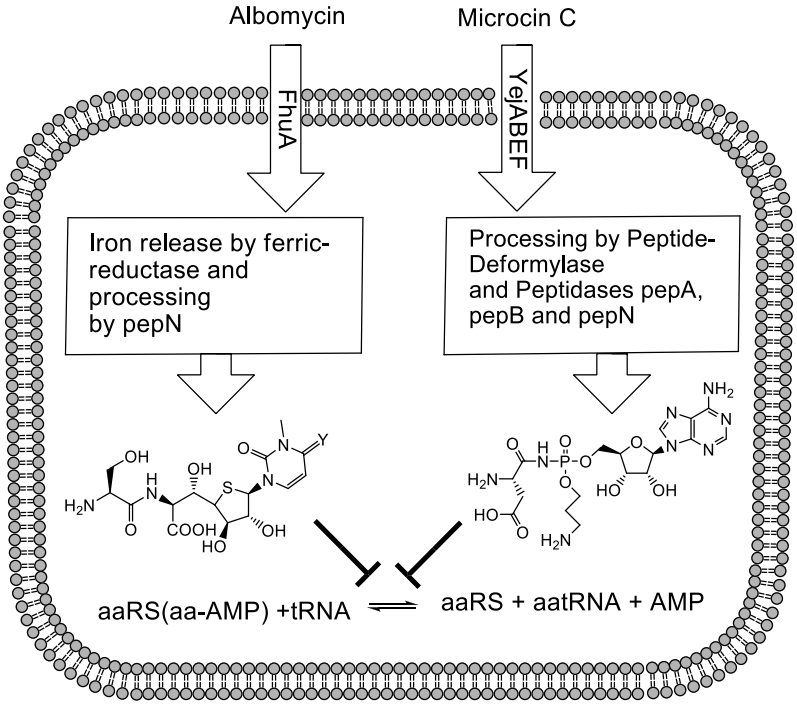



**Figure 1.6.** Schematic drawing of the Trojan horse mode of action of McC and albomycin.

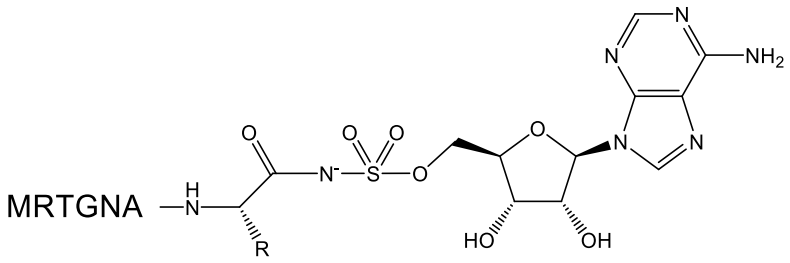
In addition, Metliskaya and co-workers recently found that processed McC binds to AspRS competitively with respect to Asp and ATP. Thereby it inhibits the first step in aminoacylation of the cognate $tRNA^{Asp}$ by blocking pyrophosphate exchange. Thus, processed McC poisons the AspRS, and in doing so it inhibits activation of the amino acid [66].

### 1.4.3 Analogues of McC as potential antibiotics

While the genetic code is recognized by the anticodons of the different tRNAs, the aminoacylation reaction of the tRNA itself requires specific aminoacyl-tRNA synthetases (aaRSs) [67]. Hence, inhibition of the latter will block the whole protein synthesis machinery. Therefore, it has been well documented already in the past that different aminoacylated sulfamoyl adenosine (aaSA) analogues and some of their congeners with isosteric linkages are strong inhibitors of aaRSs [23, 68-70].

However, while very strong inhibitory properties have been reported for such derivatives using *in vitro* evaluations, their potency as antibiotics is limited by their bioavailability. McC on the other hand was found to circumvent the problem of poor uptake by employing a peptide transporter that imports the whole compound as a kind of prodrug. New compounds that implement a signal peptide as in McC, may therefore be a solution to the problem of poor bioavailability. Hence, following the results obtained with McC, several analogues of McC were synthesized and evaluated for their ability to inhibit the aminoacylation reaction.

By means of site-directed mutagenesis in the *mccA* gene it was accomplished to produce a large set of McC analogues with amino acid substitution at the peptide moiety of McC [71]. While most of these substitutions did not significantly alter the activity of these compounds, many other substitutions did not allow for maturation leading to a functional McC. Of note, no substitution at the 7th position of peptide chain did allow for maturation [71]. In retrospect, this finding can be easily explained by the proposed mechanism for linking the peptide to adenylate, as described previously. At the same time, it was argued that the amino acid at the 7th position of the McC compound is of importance for the inhibition of its cognate aaRS. Therefore, an alternative strategy was devised for the production of McC analogues varying at the 7th position. By combining the existing methods for the synthesis of aaSAs, and classical solid-phase peptide chemistry new McC analogues could be synthesized, resulting in three reported analogues of McC with in 7th position either a Asp, Glu or Leu, respectively (**23-25**, Fig. 1.7). These compounds differed at three points from the natural McC: *i*) the *N*-terminal formyl group was omitted, *ii*) a sulfamoyl linker instead of a phosphoramidate linker was used, and *iii*) the *O*-aminopropyl group was left out. The important finding from this study was that in analogy with McC, these compounds were internalized by the YejABEF peptide transporter and subsequently metabolized by the three broad-specificity peptidases PepA, B, and N. It was further shown that the metabolized analogues could still inhibit their cognate aaRSs, proving that intracellularly the desired Asp-SA, Glu-SA and Leu-SA were obtained respectively, and thus the hypothesis of the Trojan horse mode of action was borne out [72].




**Microcin C analogues**
**23: R = -$CH_2COOH$**
**24: R = -$(CH_2)_2COOH$**
**25: R = -$CH_2CH(CH_3)_2$**

**Figure 1.7**. Microcin C analogues **23-25**, where the phosphoramidate is replaced by a sulfamoyl linker.

The same study revealed that the whole-cell antibacterial activity of MRTGNAD-SA (**23**) is about ten-fold higher than the one for the natural compound derivative which lacks both the *N*-formyl and the aminopropyl ester moiety. However, native McC still proves more active than MRTGNAD-SA. The main reason is probably the absence of the *N*-terminal formyl

group, since plain deformylated McC is also at least ten times less active in comparison to native McC. Thus, the addition of an *N*-terminal formyl group to the synthetic analogues could, besides increasing the selectivity for bacteria, also improve its activity. In addition, the absence of an aminopropyl group probably contributes to the lower observed activity of the chemical analogues as it was shown that this moiety enhances the activity [60].

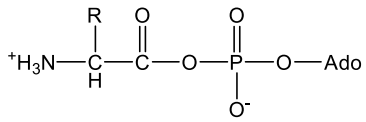
While the prospect looks promising, a cautionary note should be given though. A recent report by Messmer *et al.* confirmed Asp-SA to be the most active inhibitor of different AspRSs with stronger effects on the bacterial enzymes in comparison with human cytosolic AspRS [73]. However, for the first time likewise a strong inhibition was found on the human mitochondrial synthetase. Hence, medical applications by aaRS inhibitors should eventually consider the possible side effects on the host mitochondrial enzymes, although it is not clear yet whether the latter findings will be translated to an *in vivo* setting.

## 1.5 Recent developments in aminoacyl-AMP type inhibitors

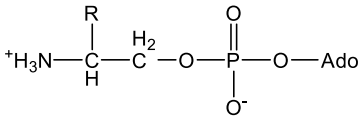
A good aaRS inhibitor has to meet several criteria. First, it should be able to interact with great affinity in the active site of the aaRS, relative to the normal substrates, *i.e.* ATP and the cognate amino acid. Second, the activation energy for hydrolysis of the analogue into AMP and the amino acid should be sufficiently high. Aside from the natural aaRS inhibitors, many analogues of aminoacyl-AMP have been created that are currently under study.

Initially it was hypothesized that in general replacement of the labile aminoacyl-phosphate (**26**) (a mixed anhydride) by a non-hydrolyzable bio-isoster, such as an aminoalkyl adenylate [34, 74, 75] (**27**), an aminoacylsulfamoyl adenosine [15, 76] (**28**) linkage or an even more simplified linkage like an amide [77] would lead to a similar interaction with the enzyme (Fig. 1.8). It was observed that the phosphoramidate linkage, as found in McC and agrocin 84, is unstable in acidic environment [78] and physiological conditions. However Moriguchi *et al*. [50] reported an unstable phosphoramidate linkage in agrocin 84 in basic conditions. Also, the phosphoramidate linkage in phosmidosine was found to be base labile [79], but the latter two findings refer to lability of the additional ester linkages.

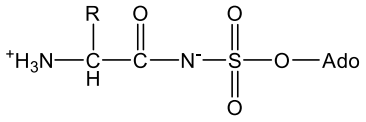
Most analogues consist of an adenosine coupled to an amino acid (analogue) *via* a stable sulfamate/ester/phosphonate linkage instead of a labile phosphoanhydride linkage. In addition, the synthesis of β-ketophosphonates (**29**) has been reported [80].



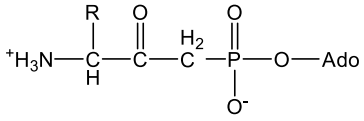
aminoacyl adenylate (**26**)



aminoalkyl adenylate (**27**)



aminoacyl sulfamoyladenosine (**28**)



*beta*-ketophosphonate derivative (**29**)

**Figure 1.8**. Possible linkages between adenosine and the amino acid as isosteres of the acyl-phosphate bond.

The most potent analogues are those bearing a sulfamoyl linkage as found in structures **17** and **28**. By X-ray analysis it was shown that the stable sulfamoyl linkage in 5'-*O*-[*N*-(L-seryl)-sulfamoyl]adenosine, a SerRS inhibitor, establishes similar hydrogen bonds inside the hydrophilic cleft of the enzyme [76]. Therefore it may be generally true that although the linkage is much more stable, the electron distributions closely resemble those of aa-AMP and the number of hydrogen bonds remains the same.

Forrest *et al*. [74] reported the presence of the carbonyl group, present in a sulfamoyl linkage but not in an aminoalkyl adenylate linkage, to be crucial for the recognition by a class II synthetase. Both acyl-phosphate mimics are negatively charged in solution due to acidity of either the phosphodiester or the NH function in the sulfamoyl moiety, respectively. Only the acyl sulfamate-containing a carbonyl, is able to delocalize this negative charge, seemingly of great importance in stabilization of the transition state.

An X-ray crystallographic conformation study of AlaSA showed the compound to be in a zwitterionic state in the crystal. The molecule was found to be in a stretched conformation, with the alanyl- and adeninyl-moieties at different sides of the molecules. Ribose puckering was in the expected C(3')-endo or North conformation, and the adenine base was in anti-position with respect to the sugar. This work also confirmed that sulfamate and phosphate are indeed close isosteres [15]. As a consequence, aaRSs bind their cognate aaSA with great affinity, but the carboxyl-sulfamate bond is considerably stronger than the carboxyl-phosphate bond in aa-AMP. This way the aaRS catalytic site becomes blocked, providing the sulfamate containing analogues the best potential toward further drug development. In general, all reported $K_i$ values are in the low nanomolar range up to 50 and 60 nM for GlySA [32] and PheSA [33].

## 1.6 Aminoacyl sulfamoyl-adenosines are not selective and display poor bioavailability

All compounds, discussed in the previous section, are reaction intermediate mimics, implying that these compounds differ only little from the natural reaction intermediates, and thus selectivity for either eukaryotic or prokaryotic aaRSs is low. As was discussed in section 1.2., during evolution extensive divergence occurred in the amino acid sequences of prokaryotic and eukaryotic aaRSs, however this did not lead to great structural difference. As a consequence, both prokaryotic and eukaryotic aaRSs use identical reaction intermediates.
By modifications at the adenine base, researchers have tried to increase the selectivity for bacterial aaRSs. The most successful example is probably CB-432 (**7**), an IleSA analogue whereby the adenine is replaced by a large apolar substituent. CB-432 showed 570 fold more affinity for the *E. coli* IleRS relative to the corresponding human enzyme [12]. Unfortunately, clinical development of this compound was discontinued as a consequence of low bioavailability, due to binding to serum albumin. Also other heterocycles like thiazole [81], and tetrazole [82] have been evaluated for activity and selectivity. Some of the thiazole derivatives have shown inhibitory activity against Gram-positive and Gram-negative bacteria as well as some selectivity over human aaRS [82].
Although aaSAs are potent inhibitors of aaRSs, their whole-cell activity is rather low, probably due to poor uptake [18]. Clues to this observation may come from the structure of any aaSA, since under physiological conditions, apart from the amino acid side chain, all aaSAs contain one negative charge at the sulfamate linkage and a positive charge at the amino terminal end. Thus, these are highly polar compounds that will not diffuse easily through the hydrophobic cell membrane. Ubukata *et al.* [51] found that L-prolyl-L-prolyl-sulfamoyl-2-chloroadenosine had an increased *in vivo* activity against both Gram-negative and Gram-positive bacteria, when compared to L-prolyl-SA. It was therefore suggested that peptide transporters are involved in increased uptake of the dipeptidyl-SA compound, leading to an increased activity, although this was not further investigated [51].
Following this example, Van de Vijver *et al*. [83] synthesized a diverse set of dipeptidyl-SAs (Fig. 1.9, **30**) that differed in physicochemical parameters such as hydrophobicity, net charge and size. These compounds were evaluated against several Gram-negative and Gram-positive bacteria. In general, whole-cell activity was shown to be relatively low. The reason why the L-Pro-L-Pro-sulfamoyl-2-chloroadenosine inhibitor did show a nice activity against a range of bacteria may be the presence of a proline-rich peptide transporter, such as SbmA, which is found in *E. coli* [84]. However, Van de Vijver *et al*. [83] found that such compounds, where proline was used as the *C*-terminal amino acid, suffered from extensive

decomposition. Several other dipeptide transporters have been described already before by different authors [85, 86].

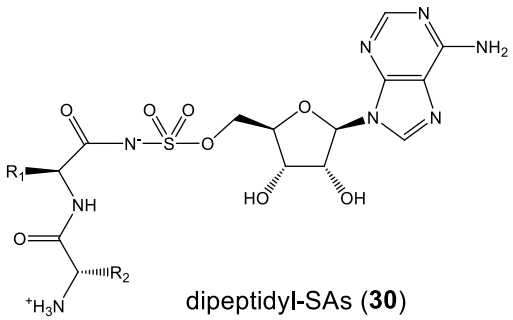



**Figure 1.9.** General structure of the dipeptidyl-sulfamoyladenosine analogues **30** ($R_1$ and $R_2$ can be any amino acid side chain, except $R_1$ cannot be proline).

## 1.7 More recent synthetic aaRS inhibitors

To address resistance development against the well known aaRS inhibitors, the search for new and further improved RS inhibitors is still ongoing. For instance, mutations in *S. aureus* MetRS have been found that conferred resistance to REP8839. However, these mutations also severely reduced bacterial fitness [22]. Therefore, many new quinolinone congeners have been prepared but unfortunately with reduced activity. Some however displayed significant inhibitory properties against enterococci [87]. Likewise, a series of 3-aryl-4-alkylaminofuran-2(5H)-ones were prepared and proved strongly inhibitory to Gram-positive organisms culminating in compound **31** (Fig. 1.10) endowed with $MIC_{50}$ of 0.42 µg/ml against *S. aureus*, but lacking activity *versus* Gram-negative bacteria [88]. Enzymatic tests indicated TyrRS to be the target and molecular docking proved a nice fit of the inhibitor with the TyrRS active site.
In addition, protozoal tRNA synthetases recently have been targeted. Benzoxaborole (see also **11**) was used as the lead structure for development of *Trypanosoma brucei* LeuRS inhibitors, resulting in compound **32** with an $IC_{50}$ of 1.6 µM. All stronger LeuRS inhibitors also afforded excellent *T. brucei* parasite growth inhibition activity, with the better results obtained for the more lipophilic congener **33** with an $EC_{50}$ of 0.37 µM [89]. Several research groups are using *in silico* strategies to uncover new leads for different tRNA synthetases, but confirmed results using such strategies are largely missing. For instance, a series of imidazolidin-2-ones as depicted in the general structure **34**, were believed to be inhibitory for IleRS following *in silico* screening, but unfortunately no activity could be detected following synthesis of the proposed structures [90]. Hoffmann and Torchala likewise proposed a series of potential LeuRS inhibitors which can be easily obtained *via* click chemistry. However, confirmation of the activity profile so far is lacking [91]. A last example proves that the strategy can be relatively fruitful as three moderately active inhibitors of *Staphylococcus epidermidis*

TrpRS were uncovered using a structure-based virtual screening effort [92]. While all three compounds displayed some common characteristics like a benzoic acid terminal end, overall structures proved quite diverse. Effective binding to the bacterial TrpRS was demonstrated by surface plasmon resonance, and low cytotoxicity to mammalian cells was reported. Bacterial growth inhibition assays however, showed only moderate activity with $MIC_{50}$ values of 6.25, 25 and 100 µM respectively.

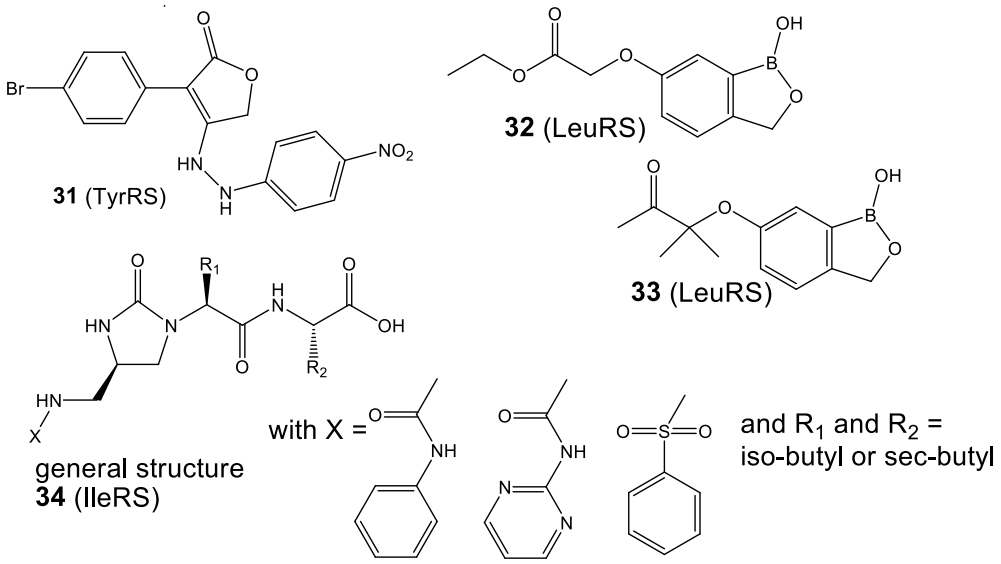



**Figure 1.10.** Some more recent developments lead to a series of furan-2(5H)-ones (**31**) and to benzoxaborole derivatives (**32, 33**) and in addition to many in silico active inhibitors represented by structure **34**.

## 1.8 Siderophores and sideromycins as tools for enhanced antibiotic uptake

Within the scope of this thesis, another interesting direction was considered to enhance the uptake of aaRS inhibitors *via* a Trojan horse concept. Current research is focused on so-called sideromycins that contain a siderophore moiety which complexes iron, an element desperately needed by virtually every organism and for which *in vivo* competition is high. A wide variety of transporters have been characterized for each class of siderophore. Specialized transporters and metabolization enzymes are needed for the uptake and release of the prodrugs, that therefore may aid in securing both bioavailability and selectivity. For example, it has been estimated that the potency of drug-siderophore conjugates (SDC) can increase up to 100-fold compared to passive diffusion [93]. Also, it has been suggested that resistance against sideromycins, although frequently observed, leads to substantial decrease in bacterial fitness. Since the *in vivo* competition for iron is high, resistance by mutations in

the iron-transport system could lead to iron starvation, thereby reducing the pathogenicity [94]. Thus, by utilizing this concept, already known compounds may be improved so that "new-old drugs" finally may be further pursued as antibiotics. Furthermore, mechanistic studies on the siderophore biosynthesis have led to an intensified search for inhibitors that may interfere in this field.

Many siderophore-drug conjugates have already been synthesized and have shown great potential. However, to the best of our knowledge none of these compounds have reached the stadium of clinical trials.

In addition to the beneficial effect on the development of antibiotics, other fields may also profit from this mechanism. For example, it is known that malignant cancer cells have a high demand for iron and therefore express higher levels of iron transporters [95]. Employing these transporters through an identical Trojan horse mechanism may thus aid in the development of anti-cancer drugs and/or cancer profiling.

To fully exploit this concept, the choice of siderophore moiety of a siderophore-drug conjugate is in this concept as pivotal as the actual drug itself. In this short introduction the already described siderophore transporters and their corresponding siderophores for the respective bacteria are compiled to better place our efforts in this area.

### 1.8.1 Siderophores as iron chelators

Possessing an ideal redox potential for carrying electrons in a physiological milieu, iron is desperately required in most living organisms, with the exclusion of a few specific bacterial strains. Therefore, a constant battle amongst different organisms is ongoing to sequester iron. In its prevalent ferric ion state ($Fe^{3+}$), iron is much less soluble than its ferrous state ($Fe^{2+}$), which can only prevail in anoxic conditions or at very low pH. Two solubility products were reported for ferric iron under physiological conditions: $K_{sp}$ $Fe(OH)_3 < 10^{-18}$ M [96] and $10^{-24}$ M in serum and tissues [97]. However, it was reported that the required concentration for passive diffusion, in order for bacteria to grow, is $>10^{-6}$ M [98]. Therefore ferric iron is commonly chelated by specialized compounds that either sequester iron inside the cell (*e.g.* heme) or are secreted to capture iron and take it back to the cell. Aside from solubilizing iron to use it as a electron carrier, this mechanism also prevents iron to take part in the Fenton reaction that produces hydroxy radicals [99]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

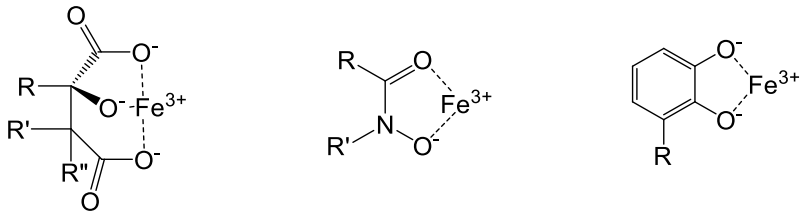
Ferric iron solubilizing compounds produced by microbes, are named siderophores, after the Greek word for iron carriers. Currently, almost 500 compounds have been found to be able to function as chelators for ferric iron, which is a relatively hard metal ion compared to its

reduced ferrous form [100]. In order to chelate such hard metal, hard ligands, such as oxygen, are required to form the preferred hexa-coordinate ligand sphere. $Fe^{2+}$ is a much softer metal and can therefore be chelated by soft ligands, such as nitrogen, that form up a tetrahedral coordination. Thus, upon reduction of $Fe^{3+}$, the affinity for the respective siderophore is drastically lowered and $Fe^{2+}$ is released [101].

Despite the large number of different siderophores, they can be divided into four categories based on their structures: α-hydroxycarboxylates (**35**), hydroxamates (**36**), catecholates (**37**) (Fig.1.11) and the so-called mixed ligand siderophores that are a combination of any of the three aforementioned iron-chelating moieties. For high affinity binding with iron a high denticity of the chelator is preferred. As the design of a siderophore is an important factor in establishing a hexadentate architecture, most siderophores consist of hydroxamate and/or catecholate moieties [100].

Indeed, in designing a potential drug, the denticity is an important factor that needs to be considered. Nevertheless, the two iron-chelating drugs on the market for clinical treatment of patients suffering from excess iron, deferiprone (marketed as Ferriprox by Apotex®) and deferasirox (marketed as Exjade, by Novartis®) show that a considerable smaller size of the latter may be as important as the requirement for high iron affinity, since deferasirox shows less side-effects when compared to deferiprone, probably due to its smaller size. Deferasirox requires two molecules to build up a hexadentate architecture which leads to a decrease in affinity for $Fe^{3+}$. Furthermore, the comparison of thermodynamic stability constants of ferric siderophores may be misleading as these values only refer to deprotonated ligands. At physiological pH, more indicative information can be obtained when comparing the negative logarithms (p[M]) of the free iron concentrations in the presence of the siderophore of interest [102]. Nevertheless, it was observed that aerobactin (pFe=25) is more readily taken up than enterobactin (pFe=37) by *E. coli* under iron-deficient conditions in a host. This may be due to the higher affinity of enterobactin to serum albumin due to its aromatic nature [103]. It is also possible that the effective free enterobactin concentration is lowered by binding to antibodies [104]. Thus, even the p[M] value may not be very indicative relatively to other factors such as stability, solubility and accessibility.

Upon sequestering of iron a new chiral center at the metal ion is introduced which is independent from the chirality of the ligands involved. Despite both isomers being in constant equilibrium with each other, the chirality is important for recognition by the outer membrane transporter. Therefore, the uptake may be stereoselective. However, the isomerization may not necessarily be a bottleneck in the uptake of siderophores as it is has been suggested that this process occurs at a higher rate than the cellular iron uptake [105]. Nevertheless, when large and bulky substituents such as drugs are going to be linked to the siderophore, fast isomerization may be impaired.



α-Hydroxy carboxylate (polycarboxylate) (**35**) Hydroxamate (**36**) Catecholate (**37**)

**Figure 1.11**. Core structures of iron-binding moieties as found in siderophores (shown as single subunits of an iron-chelating moiety).

### 1.8.2 Siderophore uptake

Aside from physico-chemical factors that need to be considered, one would preferably search for a prodrug-concept in which the siderophore contains a transporter found only in the target tissue or target organism. Currently, a wide variety of siderophore-transporting mechanisms are known, some more defined than others. Iron uptake in Gram-negative bacteria is mostly regulated by the ferric uptake regulator (Fur), which acts as a repressor (with $Fe^{2+}$ as a co-repressor), to a consensus operator sequence of the iron-regulated genes [106]. The structure and mechanism of the various outer membrane transporters and cork domains have been extensively reviewed [101, 107, 108] and do not fall within the scope of this study.

In Gram-negative bacteria, the outer membrane usually protects the bacterium from toxins, enzymes, detergents. However, trimeric β-barrel structures in the bacterial outer membrane can form porins that allow for passive diffusion of compounds of a molecular weight less than 600 kDa. Iron-chelating compounds are usually too large for these porins, however these may be actively taken up by porins consisting of 22 β-strands that form a β-barrel that allows for the uptake of siderophores. *N*-terminally these β-barrels contain a globular domain that forms a plug or cork domain, that opens the barrel if a siderophore is recognized. The proton motive force of the cytoplasmic membrane is the energy source responsible for the dislodging of the cork from the barrel. This energy is transmitted through the TonB-ExbB-ExbD-complex, that overspans the periplasm and connects to the TonB box of the cork domain [109, 110]. Studies by Scott *et al*. showed that when the cork domain of the FepA β-barrel was hybridized with the cork domain of the FhuA β-barrel, and *vice versa*, both hybrid porins could still transport each other's natural siderophore [111]. This suggests that several siderophores can be recognized by a cork domain and also be transported by several different β-barrels. Hence, selectivity for one specific siderophore may be low. This is also reflected by the so-called mixed ligand siderophores, whereby the siderophore consists of a combination of the three iron-binding moieties *(i.e.* catecholates, hydroxamates and α-

hydroxy carboxylate). Here the siderophore-drug conjugate (SDC) was designed such that several different uptake routes could be utilized, and therefore the activity could be improved and the risk of resistance be lowered.

Once the ferric siderophore is transported across the outer membrane, it is bound to a periplasmic binding protein (PBP) that shuttles the ferric-siderophore to the cytoplasmic membrane where it is further transported into the cytoplasm. For each of the three siderophore classes a specific PBP is required. In *E. coli* the catecholate PBP FhuD seems to be the most versatile, since it shows a certain flexibility in the constituence and number of hydrogen bond networks allowing it to bind a variety of different catecholate type siderophores. Also, this PBP was shown to be responsible for the shuttling of albomycin, a sideromycin (*vide infra*).

Once across the periplasm, the ferric-siderophore needs to be transported across the cytoplasmic membrane. This step is performed by a wide variety of ATP-binding cassette transporters (ABC transporters). Although these siderophore transporters show only 25% sequence identity in the different subunits that form an ABC transporter, the protein complexes are structurally *grosso modo* similar. Two distinct subunits are involved in the assembly of an ABC transporter, consisting of four subunits in total (usually two identical subunits are used). In general, bacterial ABC transporters consist of four domains: two transmembrane domains and two nucleotide binding domains that produce the required energy by hydrolyzing ATP. Hydroxamate siderophores (aerobactin, ferrichrome, coprogen, and rhodotorulic acid) are transported across the cytoplasmic membrane by common ABC transporters, while citrate and enterobactin utilize different transporters [112].

Following the release of the siderophore into the cytoplasm, the iron is released. Most microbes produce ferric reductases that free the iron from the siderophore, after which the siderophore is usually recycled by secretion into the extracellular environment. Unfortunately, the vast majority of SDCs showed lower activities relatively to the parent drug [113]. In most cases, this is probably due to absence of metabolization, so that the drug is not released and can therefore not reach its intracellular target or is unable to act as an inhibitor. A pyoverdine-quinolone antibiotic is the only example where antibacterial activity against *P. aeruginosa* is retained [114]. Currently, only in the case of albomycins, post-uptake metabolization has unambiguously been shown to act as a drug-release mechanism (*vide infra)*. It is under debate if drug release is really necessary for all SDCs to obtain activity. For example, many β-lactam antibiotics have been designed that obviously target the penicillin-binding proteins, which are located in the periplasmic space. Since binding studies showed that these complete conjugates were able to target penicillin-binding proteins, it was suggested that these compounds might not require drug release to obtain activity [114].

### 1.8.3 Ton-independent iron acquisition

In addition to the aforementioned iron uptake through specific outer-membrane transporters that require energy *via* the TonB-ExbB-ExbD complex, there are Ton-independent ways to sequester iron employed by several bacterial strains. Recently, such a transporter was characterized in *Neisseria gonorrhoeae,* which is able to transport iron-bound catecholate siderophores in a Ton-independent fashion. This FbpABC transporter is equivalent to the YfuABC found in *Yersinia pestis*, that likewise transports iron across the bacterial inner membrane into the cytoplasm [115]. Both transporters consist of three subunits, where subunit A is a periplasmic iron-binding protein, subunit B is a cytoplasmic permease and subunit C can provide the energy to transport the iron across the cytoplasmic membrane using ATP. Thus, for this type of iron transport, an energy-independent mechanism must exist to transport iron across the outer membrane, while transport across the inner membrane still requires an ABC transporter.

### 1.8.4 Host response to bacterial siderophores

Naturally, the human immune system attempts to counterbalance the fight with bacteria for iron. Neutrophils have been shown to secrete lactoferrin, which is similar to transferrin, but binds $Fe^{3+}$ even more tightly. Lactoferrin is secreted at the site of infection, so that local $Fe^{3+}$ concentrations are kept low and therefore unavailable to bacteria [116]. This prevents bacteria from forming biofilms.

Another defense mechanism consists in the secretion of lipocalin (recently renamed siderocalins by several researchers, due to its siderophore-binding properties), also produced by neutrophils. This protein was recently shown to bind several ferric-siderophore complexes, which can therefore not be taken up [117, 118]. However, it has been suggested that bacteria can escape from this innate defense mechanism by covalently attaching glucose to the siderophore [119]. Apparently, this does not affect $Fe^{3+}$ binding nor the uptake of iron-bound siderophore, and therefore might be useful as an addition to synthetically produced SDCs.

**Table 1.2. Bacterial siderophores and their respective receptors**

| Bacteria | Outer membrane transporter | Ton-dependence | Inner membrane transporter | Siderophore | Ref. |
|---|---|---|---|---|---|
| *Neisseria gonorrhoeae* | | - | FbpABC | catecholate xenosiderophores | [120] |
| | TbpA/ TbpB | + | | transferrin/lactoferrin | |
| *Yersinia pestis* | | - | YfuABC | | [115] |
| | | | YfeABCD | | [121] |
| | | | YbtP/YbtQ | yersiniabactin | [122] |
| *Yersinia pseudo-tuberculosis* | nd | | | yersiniabactin | |
| *Yersinia enterocolitica* | FyuA | | | yersiniabactin | [123] |
| | FoxA | | | ferrioxamine B | [124] |
| | FcuA | + | | ferrichrome | [125] |
| *Mycobacterium tuberculosis* | | | Rv1348 and Rv1349 | fransferrin | [126] |
| *Escherichia coli* | FhuA | + | FhuD | ferrichrome (albomycins)/ferrioxamine B (salmycins)* | [127, 128] |
| | FhuF | | | ferrioxamnine | [129] |
| | FepA | + | | enterobactin | [130] |
| | FecA | + | | citrate | |
| | FhuE | + | | coprogen/rodotorulic acid | [129] |
| | Cir | + | | dihydroxybenzoylserine | [129] |
| | Fiu | + | | dihydroxybenzoylserine | [129] |
| | IutA | + | | aerobactin | [131] |
| | IroN | + | | salmochelin | [132] |
| *Pseudomonas putida* | PupA | + | | pseudobactin | [133] |
| *Pseudomonas aerigunosa* | FpvA | + | | pyoverdine | [134] |
| | FptA | | | pyochelin | [135] |
| | PfeA | | | enterobactin | [136, 137] |
| | FiuA | | | ferrioxamine | [138] |

**Table 1.2. continued**

| | | | | | |
|---|---|---|---|---|---|
| *Salmonella* | IroN | + | FepBCDG | (glucosylated) 2,3 dihydroxybenzoylserine monomers/ salmochelin | [132] |
| | Cir | + | | (glucosylated) 2,3 dihydroxybenzoylserine monomers | [132] |
| | FepA | + | | (glucosylated) 2,3 dihydroxybenzoylserine monomers/ enterobactin | [132] |
| *Bordetella pertussis* | BfeA | | | enterobactin | [139] |
| | FauA | | | alcaligin | [140] |
| | BfrABC | | | siderophore | [141] |
| *Erwinia chrysanthemi* | Fct | | | chrysobactin | [142] |
| *Morgenella morganii* | RumAB | | | rhizoferrin | [143] |
| *Vibrio cholerae* | ViuA | | | vibriobactin | [144] |

nd: not determined

## 1.9 Sideromycins

Several natural compounds were identified as sideromycins by their ability to chelate iron and subsequently being recognized as siderophore. After recognition, the whole compound is taken up and the active moiety is released. Three inspiring sideromycins include the albomycins, salmycins, and the ferrimycins. The more recently discovered microcins are proteins, and are therefore unlikely to aid in potential drug development programs.

### 1.9.1 Albomycins

Initially discovered by Gause *et al*.[145], the structure and mechanism of albomycins (**19**) were further identified from 1982 onwards, largely by Benz *et al*.[146-151] The siderophore part in this compound consists of three $N^5$-acetyl-$N^5$-hydroxy-L-ornithines that are connected *via* amide bonds, so to mimic a ferrichrome siderophore. The peptide is *C*-terminally attached to a 4'-thio-6'-amino heptonic acid, *via* a special linker. A modified cytidine, resembling a nucleobase is attached at the alpha position (**20**). Although often named a thioribosyl pyrimidine residue [127, 152], the initial (and only) structure characterizations by Benz *et al.* show a thioxylofuranose pyrimidine moiety.

Three different albomycins have been identified, varying in the constituency of different groups attached to the cytidine moiety (Fig. 1.4).

As can be seen in Table 1.2, the various albomycins can be transported by FhuA across the bacterial outer membrane of *E. coli*. In the periplasm, albomycin is chaperoned by FhuD to the bacterial inner membrane, where it is delivered to FhuB. Upon recognition of ferrichrome-loaded FhuD by FhuB, FhuC is triggered to hydrolyze ATP so that FhuB probably opens a channel through which the siderophore or the sideromycin is transported. Once inside the cell, it is enzymatically cleaved by the serine protease peptidase N [152]. Also peptidase A in *Salmonella Typhimurium* is capable of releasing the active moiety of this sideromycin [152]. The active moiety resembles seryl-adenylate and can therefore act as a non-hydrolyzable inhibitor of seryl-tRNA synthetase.

Although resistance has been observed through point mutations in the FhuB gene, this is accompanied with an inability to transport ferrichrome and hence leads to a severe loss of bacterial fitness [127, 153, 154]. Also mutations in the ATP-binding site of FhuC confers resistance to albomycin [155]. Furthermore, the *in vivo* potential was exemplified by tests, whereby mice were infected with *Streptococcus pneumoniae* or *Yersinia enterolitica* and subsequently cured with albomycin [153]. Therefore, the use of this compound as a potential antibiotic has frequently been argued, provided sufficient amounts can be isolated or chemically synthesized.

Progress has been made in the synthesis of this compound. Benz *et al.* synthesized an analogue of albomycin $\delta_1$, containing an oxygen instead of sulfur. However, no antibacterial activity was observed with this analogue [156]. The synthesis of the peptide part was later improved by Miller *et al*.[157].

### 1.9.2 Salmycins

Originally isolated from *Streptomyces violaceus* DSM 8286, the salmycins (Fig. 1.12, **38a-d)** were found to be potent inhibitors of staphylococci and streptococci strains [158]. In contrast with the albomycins, salmycins consist of a ferrioxamine (danoxamine) siderophore moiety, that is coupled through an ester linkage to a dissacharide moiety. The exact mode of action of salmycins is not known, however it is generally assumed that a mechanism in the protein synthesis cascade is inhibited. Interestingly, in view of drug release from a siderophore, Roosenberg and Miller [159] postulated that the salmycins confer a drug release mechanism initiated by the iron reductases. Upon reduction of the iron, bound by the siderophore, an intramolecular cyclization causes cleavage of the amide or ester bond, thereby releasing the drug (Figure 1.12). Since resistant *S. pneumoniae* and *S. aureus* mutants display cross resistance against albomycin, these compounds presumably utilize the same uptake mechanism [160]. It was shown that the salmycins, when compared to albomycins, exhibit a greater antibacterial activity against efflux-mediated MRSA [114]. However, in a comparative *in*

*vivo* study by Braun *et al*.[127], salmycin proved to be less active, probably due to their relatively unstable ester linkage. The total synthesis of desferrisalmycin was reported by Dong *et al*.[161] and several danoxamine-drug conjugates that maintain identical properties in terms of uptake and drug release were prepared by Long *et al*. (manuscript in preparation, referred to in ref [114]).

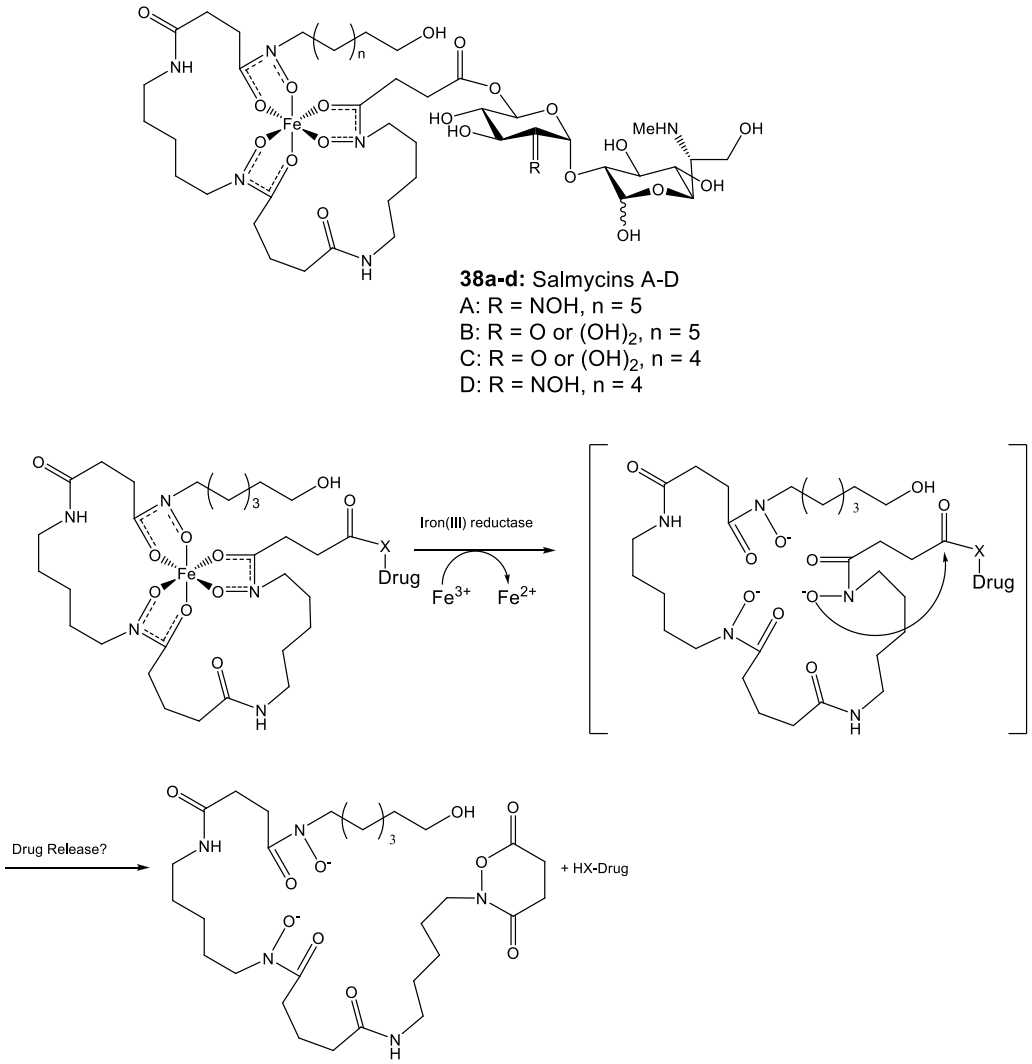



**Figure 1.12.** Upper part: Chemical structure of the salmycins.
Lower part: Ferric iron reductase-induced release of drugs conjugated to ferrioxamine siderophores (in this case danoxamine).

### 1.9.3 Ferrimycins

Less attention has been given to the ferrimycins (Fig. 1.13, **39b**), probably due to its activity which is limited to Gram-positive bacteria. Ferrimycin A consists of ferrioxamine B (**39a**) which is connected through aminohydroxybenzoic acid to an iminoester substituted lactam [162].

**39a: Ferrioxamine B: R=H**
**39b: Ferrimycin: R=**

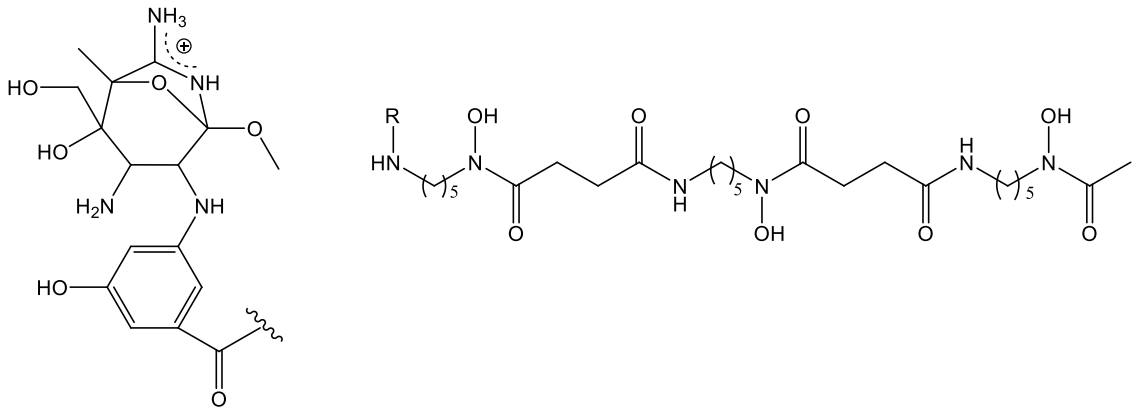


**Figure 1.13** Ferrimycin as derived from ferrioxamine.

## 1.10 Objectives of this dissertation

In the first three experimental studies of this thesis several aspects concerning the synthesis, evaluation and biological functions of McC are addressed, the fourth study includes the use of a sideromycin Trojan horse mechanism. More specifically the following objectives are studied in this doctoral work:

1) To design new analogues of McC that show that in principle virtually all amino acyl-tRNA synthetases can be inhibited.

2) To improve the synthetic strategy that lead to a better yield and extended the range of compounds that can be synthesized.

3) To design and synthezise compounds that help to elucidate the role of McC-associated enzymes such as MccE and MccF.

4) To design, synthesize and evaluate compounds carrying aryl-tetrazole moieties, which substitute adenine as found in McC. This work is based on compounds designed/synthesized and tested by Cubist Pharmaceuticals. The principal aim of this undertaking was to develop new compounds that show decreased serum albumin binding relative to the lead compounds, but show equal or increased activity, with retention of selectivity towards bacterial aaRSs relative to human aaRSs.

5) To design, synthesize and evaluate compounds containing a hydroxamate-based siderophore moiety as in albomycin. A colleague PhD student, Bharat Gadakh, developed a method to synthesize compounds of this art, which I used to link to either 3,6-anhydro-2-deoxy-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol-7-[*N*-(Ile)-sulfamoyl] or 3,6-anhydro-2-deoxy-1-[5-phenyl tetrazole]-D-allo-heptitol-7-[*N*-(Ile)-sulfamoyl].

# Chapter 2

## Microcin C analogues exhibiting altered target specificity

**Abstract:** Microcin C (McC) (**21a**) is a potent antibacterial compound produced by some *E. coli* strains. McC functions through a Trojan horse mechanism: it is actively taken up inside a sensitive cell through the function of the YejABEF transporter and then processed by cellular aminopeptidases. Processed McC (**22**) is a non-hydrolyzable aspartyl-adenylate analogue that inhibits aspartyl-tRNA synthetase (AspRS). A new synthesis is described that allows for the production of a wide variety of McC analogues in acceptable amounts. Using this synthesis a number of diverse compounds was synthesized with altered target specificity. Further characteristics of the YejABEF transporters were determined using these compounds.

This chapter was published earlier in an adapted form as:

Extended targeting potential and improved synthesis of microcin C analogs as antibacterials G. H. Vondenhoff, S. Dubiley, K. Severinov, E. Lescrinier, J. Rozenski, A. Van Aerschot, *Bioorg Med Chem*, 2011, *19*, 5462 [163].

## 2.1 Introduction

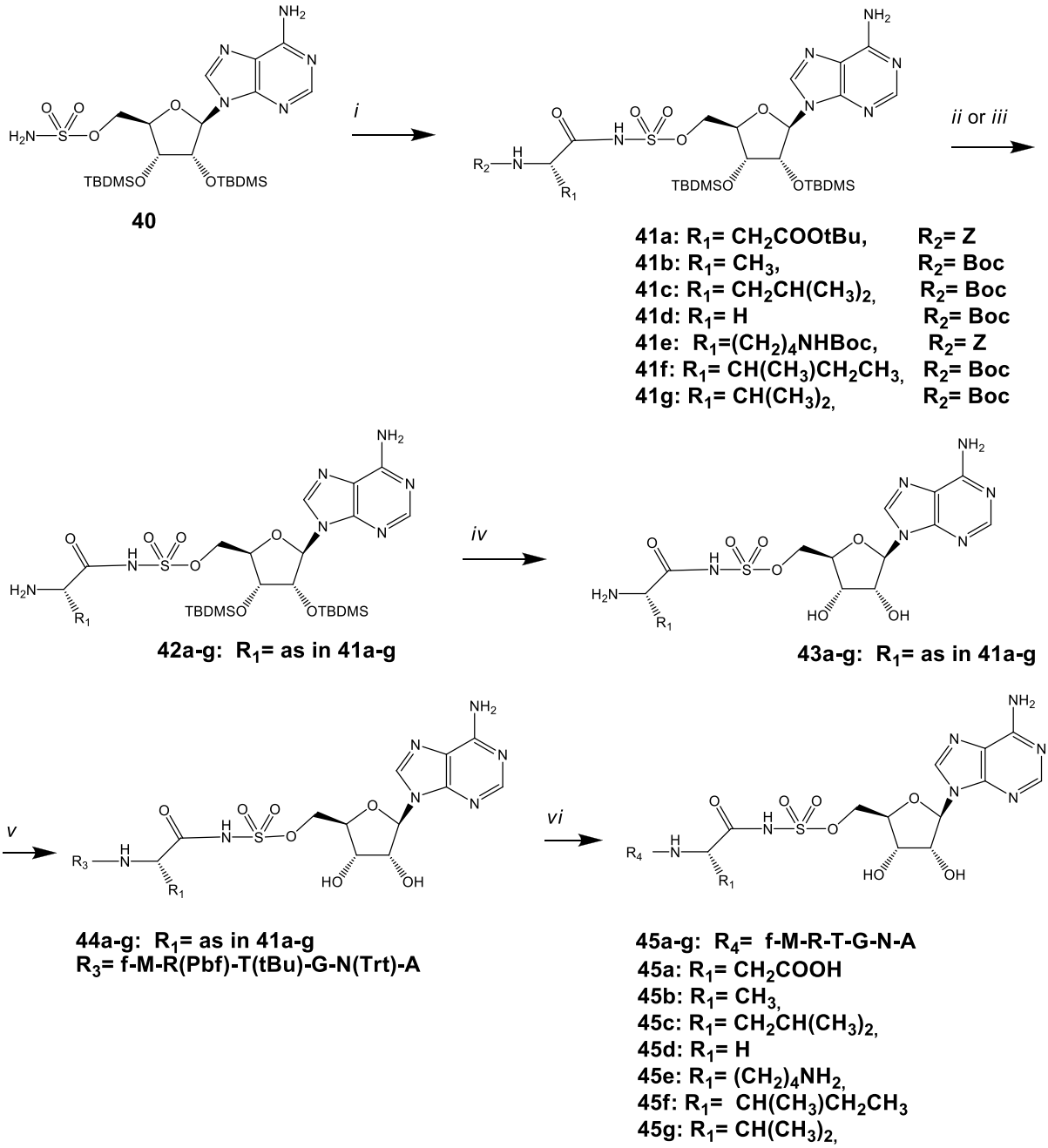
As described in Chapter 1, three McC analogues were synthesized which contained different amino acids at the seventh position of the McC analogue (**23-25**) [72]. These compounds lacked a *N*-terminal formyl group. All three proved to be active and work *via* the same mechanism as natural McC, which is through removal of the hexapeptide part following transporter mediated uptake and concomitant inhibition of the respective tRNA synthetase by the metabolite. However, synthetic yields were very low and the number of analogues that could be created using that particular scheme were limited to amino acids with non-basic side chains. Following these results, we here report on the development of a new synthetic approach to improve the synthetic yield of the synthesis and to extend the range of different McC-like compounds that could be synthesized. This improved procedure is used to generate and characterize a new series of McC analogues targeting various aaRSs and to further characterize the requirements for efficient transport by the YejABEF transporter.

## 2.2 Results

### 2.2.1 Design and synthesis of McC analogues

aaSAs were created in analogy to previously published methodology [72] with the modification that orthogonal protecting groups were used for the side chains with respect to the alpha-amine of the respective amino acids (see Scheme 2.1). Amino acylated SAs were prepared by condensation of either L-*N*-benzyloxycarbonyl-[aminoacyl(*O*-*tert*-butyl or *tert*-butyloxycarbonyl)]-*O*-succinimide analogues with the adenosine derivative **40** using DBU in DMF. The Z-group of the resulting products (**41a** and **41e**) was next removed by hydrogenation. The aaSAs containing an amino acid not requiring protection of its side chain (**41b**-**d** and **f**-**g**) were prepared by condensation of the respective L-*N*-*tert*-butyloxycarbonyl-aminoacyl-*O*-succinimide precursors with **40**. The Boc-group was removed by acidolysis and the TBDMS-protecting groups of all compounds were removed using $Et_3N{\cdot}3HF$. This strategy allowed for the synthesis of aaSAs **43a**-**g** only protected at the amino acid side chain.

The hexapeptide was assembled on a super-acid-sensitive resin, allowing the use of moderately acid-sensitive protecting groups. The fully protected, formylated hexapeptide (fM-R(Pbf)-T(tBu)-G-N(Trt)-A) was then coupled to the partially protected aaSAs using standard peptide-synthesis reagents such as DIC, HOBt, and DIPEA. The obtained products **44a**-**g** were subsequently deprotected yielding the respective McC analogues (**45a-g**).




**Scheme 2.1.** General scheme affording the various McC analogues with $R_4$ being the formylated hexapeptide. *i)* *N*-α-Cbz-L-aminoacyl-(*t*Bu or Boc)-succinimide, DBU in DMF, 6h, rt. *ii)* for $R_2$ = Z-group, $H_2$, Pd/C in MeOH, 3h, rt. *iii)* for $R_2$= Boc-group, TFA/$H_2O$ (5:2), 4h, 0 °C to rt. *iv)* $Et_3N.3HF$ in THF, 16h, rt. *v)* protected peptide (1eq.), HOBt (4eq.), DIC (4eq.) and DIEA (2eq.) in DMF, 16h, rt. *vi)* TFA/thioanisole/$H_2O$ (90/2.5/7.5), 2h, rt.

Following the here described methodology, a new set of analogues was created, in order to further extend the synthesis and evaluation of McC analogues carrying different amino acids at the seventh position (see Table 1). These compounds carried either an aspartic acid (**45a**), alanine (**45b**), leucine (**45c**), glycine (**45d**), lysine (**45e**), isoleucine (**45f**) or valine

(**45g**) as the target-determining amino acid. With these analogues also an *N*-terminal formyl group was included as it was found to improve the recognition by the YejABEF transporter.

### 2.2.2 Antibacterial activity of McC analogues

The growth-inhibitory properties on McC-sensitive *E. coli* were determined for all new compounds by measuring the optical density reached by identical cell cultures in wells of microtiter plates in the presence of various concentrations of the respective inhibitors.

As shown earlier, the intracellular target is determined by the *C*-terminal amino acid, which remains attached onto the sulfamoyl-adenosine following intracellular metabolism [72]. To facilitate the activity evaluation and the mechanism of action studies of newly synthesized compounds, an *E. coli* tester strain Ara-Yej (BW39758) was used, where the genomic *yejABEF* operon is under control of the arabinose-inducible *araBAD* promoter. In the presence of L-arabinose higher amounts of Yej transporters will be displayed at the innermembrane. The wild-type *E. coli* K-12 BW28357 and the Ara-Yej strain were either grown in Lysogeny Broth (LB) with or without 5 mM (L)-arabinose. The antibacterial activities of the various McC analogues (**45a**-**g**) were determined by monitoring the optical density of suspensions of cell cultures. In parallel, *E. coli* with disrupted *yej*, or *pepA,B,N* genes, grown on M63 agar plates were used to confirm the McC mode of action.

As can be seen in Figure 2.2, compounds **45a**, **45b**, **45e** and **45g** displayed activity at concentrations ≤0.63 µM when challenged with Ara-Yej cells cultured in arabinose-containing medium. The more hydrophobic compounds **45c, 45d, 45f** and **45g** showed reduced activity. *E. coli* cells with a disrupted *yej* gene (with concomitant loss of the active transport modality) and cells without functional aminopeptidases A, B and N (with loss of formation of the active principle) proved resistant to these compounds, indicating the same mode of action as McC.

## 2.3 Discussion

We previously suggested that deviations from the native McC structure could be quite extensive [72]. This is further exemplified here with McC analogues affording different intracellular active compounds upon metabolism. Even if more extensive alterations were incorporated, such as a lysine as the target-determining amino acid, activities were in the same range as observed for native McC. However, the potency of the compounds **45c, 45d, 45f** and **45g** was lower in comparison to that of **45a**, **45b**, **45e** and **45g**. Brown *et al*. found a $K_i$ of 0.01 nM of I-SA for IleRS [16]. Therefore the relatively low activity of the compound fXI-SA (**45f**) must be attributed to either uptake or metabolization issues.

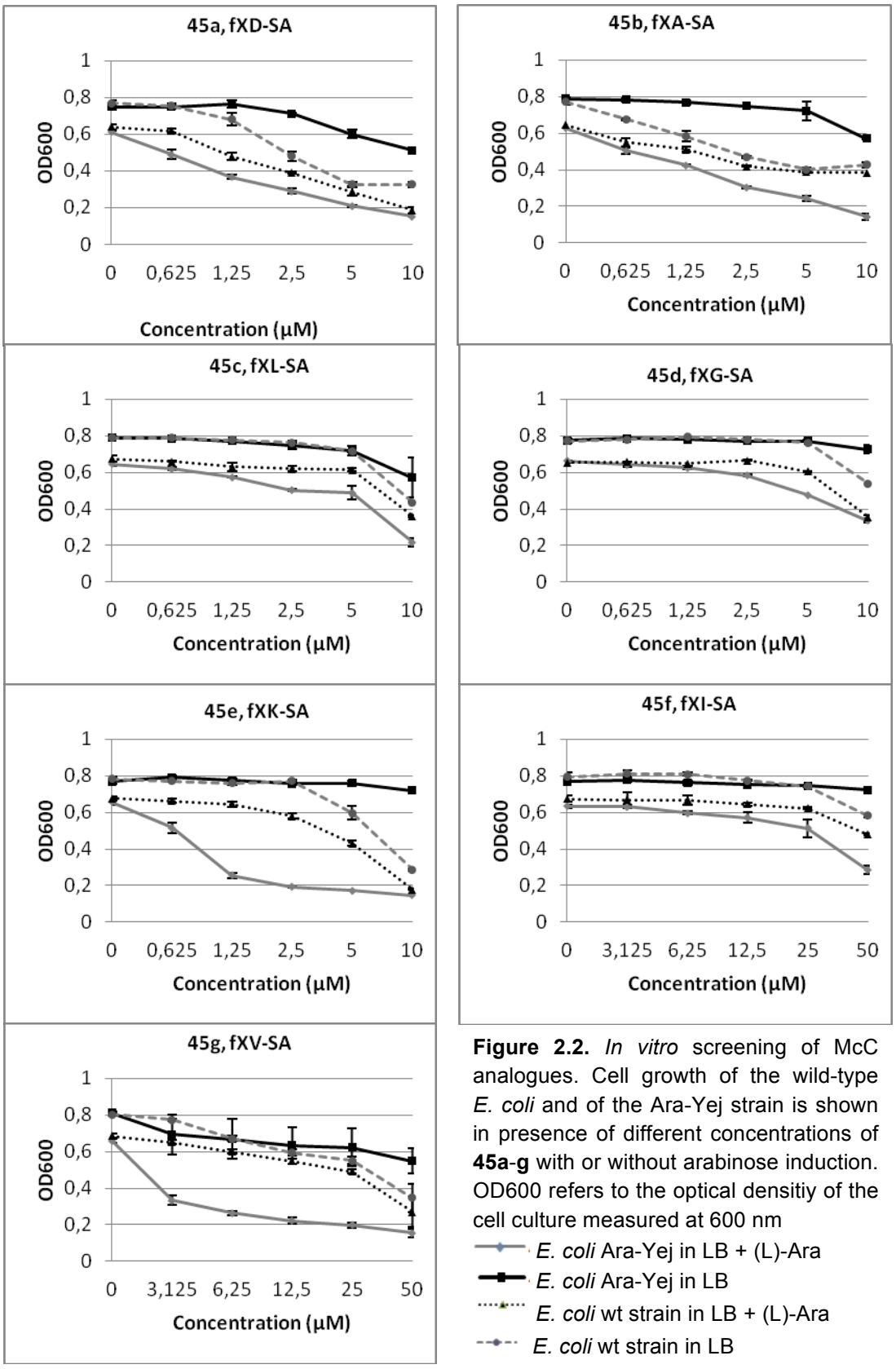



**Figure 2.2.** *In vitro* screening of McC analogues. Cell growth of the wild-type *E. coli* and of the Ara-Yej strain is shown in presence of different concentrations of **45a**-**g** with or without arabinose induction. OD600 refers to the optical densitiy of the cell culture measured at 600 nm

**Table 2.1. Lowest inhibitory concentration on arabinose-induced Ara-Yej cells**

| Entry | Lowest inhibitory concentration (μM) |
|---|---|
| fXD-SA (**45a**) | <0.63 |
| fXA-SA (**45b**) | 0.63 |
| fXL-SA (**45c**) | 2.5 |
| fXG-SA (**45d**) | 5 |
| fXK-SA (**45e**) | 0.63 |
| fXI-SA (**45f**) | 25 |
| fXV-SA (**45g**) | 3.13 |

As shown in Figure 2.2, the strain with overproduced Yej transporters is more sensitive to this compound. Therefore it may be concluded that the Yej transporter discriminates between the constituencies of the peptide chains. The polarity may be a decisive factor since compounds containing amino acids at the seventh position with more aliphatic side chains display lower activities in comparison to compounds containing aspartic acid (**45a**) or lysine (**45e**). However, compound **45c** containing leucine showed a ten-fold higher potency compared to **45f**. Therefore it may be concluded that not only the polarity, but also more specific recognition by the transporter is of importance for uptake of these compounds.

The synthetic scheme as presented here is a significant improvement upon the earlier reported synthesis with up to 15-fold improved overall yields. The new methodology allows for the preparation of sufficient amounts of different McC analogues, allowing further studies on the biology of this fascinating class of compounds and enabling the design of further improved analogues. Following this method, new compounds could be created that may not only inhibit other aaRSs, but even other targets. Furthermore, compounds that suffer from low bioavailability could be improved following the proposed synthetic scheme in order to turn these potential drugs in Trojan horse-like compounds.

## 2.4 Materials and Methods

### 2.4.1 Chemistry – general

Reagents and solvents were purchased from commercial suppliers (Acros, Sigma-Aldrich, Bachem, Novabiochem) and used as provided, unless indicated otherwise. DMF and THF were analytical grade and were stored over 4Å molecular sieves. For reactions involving Fmoc-protected amino acids and peptides, DMF for peptide synthesis (low amine content) was used. All other solvents used for reactions were analytical grade and used as provided.

Reactions were carried out in oven-dried glassware under a nitrogen atmosphere and the reaction mixtures were stirred at room temperature, unless indicated otherwise.

$^{1}H$ and $^{13}C$ NMR spectra of the compounds were recorded on a Bruker UltraShield Avance 300 MHz or 500 MHz spectrometer. Spectra were recorded in DMSO-$d_6$ or $D_2O$. The chemical shifts are expressed as δ values in parts per million (ppm), using the residual solvent peaks (DMSO: $^{1}H$, 2.50 ppm; $^{13}C$, 39.60 ppm; HOD: $^{1}H$, 4.79 ppm with dioxane added for $^{13}C$, 67.19 ppm) as a reference. Coupling constants are given in hertz (Hz). The peak patterns are indicated by the following abbreviations: bs = broad singlet, d = doublet, m = multiplet, q = quadruplet, s = singlet and t = triplet. High-resolution mass spectra were recorded on a quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard ESI interface; samples were infused in 2-propanol/$H_2O$ (1:1) at 3 μL.min$^{-1}$.

For TLC, precoated aluminium sheets were used (Merck, Silica gel 60 $F_{254}$). The spots were visualized by UV light at 260 nm. Column chromatography was performed on ICN silica gel 60 Å. For size exclusion chromatography, a 2 cm × 30 cm column of Sephadex LH-20 was used as the solid phase and MeOH/$H_20$ (7:3 v/v) as the eluent. Preparative HPLC of peptides was done using a Waters Xbridge preparative C18 (19 mm × 150 mm) column connected to a Waters 1525 binary HPLC pump and a Waters 2487 dual absorbance detector. Final products were purified using a PLRP-S 100 Å column connected to a Merck-Hitachi L6200A Intelligent pump. Eluent compositions are expressed as v/v. Purity was checked by analytical HPLC on a Inertsil ODS-3 (C-18) (4.6 mm x 100 mm) column, connected to a Shimadzu LC-20AT pump using a Shimadzu SPD-20A UV-detector. Recordings were performed at 254 nm and 214 nm.

### 2.4.2 Synthesis of 5'-*O*-[N-[L-aspartyl(*O-tert*-butyl)]-sulfamoyl] adenosine (43a)

A solution of Z-aspartyl(*O-tert*-butyl)-*O*-Su (1 g, 2.38 mmol, 1.0 eq.), 5'-*O*-sulfamoyl-2',3'-di-*O*-(*tert*-butyldimethylsilyl)-adenosine (**40**) (1.23 g, 2.14 mmol, 0.9 eq.) and DBU (356 μl, 2.38 mmol, 1.0 eq.) in DMF (7 mL) was stirred at rt for 8h under nitrogen atmosphere. DMF was evaporated under reduced pressure. Next, the reaction mixture was purified by flash chromatography ($CH_2Cl_2$, 1% $Et_3N$, 2.5 to 10% MeOH). Fractions containing the desired product were evaporated giving a yellow oil (**41a**). Yield: 1.7 g (90%). ESI-MS calcd. for $C_{38}H_{62}N_7O_{11}SSi_2$ ([M+H$^+$]): 880.4 found: 880.1

The obtained product **41a** (1.7 g) was dissolved in MeOH at 0 ºC and Pd/C (0.17 g) was added. The solution was stirred under hydrogen atmosphere for 3h. The mixture was filtered and evaporated yielding a white-yellow foam (**42a**). Yield: 1.1 g (76%). The product **42a** (1.1 g) was carefully dried and dissolved in a mixture of THF (15 mL) and $Et_3N.3HF$ (1 mL). After 3h, another 0.8 mL of $Et_3N.3HF$ was added and the reaction mixture was stirred for another

22h. The reaction mixture was evaporated and the residue was purified by flash chromatography ($CH_2Cl_2$, 5 to 20% MeOH). Fractions containing the desired product were evaporated, yielding **44a**: 670 mg (88%).

$^1$H-NMR ($D_2O$): 1.04-1.09 (t, 9H, $Et_3N$, J=7.16), 1.44 (s, 9H, tBu), 2.67-2.75 (q, 6H,$Et_3N$ $CH_2$, J=7.12), 3.68-3.72 (t, 2H, $Asp_\alpha H$, J=5.70), 4.12-4.19 (m, 4H, 3', 4', 5'), 4.63-4.67 (t, 1H, 2'H, J=5.36), 5.94-5.96 (d, 1H, 1', J=5.82), 7.31 (s, 1H, $NH_2$), 8.18 (s, 1H, 2H), 8.41 (s, 1H, 8H).
$^{13}$C-NMR ($D_2O$): 27.02 (tBu-$CH_3$), 35.71 (Asp-β-C), 51.67 (Asp-α-C), 68.14 (5'-C), 70.08 (3'-C), 74.14 (2'-C), 82.05 (4'-C), 83.88 (tBu-C), 87.37 (1'-C), 118.06 (5-C), 139.49 (8-C), 148.31(4-C), 152.19 (2-C), 154.74 (6-C), 170.50 and 173.81 (Asp- C=O).
ESI-MS calcd. for $C_{18}H_{26}N_7O_9S$ $[M-H]^-$: 516.2; found: 515.8.

### 2.4.3 Synthesis of 5'-*O*-[*N*-[L-alanyl]-sulfamoyl] adenosine (43b)

A solution of Boc-alanyl-O-Su (1 g, 3.5 mmol, 1.0 eq.), compound **40** (1.8 g, 3.2 mmol, 0.9 eq.) and DBU (450 µl, 3 mmol, 0.9 eq.) in DMF (7 mL) was stirred at rt for 8h under nitrogen atmosphere. DMF was evaporated under reduced pressure. Next, the residue was purified by flash chromatography ($CH_2Cl_2$, 1% $Et_3N$, 2.5 to 10% MeOH). Fractions containing the desired product were evaporated giving a yellow oil (**41b**). Yield: 2.09 g (90%). Compound **41b** was next treated with TFA/$H_2O$ (5/2 v/v) for 5h at rt, after which the volatiles were evaporated to yield the product **42b**. Compound **42b** was carefully dried and dissolved in a mixture of THF (15 mL) and $Et_3N$.3HF (1 mL). After 3h, another 0.8 mL of $Et_3N$.3HF was added and the reaction mixture was stirred further for 22h. The reaction mixture was evaporated and the residue was purified by flash chromatography ($CH_2Cl_2$, 5 to 20% MeOH). Fractions containing the desired product **43b** were evaporated. Yield: 710 mg (63%).

$^1$H-NMR ($D_2O$): 1.22-1.27 (t, 9H, $Et_3N$-$CH_3$, J = 7.34), 1.43-1.45 (d, 3H, Ala-β-H, J=7.2), 2.61 – 2.68 (q, 6H, $Et_3N$-$CH_2$, J = 7.35), 3.45 – 3.52 (q, 1H, Ala-α-H, J = 7.19), 4.35 – 4.41 (m, 4H, 3', 4', 5',), 4.45 – 4.48 (t, 1H, 2'-H, J = 4.7), 6.08 – 6.10 (d, 1H, 1'-H, J = 6.0), 7.28 (bs, 2H, $NH_2$), 8.22 (s, 1H, 2H), 8.36 (s, 1H, 8H).
$^{13}$C-NMR ($D_2O$): 17.07 (Ala-β-C), 52.01 (Ala-α-C), 68.89 (5'-C), 70.75 (3'-C), 74.71 (2'-C), 82.88 (4'-C), 88.03 (1'-C), 119.17 (5-C), 140.36 (8-C), 149.45 (4-C), 153.11 (2-C), 156.00(6-C), 177.12 (Asp- C=O).
ESI-MS calcd. for $C_{13}H_{18}N_7O_7S$ $[M-H]^-$: 416.4; found: 416.2.

### 2.4.4 Synthesis 5'-*O*-[*N*-[L-leucyl]-sulfamoyl] adenosine (43c)

This compound was synthesized analogously to compound **43b**.
$^1$H-NMR ($D_2O$): 0.87-0.89 (d, 3H, Leu-δ, J = 6), 0.89 – 0.90 (d, 3H, Leu-δ, J = 6), 1.64 – 1.73 (m, 3H, Leu-γ,β-H), 3.75 – 3.76(m, 1H, Leu-α-H), 4.42 – 4.45 (m, 3H, 3',,5'), 4.50 – 4.52 (t.

1H, 4'-H, J = 4.8) 4.74-4.76 (t, 1H, 2'-H, J = 4.8), 6.15 – 6.16 (d, 1H, 1'-H, J = 5.31), 8.42 (s, 1H, 2H), 8.51 (s, 1H, 8H).

$^{13}$C-NMR ($D_2O$): 20.3 (Leu-δB-$CH_3$), 21.45 (Leu-δA-$CH_3$), 23.51 (Leu-γ-CH), 39.46 (Leu-β-CH2), 53.25 (Leu-α-CH), 68.67 (5'-C), 69.49 (3'-C), 73.76 (2'-C), 81.88 (4'-C), 88.02 (1'-C), 118.29 (5-C), 142.06 (8-C), 144.17 (4-C), 147.91 (2-C), 149.50 (6-C), 174.14 (Leu- C=O).

ESI-MS calcd. for $C_{16}H_{26}N_7O_7S$ $[M+H]^+$: 460.48; found: 460.16.

### 2.4.5 Synthesis of 5'-*O*-[*N*-[glycyl]-sulfamoyl] adenosine (43d)

This compound was synthesized analogously to compound **43b**.

$^1$H-NMR ($D_2O$): 3.70 (s, 2H, α-H), 4.41–4.50 (m, 4H, 3',4',5'), 4.69–4.73 (t, 1H, 2'-H, J = 4.6), 6.12 – 6.14 (d, 1H, 1'-H, J = 5.31), 8.15 (s, 1H, 2H), 8.34 (s, 1H, 8H).

$^{13}$C-NMR ($D_2O$): 42.72 (Gly- α-C), 68.11 (5'-C), 70.06 (3'-C), 74.06 (2'-C), 82.15 (4'-C), 87.35 (1'-C), 118.43 (5-C), 139.54 (8-C), 148.73 (4-C), 152.37 (2-C), 156.15 (6-C), 172.69 (Gly - C=O).

ESI-MS calcd. for $C_{12}H_{16}N_7O_7S$ $[M-H]^-$: 402.37; found: 402.31.

### 2.4.6 Synthesis of 5'-*O*-[*N*-[L-lysyl(*N*-*tert*-butyloxycarbonyl)]-sulfamoyl]adenosine (43e)

This compound was synthesized analogously to compound **43a**.

$^1$H-NMR ($D_2O$): 1.26-1.44 (m, Lys-γ and δ), 1.38 (s, 9H, tBu), 1.77-1.84 (m, Lys-β), 2.87 (m, 2H, Lys-ε), 3.75-3.79 (t, 2H, Lys-α, J=5.77), 4.41-4.46 (m, 4H, 3',4',5'), 4.50-4.53 (t, 1H, 2', J=4.26), 6.12-6.14 (d, 1H, J=5.7), 8.26 (s, 1H, 2H), 8.43 (s, 1H, 8H)

$^{13}$C-NMR ($D_2O$): 21.89 (Lys-γ), 28.26 (tBu-$CH_3$), 29.12 (Lys-δ), 31.14 (Lys-β), 40.15 (Lys-ε), 56.03 (Lys-α), 68.97 (5'-C), 70.76 (3'-C), 74.73 (2'-C), 82.87 (4'-C), 87.69 (1'-C), 119.57 (5-C), 140.39 (8-C), 149.68 (4-C), 153.57 (2-C), 156.23 (6-C), 158.77 (Boc –C-O), 176.31 (Lys - C=O).

ESI-MS calcd. for $C_{21}H_{35}N_8O_9S$ $[M+H]^+$: 575.22; found: 575.00.

### 2.4.7 Synthesis of 5'-*O*-[*N*-[L-isoleucyl]-sulfamoyl]adenosine (43f)

This compound was synthesized analogously to compound **43b**.

$^1$H-NMR ($D_2O$): 0.84-0.89 (t, 3H, Ile-δ, J = 7.38), 0.97 – 0.99 (d, 3H, Ile-γ'-H, J = 7.02), 1.1 – 1.6 (m, 2H, Ile-$\gamma_{A/B}$-H), 1.9 – 2,1 (m, 1H, Ile-β-H), 3.75 – 3.76 (d, 1H, Ile-α-H), 4.41 – 4.50 (m, 4H, 3',4',5'), 4.54 – 4.57 (t, 1H, 2'-H, J = 4.6), 6.12 – 6.14 (d, 1H, 1'-H, J = 5.31), 8.24 (s, 1H, 2H), 8.41 (s, 1H, 8H).

$^{13}$C-NMR ($D_2O$): 11.5 (Ile-δ-$CH_3$), 15.1 (Ile-γ'-$CH_3$), 24.7 (Ile-γ-$CH_2$), 36.9 (Ile-β-CH), 60.7 (Ile-α-CH), 68.9 (5'-C), 70.79 (3'-C), 74.69 (2'-C), 82.94 (4'-C), 87.93 (1'-C), 119.13 (5-C), 140.43 (8-C), 149.47 (4-C), 153.05 (2-C), 155.79 (6-C), 175.61 (Ile- C=O).

HR-MS calcd. for $C_{16}H_{24}N_7O_9S_1$ $[M-H]^-$: 458.1548; found: 458.1450.

### 2.4.8 Synthesis of 5'-*O*-[*N*-[L-valyl]-sulfamoyl] adenosine (43g)

This compound was synthesized analogously to compound **43b**.

$^{1}$H-NMR ($D_2O$):0.96 – 0.98 ( 0.84-0.89 (t, 3H, Ile-δ, J = 7.38), 0.97 – 0.99 (d, 3H, Ile-γ'-H, J = 7.02), 1.1 – 1.6 (m, 2H, Ile-$\gamma_{A/B}$-H), 1.9 – 2,1 (m, 1H, Ile-β-H), 3.75 – 3.76(d, 1H, Ile-α-H), 4.41 – 4.50 (m, 4H, 3',4',5'), 4.54 – 4.57 (t, 1H, 2'-H, J = 4.6), 6.12 – 6.14 (d, 1H, 1'-H, J = 5.31), 8.24 (s, 1H, 2H), 8.41 (s, 1H, 8H).

$^{13}$C-NMR ($D_2O$): 16.94 (Val- $\gamma_B$-$CH_3$), 18.56 (Ile-$\gamma_A$-$CH_3$), 30.45 (Val-β-CH), 61.45 (Val-α-CH), 68.91 (5'-C), 70.78 (3'-C), 74.65 (2'-C), 82.95 (4'-C), 87.90 (1'-C), 119.20 (5-C), 140.31 (8-C), 149.54 (4-C), 153.47 (2-C), 156.15 (6-C), 176.38 (Val- C=O).

ESI-MS calcd. for $C_{15}H_{22}N_7O_7S$ $[M-H]^-$: 444.45; found: 444.30.

### 2.4.9 Synthesis of fMRTGNAD-SA (45a)

The peptide formyl-methionyl-arginyl(2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulfonyl)-threonyl(tBu)-glycyl-asparaginyl (trityl)-alanyl-OH was synthesized on a 2-chlorotrityl chloride resin using standard Fmoc-based solid phase peptide chemistry. The protected peptide was cleaved from the resin using a mixture of HOAc/trifluoroethanol/DCM (1/1/8, v/v) in 30 min. Following RP-HPLC purification, the peptide (20 mg, 16.13 µmol, 1.0 eq.) and HOBt (9 mg, 64.52 µmol, 4.0 eq.) were dissolved in DMF (500 µL) and DIC (10 µL, 64.52 µmol, 4.0 eq.) was added. This mixture was stirred for 1 h at rt under argon atmosphere. DIPEA (7.5 µL, 40.33 µmol, 2.5 eq.) was added and the mixture was added to the adenosine analogue **43a** (16.68 mg, 32.26 µmol, 2.0 eq.) and stirred for 16 h at rt under argon. Next, the volatiles were evaporated and the residue was taken up in a mixture of $CH_3CN$/water. This was purified on a PoraPak Rxn® column ($CH_3CN$ 25 to 100% in water). The fractions containing the product were evaporated yielding **44a**. The product **44a** was subsequently deprotected using a mixture of 90% TFA, 7.5% $H_2O$ and 2.5% thioanisole. The volatiles were evaporated and co-evaporated 3 times with toluene (10 mL). The remaining title product (**45a**, **fMRTGNAD-SA**) was redissolved in $H_2O$, filtered and purified by RP-HPLC (solvent A: 25 mM TEAB in $H_2O$; solvent B: 25 mM TEAB in $CH_3CN$.

Yield (as calculated over coupling and deprotection): 1.398 mg (13.9%). HR-MS calcd. for $C_{39}H_{60}N_{17}O_{18}S_2$ $[M-H]^-$: 1118.3822; found: 1118.3789.

### 2.4.10 Synthesis of fMRTGNAA-SA (45b)

Further synthesis to obtain **45b** (yield: 2.3 mg (11%)) was performed analoguously to the synthesis of **45a**. HR-MS calcd. for $C_{38}H_{62}N_{17}O_{16}S_2$ $[M+H]^+$: 1076.4002; found: 1076.3849.

### 2.4.11 Synthesis of fMRTGNAL-SA (45c)

Further synthesis to obtain **45c** (yield: 0.7 mg (7%)) was performed analoguously to the synthesis of **45a**. HR-MS calcd. for $C_{41}H_{68}N_{17}O_{16}S_2$ $[M+H]^+$: 1118.4471; found: 1118.4482.

### 2.4.12 Synthesis of fMRTGNAG-SA (45d)

Further synthesis to obtain **45d** (yield: 1.8 mg (8%)) was performed analoguously to the synthesis of **45a**. HR-MS calcd. for $C_{37}H_{58}N_{17}O_{16}S_2$ $[M-H]^-$: 1060.3689; found: 1060.3702.

### 2.4.13 Synthesis of fMRTGNAK-SA (45e)

Further synthesis to obtain **45e** (yield: 1.2 mg (2 %)) was performed analoguously to the synthesis of **45a**. HR-MS calcd. for $C_{41}H_{67}N_{18}O_{16}S_2$ $[M-H]^-$: 1131.4400; found: 1131.3250.

### 2.4.14 Synthesis of fMRTGNAI-SA (45f)

Further synthesis to obtain **45f** (yield: 1.2 mg (5.6 %)) was performed analoguously to the synthesis of **45a**. HR-MS calcd. for $C_{41}H_{66}N_{17}O_{16}S_2$ $[M-H]^-$: 1116.4314; found: 1116.4326.

### 2.4.15 Synthesis of fMRTGNAV-SA (45g)

Further synthesis to obtain **45g** (yield: 3.8 mg (19%)) was performed analoguously to the synthesis of **45a**. HR-MS calcd. for $C_{40}H_{64}N_{17}O_{16}S_2$ $[M-H]^-$: 1102.4158; found: 1102.4165.

### 2.4.16 Biological activity

The respective bacteria were grown overnight in LB medium and cultured again the following day in fresh LB medium or LB medium containing 5 mM (L)-arabinose. Compounds were titrated in a 96-well plate using either LB-medium +/- 5 mM (L)-arabinose to dilute the compounds. To each well, 85 µL LB medium +/- 5 mM (L)-arabinose was added to a total volume of 90 µL. Next, 10 µL of bacterial cell culture grown to a OD600 of 0.1 was added. The cultures were next placed into a Tecan Infinite M200® incubator and shaken at 37°C, subsequently the OD600 was determined after 8 h. All experiments have been performed in triplicate.

Bacterial strains used for the evaluations: *E. coli* Ara-Yej (BW39758), expressing the *yejABEF* transporter upon L-arabinose induction; *E. coli* K-12 (BW28357), used as the wild type control; *E. coli ΔyejA*, lacking subunit A of the YejABEF transporter; and *E.coli ΔpepABN*, lacking all three peptidases PepA, PepB and PepN. LB was prepared in the following manner: 10 g tryptone (Becton–Dickinson, cat. no. 211705), 5 g yeast extract (Becton–Dickinson, cat. no. 288620), 10 g NaCl were combined, to which 1 L deionised water was added. The pH was adjusted to 7.0 with 5 N NaOH and sterilised.

**Research Contributions**

Prof. Dr. Jef Rozenski recorded the mass spectra.

# Chapter 3

# Characterization of the peptide-chain length and constituency for YejABEF-mediated uptake of microcin C analogues

**Abstract:** Microcin C (McC), a natural antibacterial compound consisting of a heptapeptide attached to a modified adenosine, is actively taken up by the YejABEF transporter after which it is processed by cellular aminopeptidases, releasing the non-hydrolyzable aminoacyl adenylate, an inhibitor of aspartyl-tRNA synthetase. McC analogues with variable length of the peptide moiety were synthesized and evaluated in order to characterize substrate preferences of the YejABEF transporter. It was shown that a minimal peptide chain length of six amino acids and the presence of a *N*-terminal formyl-methionyl-arginyl sequence are required for transport.

This chapter was earlier published in revised form as:
Characterization of peptide chain length and constituency requirements for YejABEF-mediated uptake of microcin C analogues
G. H. Vondenhoff, B. Blanchaert, S. Geboers, T. Kazakov, K. A. Datsenko, B. L. Wanner, J. Rozenski, K. Severinov, A. Van Aerschot, *J Bacteriol*, 2011, *193*, 3618 [164].

## 3.1 Introduction

McC penetrates the outer membrane of the *E. coli* cell mostly through the OmpF porin but also through other, yet unidentified transport systems (Novikova, Metlitskaya, Severinov, unpublished data) and as is described in section 1.4.2, is subsequently transported through the inner membrane by the YejABEF transporter [165]. YejABEF is the only complex responsible for McC transport since *yej* mutants are highly resistant to McC, its maturation intermediates, and chemical analogues. While intact McC inhibits the growth of sensitive *E. coli* cells at low micromolar concentrations, processed McC does not affect cell growth even at millimolar concentrations. Thus, the peptide chain enables McC to function through a Trojan horse mechanism by promoting active uptake via the YejABEF transporter. The recently improved synthetic approach for the production of McC analogues has led us to investigate the uptake properties of the Yej transporter in more detail. The obtained results could be of importance for further drug development whereby peptides function as carrier-moieties for drugs that otherwise would not be able to penetrate the bacterial membranes. Here, we used a number of McC analogues truncated either from their *C*- or *N*-terminal sides, or otherwise modified, to determine the minimal peptide chain length sufficient for facilitated transport by Yej.

## 3.2 Results

### 3.2.1 Design, synthesis and inhibitory activity of McC analogues in cell extracts

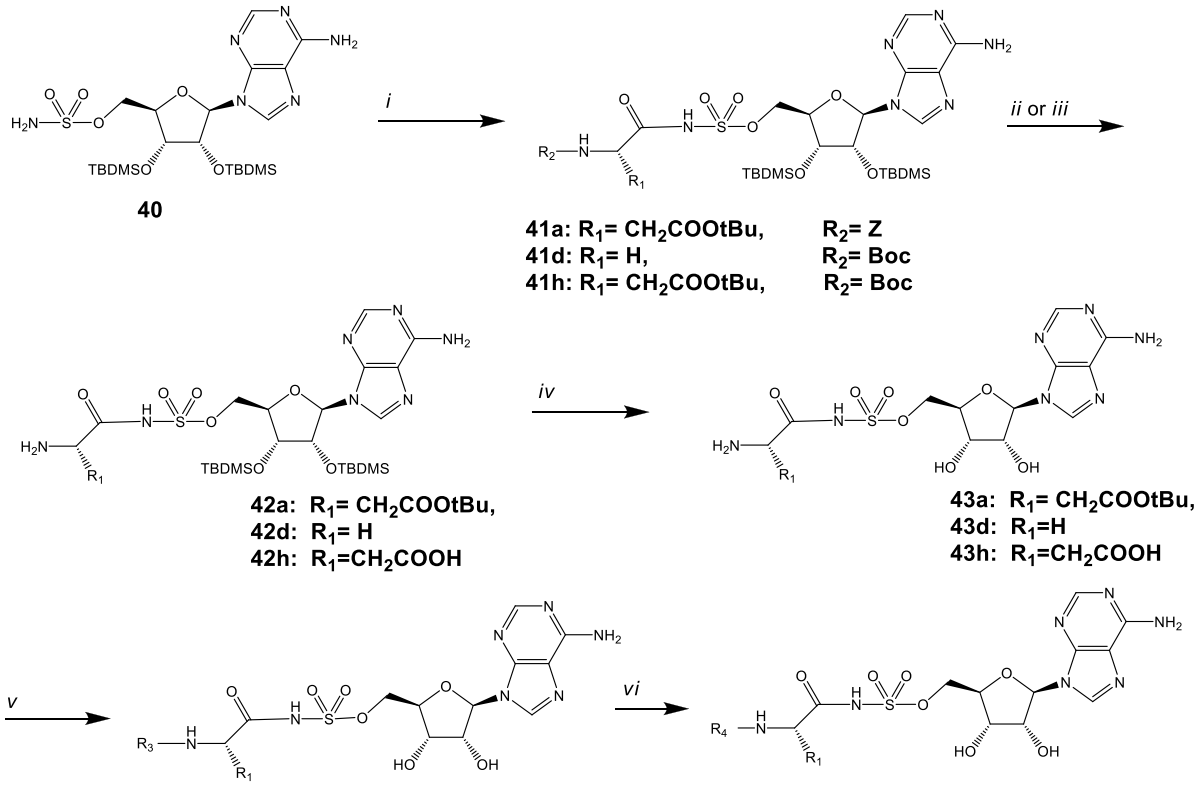
Acyladenylates are the natural reaction intermediates of aminoacyl-tRNA synthetases (aaRSs). Therefore, mimicking these analogues, the charging of tRNAs with their cognate amino acid and hence mRNA translation will be inhibited. Crucial portion of the inhibitor scaffold is the linker between the amino acid and the adenosine moiety, which needs to be metabolically stable [166]. Many different linkers have been developed before as surrogates for the labile acylphosphate linkage [167-169], however, aminoacyl-sulfamoyl adenosines on many occasions were found to be the most potent analogues, and proved to be nanomolar inhibitors of their corresponding aaRSs [170, 171]. When trying to mimick the natural McC, we therefore opted for aminoacyl-sulfamoyl adenosines, as these are more stable and readily synthesized relative to aminoacyl-phosphoramidate-adenosine analogues, as found in natural McC. Following a recently developed method for the synthesis of McC analogues (Scheme 3.1), a number of compounds were created that were truncated at their *C*-terminus (compounds **55-59**, Scheme 3.1), each compound containing an aspartate as the ultimate *C*-

terminal residue and lacking the *N*-formyl group, or from their *N*-terminus (compound **60**). To evaluate the role of arginine at the second position of the peptide part, compound **61** was created. The expected positive effect of the presence of an *N*-formyl moiety could be studied *via* the "full analogue" (compound **45a**).

In cell extracts prepared from mutant *E. coli*, lacking RimL, the addition of these compounds inhibited the $tRNA^{Asp}$ aminoacylation reaction (Fig. 3.1). No inhibition was observed in extracts prepared from cells lacking aminopeptidases A, B, and N with compounds **21a, 21b, 23** or **55-62**. In contrast, the addition of compound **46h** to mutant cell extracts led to $tRNA^{Asp}$ aminoacylation inhibition. This is an expected result since D-SA, is a non-hydrolyzable aspartyl-adenylate that can inhibit AspRS directly, *i.e*, without processing. McC analogues targeting AspRS and carrying peptide chains longer than seven amino acids were found to be significantly less stable to acidic deprotection conditions during their synthetic assembly and proved thus hard to synthesize, which could be attributed on intramolecular cyclization resulting in $N^3$-5'-cycloadenosine [170]. Hence, to evaluate McC analogues carrying a peptide longer than the usual heptapeptide, fMRTGNAAG-SA (compound **54**) was synthesized, with incorporation of an additional internal alanine and substitution of glycine for the *C*-terminal aspartate. This compound could be synthesized using a non-protected peptide, albeit with a low yield. In cell extracts, the addition of this compound inhibited the $tRNA^{Gly}$ aminoacylation reaction (Fig. 3.1, lower panel). No inhibition was observed in extracts prepared from cells lacking aminopeptidases A, B, and N, compound **43d** excluded. Again, compound **43d** does not require processing and can therefore inhibit $tRNA^{Gly}$ formation directly. The results thus indicate that compound **54** targets GlyRS through formation of glycyl sulfamoyl adenosine generated by processing in the RimL deficient *E. coli* cell extracts. The control heptapeptidic analogue (fMRTGNAG-SA, compound **62**) likewise targets GlyRS.

### 3.2.2 Antibacterial activity of McC analogues with truncated or elongated peptide chains

The ability of new compounds to inhibit the growth of McC-sensitive *E. coli* was evaluated next. The assay used to determine the sensitivity to various compounds consisted of measuring the optical density reached by identical cell cultures in wells of microtiter plates in the presence of various concentrations of inhibitors. The assay has proven to be highly sensitive and reproducible, and superior to standard tests based on determination of growth inhibition zones on plates.




**40**

**41a: $R_1$= $CH_2COOtBu$, $R_2$= Z**
**41d: $R_1$= H, $R_2$= Boc**
**41h: $R_1$= $CH_2COOtBu$, $R_2$= Boc**

**42a: $R_1$= $CH_2COOtBu$,**
**42d: $R_1$= H**
**42h: $R_1$=$CH_2COOH$**

**43a: $R_1$= $CH_2COOtBu$,**
**43d: $R_1$=H**
**43h: $R_1$=$CH_2COOH$**

**46-54: $R_1$=$CH_2COOtBu$**
**46: $R_3$= Boc-M-R(Pbf)-T(tBu)-G-N(Trt)-A**
**47: $R_3$= Boc-M-R(Pbf)-T(tBu)-G-N(Trt)**
**48: $R_3$= Boc-M-R(Pbf)-T(tBu)-G**
**49: $R_3$= Boc-M-R(Pbf)-T(tBu)**
**50: $R_3$= Boc-M-R(Pbf)**
**51: $R_3$= Boc-M**
**52: $R_3$= Boc-R$(Boc)_2$-T(tBu)-G-N(Trt)-A**
**53: $R_3$=f-M-T(tBu)-G-N(Trt)-A**
**44a: $R_3$=f-M-R(pbf)-T(tBu)-G-N(Trt)-A**
**54: $R_1$= H, $R_3$= fMRTGNAA**

**23 and 55-61: $R_1$= $CH_2COOH$**
**23: $R_4$= M-R-T-G-N-A**
**55: $R_4$= M-R-T-G-N**
**56: $R_4$= M-R-T-G**
**57: $R_4$= M-R-T**
**58: $R_4$= M-R**
**59: $R_4$= M**
**60: $R_4$= R-T-G-N-A**
**61: $R_4$= f-M-T-G-N-A**
**45a: $R_4$= f-M-R-T-G-N-A**
**62: $R_1$=H $R_4$= f-M-R-T-G-N-A**

**Scheme 3.1.** General scheme affording the various McC analogues. *i*) *N*-α-Cbz-L-aminoacyl-(*t*Bu or Boc)-succinimide, DBU in DMF, 6h, rt. *ii*) for $R_2$ = Z-group, $H_2$, Pd/C in MeOH, 3h, rt. *iii*) for $R_2$= Boc-group of TFA/$H_2O$ (5:2), 4h, 0 °C to rt. *iv*) $Et_3N.3HF$ in THF, 16 h, rt. *v*) protected peptide (1 eq.), HOBt (4 eq.), DIC (4 eq.) and DIEA (2 eq.) in DMF, 16h, rt. *vi*) TFA/thioanisole/$H_2O$ (90/2.5/7.5), 2h, rt.

Since intracellular transport by YejABEF may be limiting cell sensitivity to McC and its derivatives, a new *E. coli* tester strain Ara-Yej (BW39758) was developed (K. A. Datsenko and B. L. Wanner, personal communication). In this strain, the genomic *yejABEF* operon is under control of the arabinose-inducible *araBAD* promoter. We expected that this strain will be relatively resistant to McC in the absence of inducer but may be hypersensitive in its presence. To test this expectation, the sensitivity of Ara-Yej cells and control wild-type *E. coli* K-12 BW28357 cells to McC (compound **21a**) was determined. All results discussed in this Chapter are summarized in Table 3.1, but for clarity, full details are to be found in Fig. 3.2 and Fig. 3.3. Uninduced Ara-Yej cells were practically resistant to up to 1 mM of McC, the highest concentration used in this experiment. In contrast, the growth of wild-type cells was

visibly inhibited at these conditions. The addition of 5 mM (L)-arabinose to the medium had little effect on McC sensitivity of wild-type cells but dramatically increased the sensitivity of Ara-Yej strain to a level above that of wild-type cells at matching McC concentrations (pronounced growth inhibition in the presence of 0.25 μM McC). The results thus indicate that *i*) induced Ara-Yej *E. coli* is a superior strain for measuring sensitivity to McC and related compounds (by comparing induced and uninduced cell growth in the presence of a given concentration of inhibitor tested) and *ii*) the amount of the YejABEF transporter produced by wild-type *E. coli* during growth in rich medium indeed limits cell sensitivity to McC and, presumably, the natural YejABEF substrates are taken up less efficiently as well. The latter finding is of potential interest since it has been shown that the levels of the *yejABEF* transcript are subject to negative regulation by a small RNA rydC [172], which is involved in stress response.

In agreement with previous findings, we also observed that the McC derivative lacking the *N*-terminal formyl group (compound **21b**) was less effective against wild-type cells than the formylated variant **21a** (Fig. 3.2). This was likewise demonstrated with the formylated synthetic McC analogue (compound **45a**) versus the non-formylated variant (compound **23**). The effect was also observed (though less pronounced) in the case of the induced Ara-Yej strain.

The antibacterial activities of various McC-analogues were next determined. The activities of new compounds were compared to that of MRTGNAD-SA (compound **23**, Fig. 3.3), a previously characterized synthetic McC analog containing a sulfamoyl bond instead of the natural phosphoamide and lacking the aminopropyl moiety. Both formylated (compound **45a**) and non-formylated MRTGNAD-SA (compound **23**) variants were approximately 10 times less effective than McC. This difference is likely due to the lack of the aminopropyl group, which was shown to increase the efficiency of target inhibition by processed McC [173]. Interestingly, uninduced *E. coli* Ara-Yej cells were partially inhibited by compound **23** at concentrations above 2.5 μM. This could be explained by low expression levels of the $P_{BAD}$*:yejABEF* fusion caused by traces of arabinose in the LB medium or by the presence of a so far unknown additional, low-affinity transporter.

As can be seen, removal of penultimate McC peptide amino acid ($Ala^6$) (compound **55**) had little effect on antibacterial activity. The result thus indicates that the Yej transporter can recognize McC derivatives with a six-amino acid peptide rather than the seven-amino acid peptide found in natural McC. Removal of two amino acids ($Asn^5$ and $Ala^6$, compound **56**) strongly decreased antibacterial activity, which however remained *yejABEF*-dependent. Removal of additional internal amino acids of the McC peptide abolished the activity (compounds **57-59**, not shown).

In contrast to the single amino acid deletion at the *C*-terminus, a variant lacking the *N*-terminal methionine (compound **60**) was biologically inactive, indicating that YejABEF recognizes not just the length but also the sequence of the peptide attached to the AMP-analogue. Compound **61** lacking $Arg^2$ and containing formylated *N*-terminal methionine likewise was very poorly active.

For each compound, control experiments with McC-resistant cells lacking one of the *yej* genes or *pepA,B,N* genes were also performed (sensitivities of these cells were determined in a standard plate assay) on M63 agar plates. Both control cells were fully resistant to all compounds, with the exception of compound **59** (MD-SA) which showed minor activity against *Δyej* cells, but no activity against *ΔpepABN* cells, and compound **43h** (D-SA) which is active at concentrations > 100 μM against both the wild-type and mutant cells, as expected [72].

Elongation of the peptide chain by a single amino acid residue as in compound **54** did not affect its potency. In fact, this compound was slightly more active than the corresponding compound **62** containing the heptapeptide (Fig. 3.3). As expected, compound **54** proved inactive against *E. coli* cells with a disrupted *yej* gene, or cells without functional aminopeptidases A, B and N and is therefore acting through a Trojan horse mechanism.

To confirm the mechanism of action, a series *of in vitro* aminoacylation experiments were performed. As can be observed from Fig. 3.1, all shortened compounds inhibit tRNA aminoacylation reactions upon 15 min. in cell extract, the time sufficient for processing of natural McC. On the other hand, no inhibition was observed in cell extracts prepared from *E. coli ΔpepABN* cells. This demonstrates again that all of the shortened compounds, D-SA and G-SA excluded, required the action of PepA, PepB or PepN for release of the inhibitor.

The physiological substrate of the YejABEF transporter (other than McC) is not yet known. Since our data indicate that McC uptake is peptide length-dependent, we attempted to estimate the optimal length of peptide substrates transported by YejABEF by setting up *in vivo* competition between McC and several MccA-based peptides. To this end, fixed amounts of McC were deposited on lawns of sensitive cells along with increasing concentrations of peptides. The expectation was that at some point the peptide will outcompete McC, rendering the cells resistant and leading to the disappearance of growth inhibition zones. In all, five peptides were tried (MRTGNAN, GMRTGNAN, GGMRTGNAN, $G_3$MRTGNAN, and $G_6$MRTGNAN). The results indicated the minimal peptide concentrations needed for complete protection from 13 μM McC (no inhibition zone observed) to be ~7 mM for the peptides MRTGNAN and $G_6$MRTGNAN, but three times lower or ~2 mM for the other three peptides. The results are consistent with increased potency of octapeptide-based G-SA inhibitor and indicate that preferred peptide length for YejABEF-mediated uptake is above 7 but below 13 amino acids.

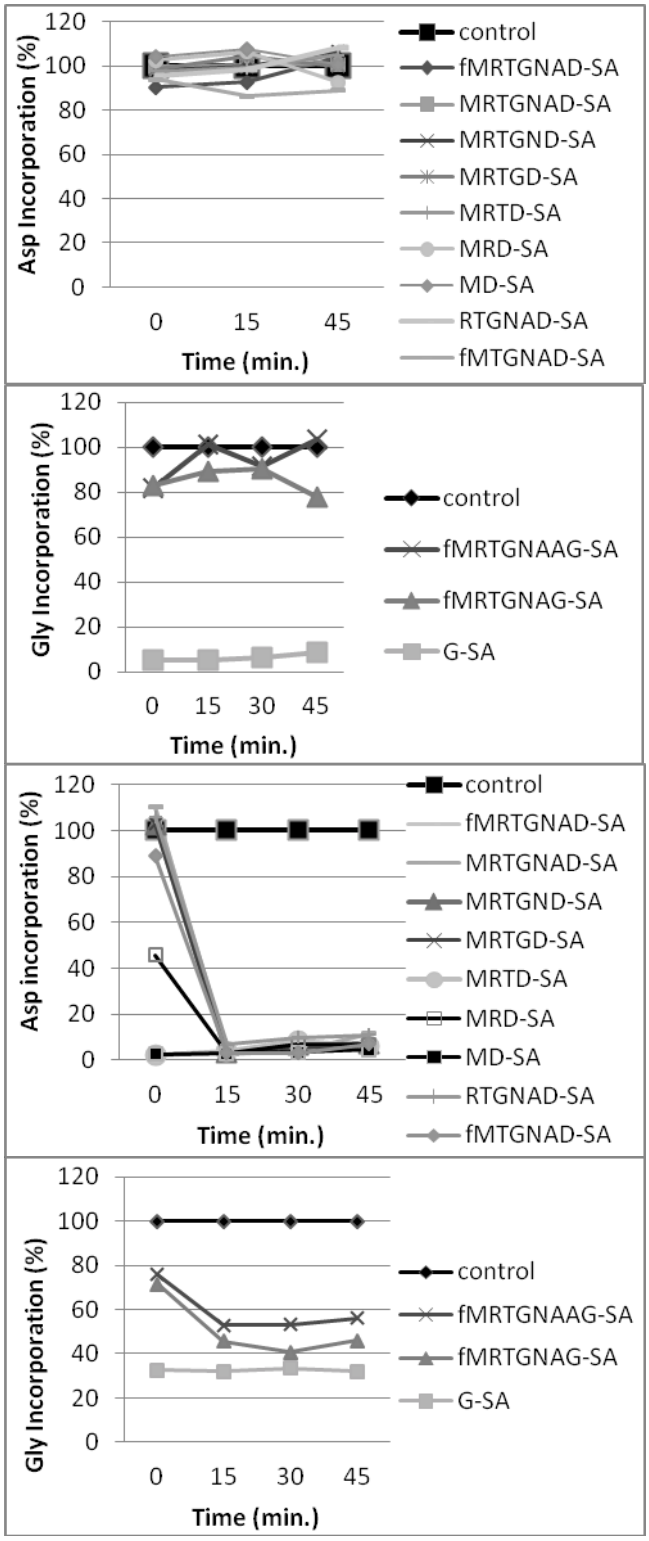



**Figure 3.1. Upper two panels**: Asp-RS and Gly-RS inhibition in S30 extracts, lacking peptidase A, B and N. The different extracts were incubated with the respective AspRS or GlyRS inhibitors. At different time-points the aminoacylation reaction was carried out. The amount of aminoacylated $tRNA^{Asp}$ or $tRNA^{Gly}$ was measured by liquid scintillation counting.

**Lower two panels:** Asp-RS and Gly-RS inhibition in S30 extracts, lacking the acetylase RimL.

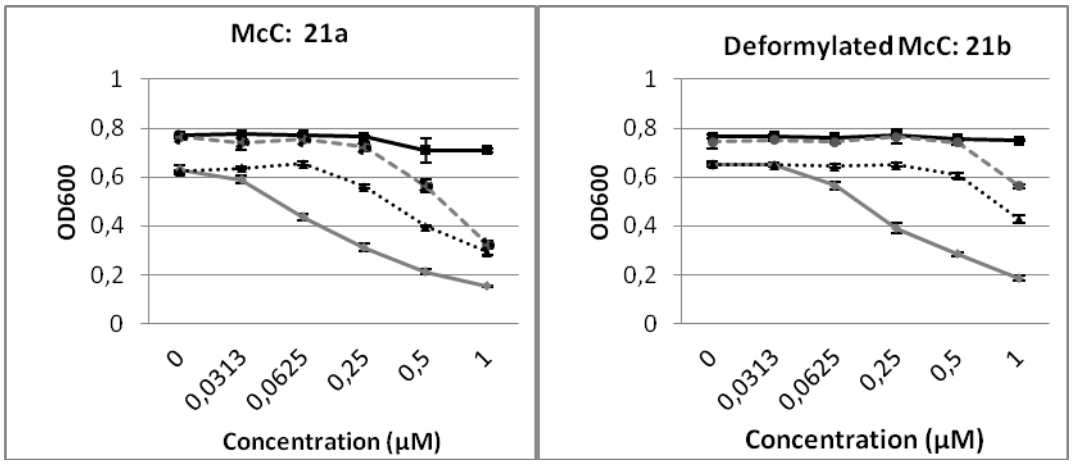
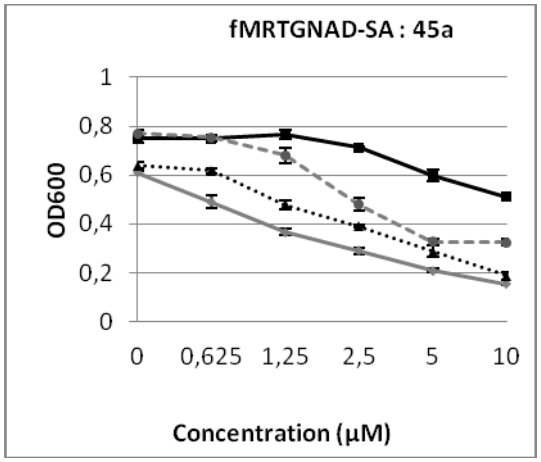
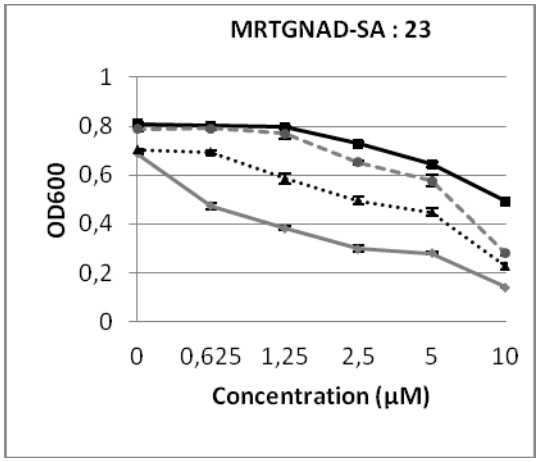


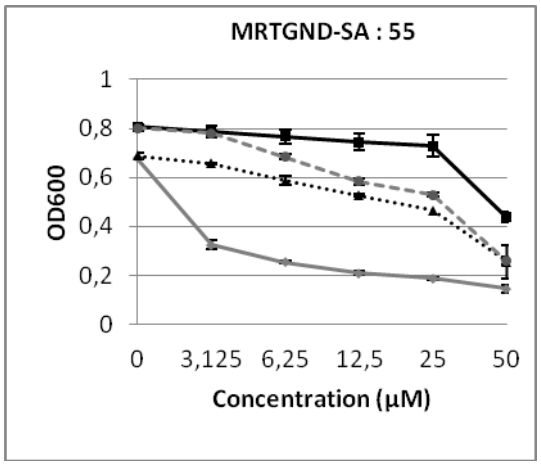

**Figure 3.2.** Antibacterial activity of McC (compound **21a**) and its non-formylated variant (compound **21b**) assayed with wild-type *E. coli* and with induced and uninduced Ara-Yej cells. OD600 refers to the optical densitiy of the cell culture measured at 600 nm.

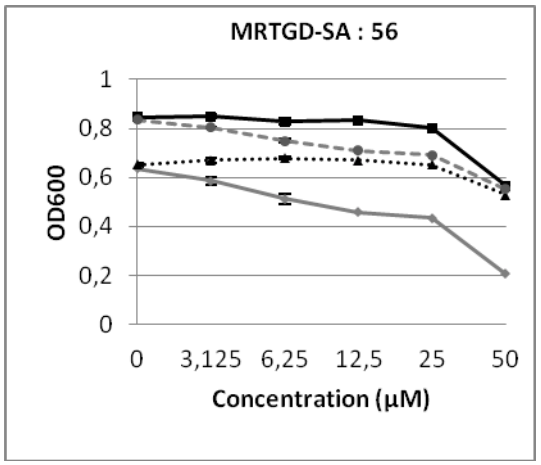
## 3.3 Discussion

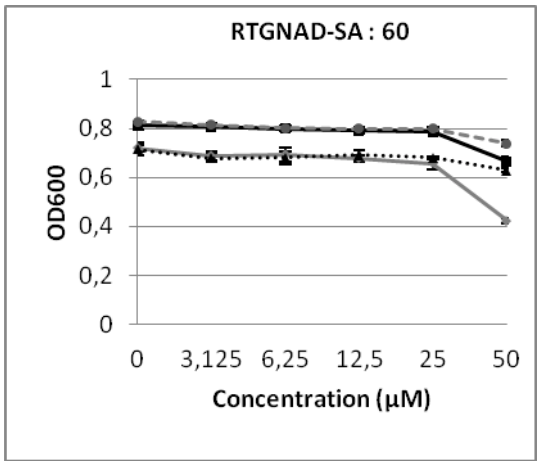
As shown before, the McC peptide part plays an important role in endowing the intracellularly active compound **21a** with its antibacterial activity. Without the peptide recognized by the YejABEF transporter, no activity at micromolar concentrations can be demonstrated, as the phosphoamidated nucleoside analogue is not efficiently taken up by the target cells. This has been demonstrated clearly using cells carrying mutations in the *yej* genes coding for the transporter, which proved resistant to McC, highlighting the necessity of the transporter for internalization and antibacterial activity of compounds **21a** and **21b** [165]. Likewise, this has been demonstrated for different sulfamoylated nucleoside analogues like compound **23**, which display strong inhibitory activity *in vitro* against cognate aaRSs, but proved to be rather weak inhibitors in cellular antibacterial screens. Site-specific mutagenesis of the *mccA* gene coding for the peptide moiety of McC and providing substitution of the internal amino acid positions generated multiple active McC variants. Only the *C*-terminal asparagine, which is converted to aspartic acid upon maturation of McC, proved indispensable [174].

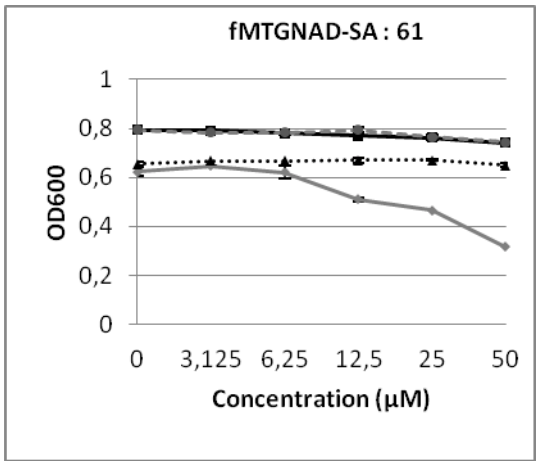

fMRTGNAD-SA : 45a
OD600
1
0,8
0,6
0,4
0,2
0
0
0,625
1,25
2,5
5
10
Concentration (µM)


MRTGNAD-SA : 23
OD600
1
0,8
0,6
0,4
0,2
0
0
0,625
1,25
2,5
5
10
Concentration (µM)


MRTGND-SA : 55
OD600
1
0,8
0,6
0,4
0,2
0
0
3,125
6,25
12,5
25
50
Concentration (µM)


MRTGD-SA : 56
OD600
1
0,8
0,6
0,4
0,2
0
0
3,125
6,25
12,5
25
50
Concentration (µM)


RTGNAD-SA : 60
OD600
1
0,8
0,6
0,4
0,2
0
0
3,125
6,25
12,5
25
50
Concentration (µM)


fMTGNAD-SA : 61
OD600
1
0,8
0,6
0,4
0,2
0
0
3,125
6,25
12,5
25
50
Concentration (µM)

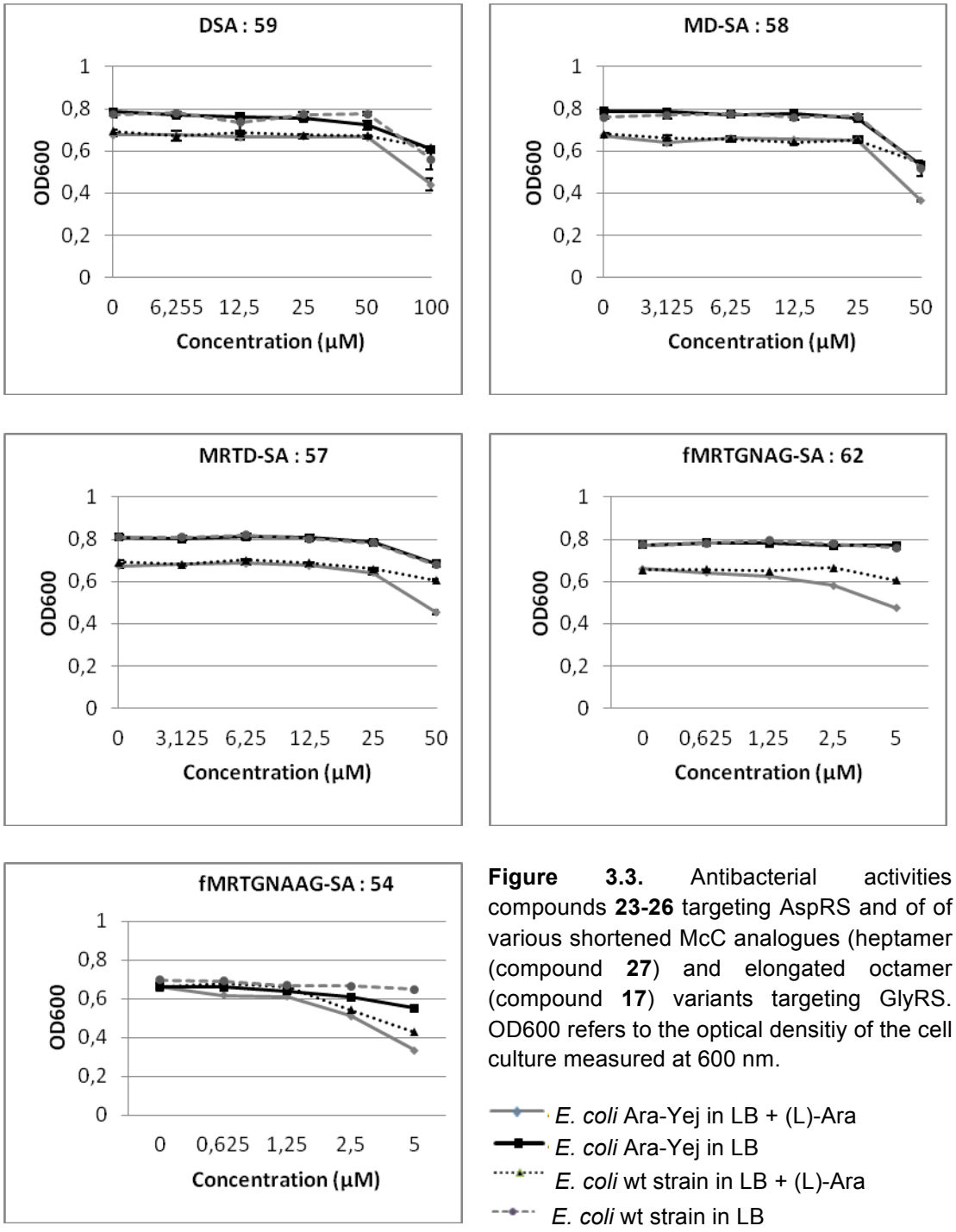



**Figure 3.3.** Antibacterial activities compounds **23-26** targeting AspRS and of of various shortened McC analogues (heptamer (compound **27**) and elongated octamer (compound **17**) variants targeting GlyRS. OD600 refers to the optical densitiy of the cell culture measured at 600 nm.

*E. coli* Ara-Yej in LB + (L)-Ara
*E. coli* Ara-Yej in LB
*E. coli* wt strain in LB + (L)-Ara
*E. coli* wt strain in LB

**Table 3.1. Lowest concentration at which clear inhibition could be observed against wt *E. coli* K-12 (BW28357) cells**

| Entry | Lowest inhibitory concentration (µM) |
|---|---|
| McC (**21a**) | < 0,25 |
| Deformylated McC (**21b**) | 0,25 |
| fMRTGNAD-SA (**45a**) | < 0,63 |
| MRTGNAD-SA (**23**) | 0,63 |
| MRTGND-SA (**55**) | 3,13 |
| MRTGD-SA (**56**) | 6,25 |
| RTGNAD-SA (**60**) | 50 |
| fMTGNAD-SA (**61**) | 12,5 |
| D-SA (**43h**) | >100 |
| MD-SA (**59**) | 50 |
| MRTD-SA (**57**) | 50 |
| fMRTGNAG-SA (**62**) | 5 |
| fMRTGNAAG-SA (**54**) | 5 |

The *N*-terminal formyl methionine moiety was not included in the analysis, since it is essential for initiation of translation of MccA. Chemical manipulation of sulfamoylated congeners of McC allows to substitute the last aspartic acid, enabling inhibition of different aaRSs [72], and to probe the importance of the *N*-terminal methionine residue. Hence, to further delineate the recognition elements for uptake by the YejABEF transporter, different truncated and modified peptides were introduced here to determine the minimal chain length and best composition for efficient uptake.

Four important results were obtained in this work. First, it was shown that the YejABEF-transporter is able to transport larger McC analogues across the bacterial inner membrane, but there seems to be no recognition for compounds with peptide chains less than six amino acids. We were not able to investigate to which extent the maximal chain length can be extended, due to difficulties in synthesizing larger compounds. Obviously, from a pharmaceutical perspective this is of minor importance. Secondly, if the *N*-terminal formyl group is excluded from the McC analogue, a slightly lower activity is observed. This observation is also consistent with these for McC (compound **21a**) and non-formylated McC (compound **21b**), where **21b** is somewhat less active than **21a**. Third, if the *N*-terminal amino acid of the hexapeptidyl McC analogue is different from methionine, little or no activity is

observed. In contrast, a hexapeptidyl analogue carrying methionine at the *N*-terminus (compound **55**) proved active, albeit at five-fold higher concentration when compared to compound **45a**. This suggests that the *N*-terminal methionine or more exactly formyl-methionine (see previous point) is of importance to the recognition by the YejABEF transporter. Finally, upon exclusion of arginine from the hexapeptide moiety as in compound **61**, the activity is dramatically decreased compared to compound **45a**. Therefore, the presence of an *N*-terminal formyl-methionine in combination with arginine seems to be important for uptake by the YejABEF transporter. One therefore could speculate on a positive charge being beneficial at this position. However, the latter result is in contrast with the McC variants obtained by mutagenesis, where $Ala^2$, $Ser^2$ and $Trp^2$ variants also proved active [174]. In addition and more curiously, compounds **59** and **43h** displayed antibacterial activity albeit at high concentrations, which may be explained by their small size and hence their ability to diffuse through the bacterial membrane. Although clear sigmoidal inhibition curves were not obtained, the different profiles nicely show the sensitivity of the tested *E. coli* strains to all new compounds which were synthesized. An approximate $MIC_{50}$ value taking into account the inhibition of the wt *E. coli* strain in presence of arabinose can be found in Table 3.1. This highlights the different points made in the previous paragraph. The results obtained here delineate the recognition properties of the YejABEF transporter, which is proved indispensable for the antibacterial potential of different McC analogues. The Trojan horse mechanism of McC action, mediated by the transporter, also paves the way for improving the uptake and hence the biological activity of different toxic entities with otherwise low *in vitro* and/or *in vivo* activity.

## 3.4 Materials and methods

### 3.4.1 Chemistry

Reagents and solvents were purchased from commercial suppliers (Acros, Sigma-Aldrich, Bachem, Novabiochem) and used as provided, unless indicated otherwise. DMF and THF were analytical grade and were stored over 4Å molecular sieves. For reactions involving Fmoc-protected amino acids and peptides, DMF for peptide synthesis (low amine content) was used. All other solvents used for reactions were analytical grade and used as provided. Reactions were carried out in oven-dried glassware under a nitrogen atmosphere and the reaction mixtures were stirred at room temperature, unless indicated otherwise.

$^1H$ and $^{13}C$ NMR spectra of the compounds dissolved in DMSO-$d_6$ or $D_2O$ were recorded on a Bruker UltraShield Avance 300 MHz or 500 MHz spectrometer. The chemical shifts are expressed as δ values in parts per million (ppm), using the residual solvent peaks (DMSO:

$^{1}$H, 2.50 ppm; $^{13}$C, 39.60 ppm; HOD: $^{1}$H, 4.79 ppm) as a reference. Coupling constants are given in hertz (Hz). The peak patterns are indicated by the following abbreviations: bs = broad singlet, d = doublet, m = multiplet, q = quadruplet, s = singlet and t = triplet. High resolution mass spectra were recorded on a quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard ESI interface; samples were infused in 2-propanol/$H_2O$ (1:1) at 3 µL.min$^{-1}$.

For TLC, precoated aluminium sheets were used (Merck, Silica gel 60 $F_{254}$). The spots were visualized by UV light at 260 nm. Column chromatography was performed on ICN silica gel 60 Å. Preparative HPLC of peptides was done using a Waters Xbridge prep. C18 (19 mm × 150 mm) column connected to a Waters 1525 binary HPLC pump and a Waters 2487 dual absorbance detector. Final products were purified using a PLRP-S 100Å column connected to a Merck-Hitachi L6200A Intelligent pump. Eluent compositions are expressed as v/v. Purity was checked by analytical HPLC on a Inertsil ODS-3 (C-18) (4.6 mm x 100 mm) column, connected to a Shimadzu LC-20AT pump using a Shimadzu SPD-20A UV-detector. Recordings were performed at 254 nm and 214 nm.

### 3.4.2 Synthesis of 5'-*O*-[*N*-[L-aspartyl(*O*-*tert*-butyl)]-sulfamoyl]adenosine (43a)

A solution of Z-aspartyl(*O*-*tert*-butyl)-*O*-Su (1 g, 2.38 mmol, 1.0 eq.), 5'-O-sulfamoyl-2',3'-di-*O*-(*tert*-butyldimethylsilyl)-adenosine (**40**) (1.23 g, 2.14 mmol, 0.9 eq.) and DBU (356 µl, 2.38 mmol, 1.0 eq.) in DMF (7 mL) was stirred at rt for 8h under nitrogen atmosphere. DMF was evaporated under reduced pressure. Next, the residue was purified by flash chromatography ($CH_2Cl_2$, 1% $Et_3N$, 2.5 to 10% MeOH). Fractions containing the desired product were evaporated giving a yellow oil (**41a**). Yield: 1.7 g (90%). ESI-MS calcd. for $C_{38}H_{62}N_7O_{11}SSi_2$ $[M+H]^+$: 880.4; found: 880.1

Compound **41a** (1.7 g) was dissolved in MeOH at 0 ºC while Pd/C (0.17 g) was added. The solution was stirred under hydrogen atmosphere for 3h. The mixture was filtered and evaporated yielding a white-yellow foam (**42a**). Yield: 1.1g (76%)

The product **42a** (1.1 g) was carefully dried and dissolved in a mixture of THF (15 mL) and $Et_3N.3HF$ (1 mL). After 3h, another 0.8 mL of $Et_3N.3HF$ was added and the reaction mixture was stirred for another 22h. The reaction mixture was evaporated and the reaction mixture was purified by flash chromatography ($CH_2Cl_2$, 5 to 20% MeOH). Fractions containing the desired product were evaporated, yielding the title product **43a**: 670 mg (88% yield).

$^{1}$H-NMR ($D_2O$): 1.04-1.09 (t, 9H, $Et_3N$, J=7.16), 1.44 (s, 9H, tBu), 2.67-2.75 (q, 6H,$Et_3N$ $CH_2$, J=7.12), 3.68-3.72 (t, 2H, $Asp_\alpha H$, J=5.70), 4.12-4.19 (m, 4H, 3', 4', 5'), 4.63-4.67 (t, 1H, 2'H, J=5.36), 5.94-5.96 (d, 1H, 1', J=5.82), 7.31 (s, 1H, $NH_2$), 8.18 (s, 1H, 2H), 8.41 (s, 1H, 8H).

$^{13}$C-NMR ($D_2O$): 27.02 (*t*Bu-$CH_3$), 35.71 (Asp-β-C), 51.67 (Asp-α-C), 68.14 (5'-C), 70.08 (3'-C), 74.14 (2'-C), 82.05 (4'-C), 83.88 (tBu-C), 87.37 (1'-C), 118.06 (5-C), 139.49 (8-C), 148.31(4-C), 152.19 (2-C), 154.74 (6-C), 170.50 and 173.81 (Asp- C=O).
ESI-MS calcd. for $C_{18}H_{26}N_7O_9S$ $[M-H]^-$: 516.2; found: 515.8.

### 3.4.3 Synthesis of 5'-*O*-[*N*-[L-aspartyl]-sulfamoyl]adenosine (43b)

Boc-Asp(*O*-*t*Bu)-*O*-succinimide and (**40**) (1.89 g, 3.3 mmol, 1 eq.) were dissolved in DMF (1.5 mL). After 15 min, DBU (511 µL, 3.3 mmol, 1 eq.) was added. The reaction was continued for 5h at rt. The volatiles were evaporated and the residue purified by flash chromatography (5% MeOH, 1% $Et_3N$ in EtOAc). Fractions containing the product were identified by TLC analysis, pooled and evaporated yielding 2.23 g (78%) of **41b**.
$^1$H-NMR (DMSO-$d_6$) : 0.91 (s, 9H, tBu TBDMS), 1.14 - 1.19 (t, 9H, $Et_3N$-$CH_3$, J = 7.28), 1.36-1.37 (s, 18H, tBu/Boc), 2.34 – 2.42 (dd, 1H, Asp-β-$H_A$), 2.62 – 2.68 (dd, 1H, Asp-β-$H_B$), 3.05 – 3.13 (q, 6H, $Et_3N$-$CH_2$, J = 7.25), 4.01 – 4.09 (m, 4H, 4'H, 5'H), 4.31-4.32 (d, 1H, 3'H, J = 6), 4.89 – 4.93 (t, 1H, 2'H, J = 9), 5.93 – 5.96 (d, 1H, 1'H, J = 9), 6.13 – 6.16 (d, 1H, NH, J= 9), 7.28 (bs, 2H, $NH_2$, exchangable with $D_2O$), 8.13 (s, 1H, 2H), 8.44 (s, 1H, 8H).
$^{13}$C-NMR (DMSO-$d_6$): 9.56 ($Et_3N$-$CH_3$), 17.47 – 17.82 (TBDMS, -*C*$H_2$-$CH_3$), 25.52 – 25.82 (TBDMS, -$CH_2$-*C*$H_3$), 27.78 (Boc, -$CH_2$-*C*$H_3$), 28.27 (*O*tBu), 36 (Asp-β-C), 39.60 ($Et_3N$-$CH_2$), 52.70 (Asp-α-C), 66.82 (5'-C), 73.37 - 74.63 (2'-C and 3'-C), 77.61 – 79.47 (Boc, -*C*$H_2$-$CH_3$ and *O*tBu), 84.06 (4'-C), 86.03 (1'-C), 118.90 (5-C), 139.54 (8-C), 149.89 (4-C), 152.71 (2-C), 154.80 (6-C), 56.08 (Boc, C=O), 170.15-174.63 (Asp- C=O and *O*tBu C=O).
HRMS calcd. for $C_{35}H_{64}N_7O_{11}SSi_2$ $[M+H]^+$: 846.3923; found: 846.3908.

Compound **41b** (2.23 g, 2.35 mmol, 1.0 eq.) was dissolved in TFA/$H_2O$ (5:2 v/v) (20 mL) and the resulting solution was stirred for 4h. Next the volatiles were removed by rotary evaporation, followed by coevaporation with EtOH (2×). Next, the residue was dissolved in EtOH (10 ml) and $Et_3N$ (1 ml) was added. The volatiles were evaporated again and the residual oil was dried ($P_2O_5$, vacuum, rt, 48h). To this oily residue was added $Et_3N$.3HF (1.2 ml, 7.36 mmol, 9.4 eq.) and THF (100 mL, added in portions). After 4h, the volatiles were removed by rotary evaporation and the residue was adsorbed onto silica (10 g). The product was partly purified on a short silica gel column, with stepwise elution (MeOH 20 to 99%, $Et_3N$ 1% in $CHCl_3$). The product **43b** was further purified by ion exchange chromatography (DEAE-cellulose; elution with TEAB 0.05 M to 1.0 M in $H_2O$), to yield 1.10 g (1.96 mmol, 83%) of **43b**.
$^1$H-NMR (DMSO-$d_6$): δ 1.08 (t, 9H, $Et_3N$-$CH_3$, J = 7.2 Hz), 2.38 (dd, 1H, Asp-β-$H_A$, J = 8.7 Hz, J = 16.7 Hz), 2.57 (dd, 1H, Asp-β-$H_B$, J = 3.9 Hz, J = 16.7 Hz), 2.86 (q, 6H, $Et_3N$-$CH_2$, J = 7.2 Hz), 3.57 (dd, 1H, Asp-α-H, J = 4.0 Hz, J = 8.6 Hz), 4.03 – 4.18 (m, 4H, 3'-H,4'-H, 5'-$H_2$),

4.58 (t, 1H, 2'-H, J = 5.3 Hz), 5.91 (d, 1H, 1'-H, J = 5.8 Hz), 7.28 (bs, 2H, Ade-$NH_2$, $D_2O$-exchangeable), 8.15 (s, 1H, Ade-2-H), 8.39 (s, 1H, Ade-8-H).
$^{13}$C-NMR (DMSO-$d_6$): δ 9.6 ($Et_3N$-$CH_3$), 36.4 (β-C), 45.5 ($Et_3N$-$CH_2$), 52.70 (α-C), 67.6 (5'-C), 70.7 (3'-C), 73.6 (2'-C), 82.5 (4'-C), 87.1 (1'-C), 118.9 (5-C), 139.5 (8-C), 152.8 (2-C), 156.1 (6-C), 172.3 & 173.4 (C=O).
HRMS calcd. for $C_{14}H_{20}N_7O_9S$ $[M+H]^+$ : 462.1043; found: 462.1027.

### 3.4.4 Synthesis of MRTGNAD-SA (23)

The peptide Boc-methionyl-arginyl(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-threonyl(*t*Bu)-glycyl-asparaginyl(trityl)-alanyl-OH was synthesized on a 2-chlorotrityl chloride resin using standard Fmoc-based solid phase peptide chemistry. The peptide was cleaved from the resin using a mixture of HOAc/TFE/DCM (1/1/8, v/v) in 30 min.
The peptide (25 mg, 18.94 µmol, 1.0 eq.) and HOBt (10 mg, 66.6 µmol, 4.0 eq.) were dissolved in DMF (700 µL) and DIC (12µL, 75 µmol, 4.0 eq.) was added. This mixture was stirred for 1 h at rt under argon atmosphere. DIPEA (6.7 µL, 37.88 µmol, 2.0 eq.) was added and the mixture was further added to **43a** (16 mg, 30 µmol, 1.6 eq) and stirred for 16 h at rt under argon. The volatiles were next evaporated and the residue was taken up in a mixture of $CH_3CN$/water. This was then purified on a PoraPak Rxn® column ($CH_3CN$ 25 to 100% in water). The fractions containing the product were evaporated yielding **46**. The product **46** was subsequently deprotected using a mixture of 90% TFA, 7.5% $H_2O$ and 2.5% thioanisole. The volatiles were evaporated and co-evaporated 3 times with toluene (3×10 mL each). The remaining product was re-dissolved in $H_2O$ and filtered. The product **23** was finally purified by RP-HPLC (solvent A: 25 mM TEAB in $H_2O$; solvent B: 25 mM TEAB in $CH_3CN$) affording 1.1 mg (5% total yield).
HR-MS calcd. for $C_{38}H_{60}N_{17}O_{17}S_2$ $[M-H]^-$: 1090.3795; found: 1090.3832.

### 3.4.5 Synthesis of MRTGND-SA (55)

Further synthesis was performed analogously to the preparation of **23**, affording 1.0 mg (4% total yield) of the title compound.
HR-MS calcd. for $C_{35}H_{55}N_{16}O_{16}S_2$ $[M-H]^-$: 1019.3424; found: 1019.3425.

### 3.4.6 Synthesis of MRTGD-SA (56)

Further synthesis was performed analogously to the preparation of **23**, affording 5.9 mg (12% total yield) of the title compound.
HR-MS calcd. for $C_{31}H_{51}N_{14}O_{14}S_2$ $[M+H]^+$: 907.3072; found: 907.3152.

### 3.4.7 Synthesis of MRTD-SA (57)

Further synthesis was performed analogously to the preparation of **23**, affording 1.8 mg (4% total yield) of the title compound.

HR-MS calcd. for $C_{29}H_{46}N_{13}O_{13}S_2$ $[M-H]^-$: 848.2780; found: 848.2781.

### 3.4.8 Synthesis of MRD-SA (58)

Further synthesis was performed analogously to the preparation of **23**, affording 4.6 mg (11% total yield) of the title compound.

HR-MS calcd. for $C_{25}H_{41}N_{12}O_{11}S_2$ $[M+H]^+$: 749.2459; found: 749.2511.

### 3.4.9 Synthesis of MD-SA (59)

Further synthesis was performed analogously to the preparation of **23**, affording 100 mg (38% total yield) of the title compound.

HR-MS calcd. for $C_{19}H_{29}N_8O_{10}S_2$ $[M+H]^+$: 593.1448; found: 593.1450.

### 3.4.10 Synthesis of RTGAND-SA (60)

Further synthesis was performed analogously to the preparation of **23**, affording 8.4 mg (32% total yield) of the title compound.

HR-MS calcd. for $C_{33}H_{51}N_{16}O_{16}S$ $[M-H]^-$: 959.3390; found: 959.3367.

### 3.4.11 Synthesis of fMTGNAD-SA (61)

Further synthesis was performed analogously to the preparation of **23**, affording 1.0 mg (4% total yield) of the title compound.

HR-MS calcd. for $C_{33}H_{50}N_{13}O_{17}S_2$ $[M+H]^+$: 964.2889; found: 964.2842.

### 3.4.12 Synthesis of fMRTGNAD-SA (45a)

Further synthesis was performed analogously to the preparation of **23**, affording 1.4 mg (14% total yield) of the title compound.

HR-MS calcd. for $C_{39}H_{60}N_{17}O_{18}S_2$ $[M-H]^-$: 1118.3822; found: 1118.3789.

### 3.4.13 Synthesis of fMRTGNAAG-SA (54)

Compound 5'-*O*-[*N*-[ glycyl]-sulfamoyl]adenosine (**43c**) was prepared analogously as for **43**.

$^1$H-NMR ($D_2O$): 3.70 (s, 2H, α-H), 4.41 – 4.50 (m, 4H, 3',4',5'), 4.69 – 4.73 (t, 1H, 2'-H, J = 4.6), 6.12 – 6.14 (d, 1H, 1'-H, J = 5.31), 8.15 (s, 1H, 2H), 8.34 (s, 1H, 8H).

$^{13}$C-NMR ($D_2O$): 42.72 (Gly- α-C), 68.11 (5'-C), 70.06 (3'-C), 74.06 (2'-C), 82.15 (4'-C), 87.35 (1'-C), 118.43 (5-C), 139.54 (8-C), 148.73 (4-C), 152.37 (2-C), 156.15 (6-C), 172.69 (Gly -C=O).

ESI-MS calcd. for $C_{12}H_{16}N_7O_7S$ $[M-H]^-$ : 402.37; found: 402.31.

The peptide fMRTGNAA-OH was synthesized on a Wang-resin using standard Fmoc-based solid phase peptide chemistry. The peptide was next cleaved from the resin using a mixture of TFA/$H_2O$/thioanisole (95/2.5/2.5 v/v). The obtained product was coupled to **43c,** again using the same conditions as used for **23**. The product was purified by RP-HPLC, affording 0.1 mg (0.5% total yield) of the title product **54**.
HR-MS calcd. for $C_{40}H_{65}N_{18}O_{17}S_2$ $[M+H]^+$: 1133.4116; found: 1133.4238.

### 3.4.14 Synthesis of fMRTGNAG-SA (62)

Further synthesis to obtain **62** was performed analogously to the preparation of **23**, affording 1.8 mg (8% total yield) of the title compound.
HR-MS calcd. for $C_{37}H_{58}N_{17}O_{16}S_2$ $[M-H]^-$: 1060.3689; found: 1060.3702.

### 3.4.15 Biological activity of truncated compounds

#### 3.4.15.1 Whole-cell activity determinations

Whole-cell activity screenings were performed as described in section 2.4.16.

#### 3.4.15.2 Aminoacylation experiments

To assess the degree of inhibition of the aminoacylation reaction, *in vitro* tests were performed using the relevant S30 cell extracts.

*Preparation of S30 cell extracts.* Cells were grown in 50 mL LB-medium. After centrifution at 3000 × g for 10 min the supernatant was discarded and the pellet was resuspended in 40 mL buffer containing: Tris.HCl or Hepes.KOH (pH = 8.0) (20 mM), $MgCl_2$ (10 mM), KCl (100 mM). The cellsuspension was centrifuged again at 3000 × g. This procedure was repeated twice. The pellet was resuspended in 1 mL of the following buffer Tris.HCl or Hepes.KOH (pH = 8.0) (20 mM), $MgCl_2$ (10 mM), KCl (100 mM), DTT (1 mM) and kept at 0 °C. Subsequently, the cells were sonicated for 10 s and left at 0 °C for 10 min. This procedure was repeated 5-8 times. The lysate was centrifuged at 15000 g for 30 min at +4 °C.

*tRNA aminoacylation reaction.* To 1 µL of solution containing inhibitor, 3 µL of *E. coli* S30 extracts was added. Next, 16 µL of the following aminoacylation mixture was added: Tris.HCl (30mM, pH 8.0), DTT (1 mM), bulk of *E. coli* tRNA (5 g/l), ATP (3 mM), KCl (30 mM), $MgCl_2$ (8 mM), and the specified, $^{14}$C-radiolabeled amino acid (40 µM). The reaction products were precipitated in cold 10% TCA on Whatman 3MM papers, 5 min after the aminoacylation mixture was added. The aminoacylation reaction was carried out at room temperature. Depending on whether or not processing was needed, variable time intervals were included between the addition of the cell extract and the addition of the aminoacylation mixture. After thorough washing with cold 10% TCA, the papers were washed twice with acetone and dried

on a heating plate. Following the addition of scintillation liquid (12 mL), the amount of radioactivity was determined with a Tri-card 2300 TR liquid scintillation counter. $^{14}$C-Radiolabeled amino acids (200 mCi/mmol) and scintillation liquid (Hisafe 2, Cat. No. 1200-436) were purchased from Perkin Elmer.

Because of the high costs and environmental issues, all experiments involving radiolabeled amino acids were performed only once for every compound at a given concentration. However, cross-confirmation (*i.e.* aminoacylation experiments for the same compounds at different concentrations) was performed multiple times. These assays have earlier been shown to be highly reproducible.

**Research Contributions:**

Bart Blanchaert assisted during the synthesis of some of the compounds, Svetlana Dubiley tested the effect of elongated peptides on the uptake and Teymur Kazakov tested some of the compounds for their ability to inhibit their respective aaRS. Kiril Datsenko constructed the *E. coli* tester strain Ara-Yej (BW39758) in the laboratory of B. L. Wanner, Purdue University, Indiana, USA.

# Chapter 4

## *N*-alkylated aminoacyl sulfamoyladenosines as potential inhibitors of the aminoacylation reaction and incorporation of D-amino acids in the peptide moieties of microcin C analogues

**Abstract:** *N*-methylated aaSAs have been synthesized to investigate their potential as aaRS inhibitors and to establish whether these *N*-alkylated analogues would escape the natural inactivation mechanism *via* acetylation of the alpha amine. It was shown however, that these compounds are not able to effectively inhibit their respective aaRSs. In addition we showed that (D)D-SA (*i.e.* Asp-SA with a D-configuration of the Asp), is a potent inhibitor of AspRS. However, we also showed that the inhibitory effect of (D)D-SA is relatively short-lasting. This was proven to be attributable to a large extent to acetylation by RimL, although it was shown that other unknown factors also play a role in the inactivation of this compound. McC analogues with D-amino acids at positions two to six proved inactive. They were shown to be resistant against metabolism by the different peptidases and therefore are not able to release the active moiety. This observation could not be reversed by incorporation of L-amino acids at position six, showing that none of the available peptidases does exhibit endopeptidase activity.

## 4.1 Introduction

As described in Chapter 1, several McC associated proteins are encoded on the plasmid also encoding the McC peptide. During the course of this PhD project a number of interesting biological findings were done, related to the self-immunity of McC-producing bacteria against the McC product itself. More specifically, the functions of MccE and MccF have been studied further by Novikova *et al*.[175] and Tikhonov *et a*l.[176], respectively.

In short, MccF is a serine protease that cleaves the *C*-terminal carboxamide bond of McC, D-SA and E-SA, but not other aminoacyl sulfamoyl adenosines [176]. In this way MccF is more specific than MccE which inactivates both processed McC and, with the exception of P-SA, all other tested aaSAs. MccE is an acetyltransferase acetylating the α-amine of the aminoacyl moiety. In response to these recent findings we set out to develop modified McC analogues that would be more resistant to MccE and/or MccF.

In this chapter, two approaches were examined to prevent potential resistance to compounds such as aaSAs and other aaRS inhibitors as isosters of the aminoacyl-adenylates, by acetylases and proteases such as MccE and MccF. We therefore focused on modifying the aminoacyl moiety so that it would become resistant against acetylation. Secondly, the use of D-amino acids in aaSAs was explored to examine if this would still be recognized by the corresponding aaRS and, subsequently, whether these could escape inactivation by MccE/MccF.

It is well known that during evolution, cells developed specialized mechanisms to prevent the incorporation of D-amino acids in their proteins and ribosomally synthesized peptides. However, several L-aminoacyl-tRNA synthetases can transfer D-amino acids onto tRNA. This mis-esterification can however be corrected by D-aminoacyl-tRNA deacylases (DTD), which hydrolyze the ester bond [177]. Eukaryotes generally contain DTD1, while plants have DTD2 homologues [177]. Some bacteria, including most cyanobacteria lack genes encoding DTD1 homologues. It has also been reported that the editing site of ThrRS functions as a deacylase, removing non-cognate D-Thr [178]. In addition several racemases, or in case of D-Glu a transaminase, can convert D-amino acids into L-amino acids. Alternatively, D-Ala, which is used for the formation of peptidoglycan in the bacterial cell wall, results from L-Ala by conversion *via* a racemase. Despite the action of these corrective mechanisms, significant amounts of D-aminoacylated tRNA have been observed *in vitro* for D-Trp-tRNA$^{Trp}$, D-Asp-tRNA$^{Asp}$ [179], and D-Tyr-tRNA$^{Tyr}$ [180, 181].

The introduction of D-amino acids in the peptide chain of McC, at other positions than the *C*-terminal position, is also from a pharmaceutical perspective interesting as it has been shown that the plasma half-life of peptides can be considerably increased through the introduction of D-amino acids [182]. Provided the bacterial cells can still release the active principle, this

increase in plasma half-live would render the McC analogues much more interesting from therapeutic perspective.

## 4.2. Results

### 4.2.1. Design and synthesis

As mentioned before, P-SA could escape both immunity mechanisms of MccF and MccE. Therefore a McC analogue with proline at position seven would seem desirable. However, such compounds could not be synthesized, due to instablilty of such compounds. This problem was also encountered earlier by Van de Vijver *et al.*[183] when studying dipeptidyl-sulfamoyl adenosines containing proline at the *C*-terminal position.

Nevertheless, the fact that P-SA was stable against MccE, suggests that the presence of a secondary amine is sufficient to circumvent acetylation by this enzyme. Hence, we hypothesized that *N*-methylated aaSAs could also be resistant against acetylation by MccE. To further assess this, 5'-*O*-[*N*-[L-*N*-methyl-leucyl]-sulfamoyl]adenosine (**75**) and 5'-*O*-[*N*-[sarcosyl]-sulfamoyl]adenosine (**76**) were synthesized and tested for their inhibitory properties. Compound **75** was selected for its straightforward synthesis, while compound **76** was selected for its availability and its size. We hypothesized that the latter might be active against either GlyRS, ProRS and/or AlaRS.

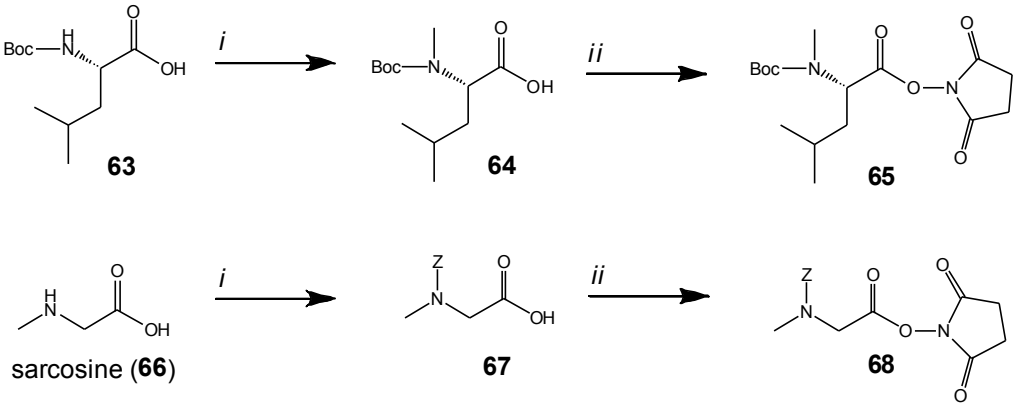
Furthermore, McC analogues comprising D-amino acids were synthesized to investigate if these would show activity and at the same time be resistant against the action of MccE and MccF. In addition these compounds allowed us to assess the ability of the different peptidases to metabolize D-aminoacylated McC analogues.

For the preparation of **75**, compound **65** was synthesized starting from Boc-Leu-OH (**63**), followed by methylation according to Cheung *et al.*[184] (Scheme 4.1). Further synthesis to obtain the *N*-methylated Leu-SA (**75)** was carried out using similar methods as described in the previous two chapters for the other aminoacyl sulfamoyladenosines (Scheme 4.2). Likewise, sarcosyl-SA (**76**) was prepared starting from sarcosine, which was protected with a Cbz group and *C*-terminally activated with succinimide.

A number of McC analogues was created containing either D-amino acids in the peptide tail (**83**-**86**) or as the *C*-terminal amino acid (**87**). Compounds **83**-**86** were created to investigate the ability of the peptidases to metabolize these compounds and to evaluate whether the YejABEF transporter would be able to recognize and transport these compounds. Compounds **77b** ((D)D-SA) and **87** (McC analogue with D-Asp at position 7) were synthesized for the same reasons as mentioned before. In addition we were interested to investigate if compounds **77b** and **87**, having a D-configuration, would still inhibit the

aminoacylation reaction and the potential to escape hydrolysis/acetylation by MccF and MccE, respectively. To rule out the possibility of racemisation during the synthesis of these compounds, the optical rotation of compounds **43h** and **77b** was measured by a Perkin Elmer polarimeter with a Na lamp at 589 nm. For compound **43h**, the optical rotation was determined to be -0.010 °, while for compound **77b** this was +0.007 °.

The synthesis of the McC analogues carrying D-amino acids was (obviously) carried out analogously to methods described in the previous two chapters.

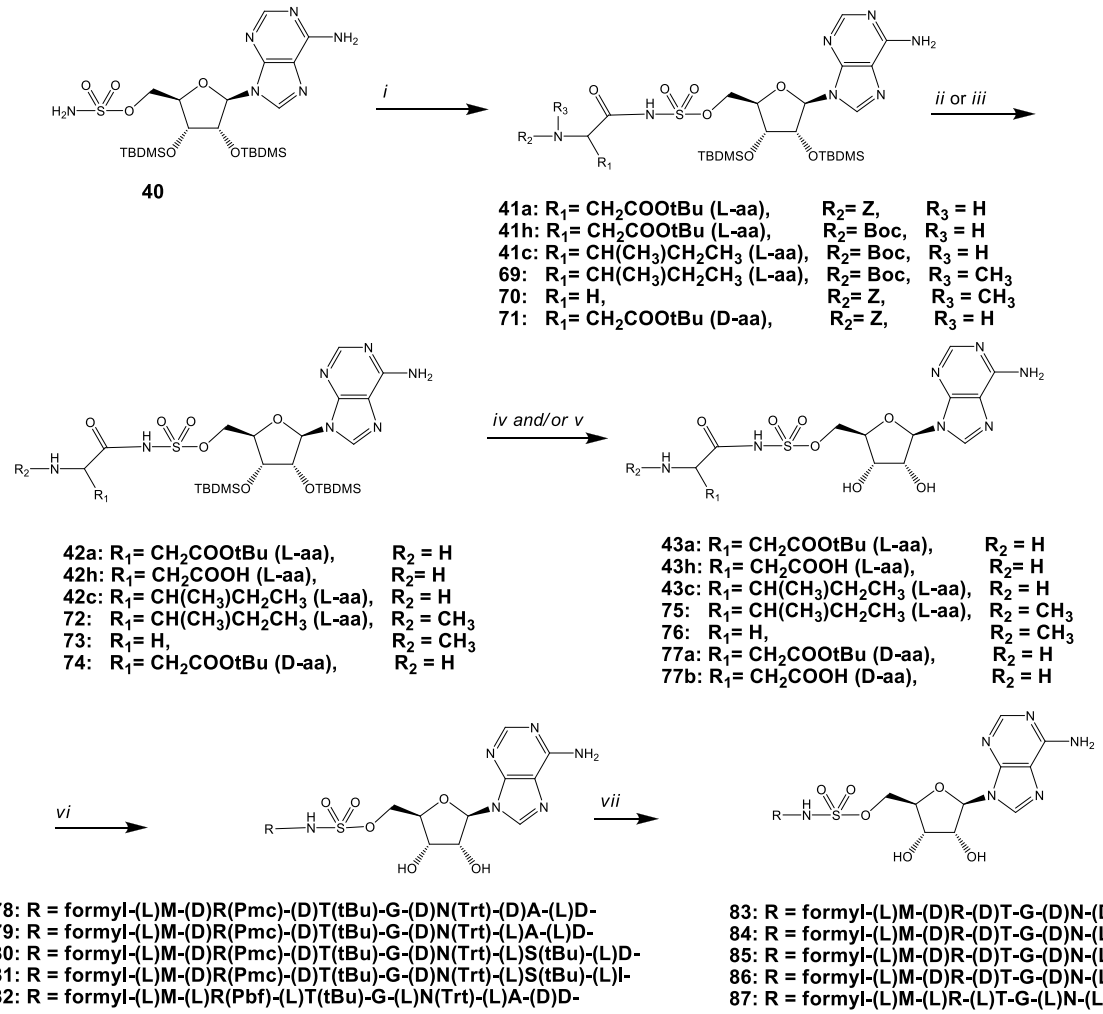



**Scheme 4.1**.
Upper part: Synthesis of *N*-methylated and succinimide-activated Boc-Leu-OH. *i)* NaH, MeI in THF, 0 °C, 30 min. *ii)* EDCI·HCl, HOSu, DIPEA in DMF, rt, 16 h. Lower part: Synthesis of Cbz-protected and succinimide activated sarcosine. *i*) benzyloxycarbonyloxy succinimide, $NaHCO_3$ in $H_2O$/dioxane, 0 °C to rt, 7 h. *ii*) HOSu and EDCI·HCl in DMF, rt, 16 h.

### 4.2.2. Biological activity

The antibacterial activities of the various McC-analogues (**83-87**) were determined by methods described earlier in section 2.2.2. In addition, to further confirm the Trojan horse mode of action, cells in which either the *yej* gene or the genes for peptidases A, B and N had been disrupted, were screened for activity. Compound **45a** served as a positive control.

The *N*-methylated aaSAs (**75** and **76)** proved inactive in this whole-cell activity assay (data not shown). In *in vitro* aminoacylation experiments compound **76** was tested for its potential to inhibit ProRS, AlaRS and GlyRS and compound **75** was tested against IleRS in a wild type extract. Unfortunately, no activity was observed at concentrations of ≤ 50 µM (data not shown).




**40**

**41a:** $R_1$= $CH_2COOtBu$ (L-aa), $R_2$= Z, $R_3$ = H
**41h:** $R_1$= $CH_2COOtBu$ (L-aa), $R_2$= Boc, $R_3$ = H
**41c:** $R_1$= $CH(CH_3)CH_2CH_3$ (L-aa), $R_2$= Boc, $R_3$ = H
**69:** $R_1$= $CH(CH_3)CH_2CH_3$ (L-aa), $R_2$= Boc, $R_3$ = $CH_3$
**70:** $R_1$= H, $R_2$= Z, $R_3$ = $CH_3$
**71:** $R_1$= $CH_2COOtBu$ (D-aa), $R_2$= Z, $R_3$ = H

**42a:** $R_1$= $CH_2COOtBu$ (L-aa), $R_2$ = H
**42h:** $R_1$= $CH_2COOH$ (L-aa), $R_2$= H
**42c:** $R_1$= $CH(CH_3)CH_2CH_3$ (L-aa), $R_2$ = H
**72:** $R_1$= $CH(CH_3)CH_2CH_3$ (L-aa), $R_2$ = $CH_3$
**73:** $R_1$= H, $R_2$ = $CH_3$
**74:** $R_1$= $CH_2COOtBu$ (D-aa), $R_2$ = H

**43a:** $R_1$= $CH_2COOtBu$ (L-aa), $R_2$ = H
**43h:** $R_1$= $CH_2COOH$ (L-aa), $R_2$= H
**43c:** $R_1$= $CH(CH_3)CH_2CH_3$ (L-aa), $R_2$ = H
**75:** $R_1$= $CH(CH_3)CH_2CH_3$ (L-aa), $R_2$ = $CH_3$
**76:** $R_1$= H, $R_2$ = $CH_3$
**77a:** $R_1$= $CH_2COOtBu$ (D-aa), $R_2$ = H
**77b:** $R_1$= $CH_2COOH$ (D-aa), $R_2$ = H

**78:** R = formyl-(L)M-(D)R(Pmc)-(D)T(tBu)-G-(D)N(Trt)-(D)A-(L)D-
**79:** R = formyl-(L)M-(D)R(Pmc)-(D)T(tBu)-G-(D)N(Trt)-(L)A-(L)D-
**80:** R = formyl-(L)M-(D)R(Pmc)-(D)T(tBu)-G-(D)N(Trt)-(L)S(tBu)-(L)D-
**81:** R = formyl-(L)M-(D)R(Pmc)-(D)T(tBu)-G-(D)N(Trt)-(L)S(tBu)-(L)I-
**82:** R = formyl-(L)M-(L)R(Pbf)-(L)T(tBu)-G-(L)N(Trt)-(L)A-(D)D-

**83:** R = formyl-(L)M-(D)R-(D)T-G-(D)N-(D)A-(L)D-
**84:** R = formyl-(L)M-(D)R-(D)T-G-(D)N-(L)A-(L)D-
**85:** R = formyl-(L)M-(D)R-(D)T-G-(D)N-(L)S-(L)D-
**86:** R = formyl-(L)M-(D)R-(D)T-G-(D)N-(L)S-(L)I-
**87:** R = formyl-(L)M-(L)R-(L)T-G-(L)N-(L)A-(D)D-

**Scheme 4.2.** General scheme affording the various McC analogues. *i*) *N*-α-Cbz-L-aminoacyl-(*t*Bu or Boc)-succinimide, DBU in DMF, 6h, rt. *ii*) for $R_2$ = Z-group, $H_2$, Pd/C in MeOH, 3h, rt. *iii*) for $R_2$ = Boc-group of TFA/$H_2O$ (5:2), 4h, 0 °C to rt. *iv*) $Et_3N.3HF$ in THF, 16h, rt. *v*) protected peptide (1eq.), HOBt (4eq.), DIC (4eq.) and DIEA (2eq.) in DMF, 16h, rt. *vi*) TFA/thioanisole/$H_2O$ (90/2.5/7.5), 2h, rt.

Of the newly synthesized McC analogues (**83-87**), only compound **87** showed significant activity in whole-cell activity screenings. To further corroborate these results, and to explain the absence of activity, *in vitro* studies were performed where all compounds were tested in an aminoacylation experiment using two cell extracts prepared from either Δ*rimL E. coli* cells or from the Δ*pepABN* mutant *E. coli* strain. From Figure 4.1 it can be observed that also here, only compound **87** was active in extracts deficient of RimL, but not in extracts lacking PepA, PepB and PepN, confirming the requirement of these three peptidases to release the active moiety. However, none of the other McC analogues, containing D-amino acids at positions two to six, showed clear inhibition of AspRS or IleRS, relative to compounds **43h** and **77b** which do not require metabolism.

To investigate if compound **87** would be more resistant than compound **45a** against hydrolysis/acetylation and to investigate a possible role of DTD, a time-dependent

experiment was carried out, using two different cell extracts, in which either RimL or DTD was lacking (K. Severinov, personal communications). From Figure 4.1 (lower panel) it can be observed that at timepoint zero, the compounds were (obviously) not metabolized yet and therefore could not inhibit AspRS. After 15 min, both compounds were metabolized and almost fully inhibited AspRS, again confirming their mode of action as previously observed for McC. However, 30 min after starting the experiment, and more clearly after 60 min, both compounds showed less effective inhibition of AspRS. For compound **87** this effect is more profound than for compound **45a**, especially in the *E. coli* cell extract lacking DTD. After 60 min, esterification of Asp-$C^{14}$ to $tRNA^{Asp}$ was reduced to only 44% (compared to 89% inhibition at 15 min). In case RimL was absent, both compounds largely remained inhibitory.
In case DTD was absent, compound **87** could still reach full activity, comparable to the (L)D-SA analogue, suggesting that DTD does not rescue AspRS from inhibition by (D)D-SA. This is in agreement with the observation of Soutourina *et al.* [179] and others, that deacylases hydrolyze the ester bond of aminoacylated tRNA, rather than preventing esterification of D-amino acids to tRNA. One also can notice in Figure 4.1 (lower panel) that compound **87** suffers more from inactivation than compound **45a** in extracts lacking RimL.
Compound **87** also showed whole-cell activity against L-arabinose induced Ara-Yej cells, albeit to a lower extent than the L-analogue (Figure 4.2). None of the compounds **87** or **45a** showed activity against Δ*yej* or Δ*pepABN* cells (data not shown), confirming their Trojan horse mode of action.

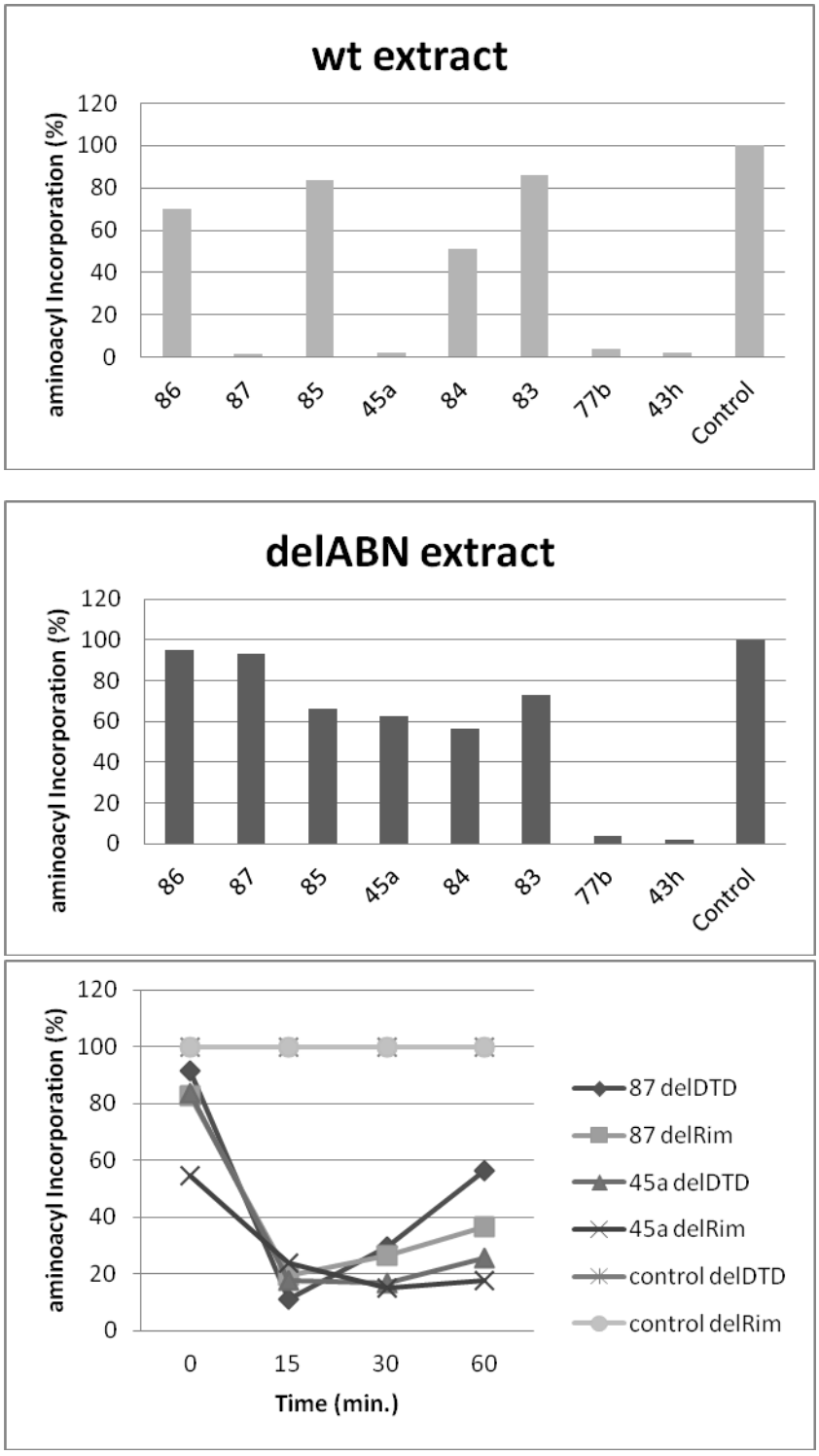



**Figure 4.1.** Aminoacylation experiments of the McC analogues containing D-amino acids at 50 µM.
Upper panel: 30 min incubation with an cell extract of *E. coli ΔrimL.*
Middle panel: 30 min incubation with an cell extract of *E. coli ΔpepABN.*
Lower panel: Comparison of two McC analogues targeting AspRS, compound **87** having a *C*-terminal D-configuration, compound **45a** having a L-configuration. Incubation was done with two different cell extracts, lacking either DTD or RimL.
The amount of aminoacylated $tRNA^{Asp}$ was measured by liquid scintillation counting.

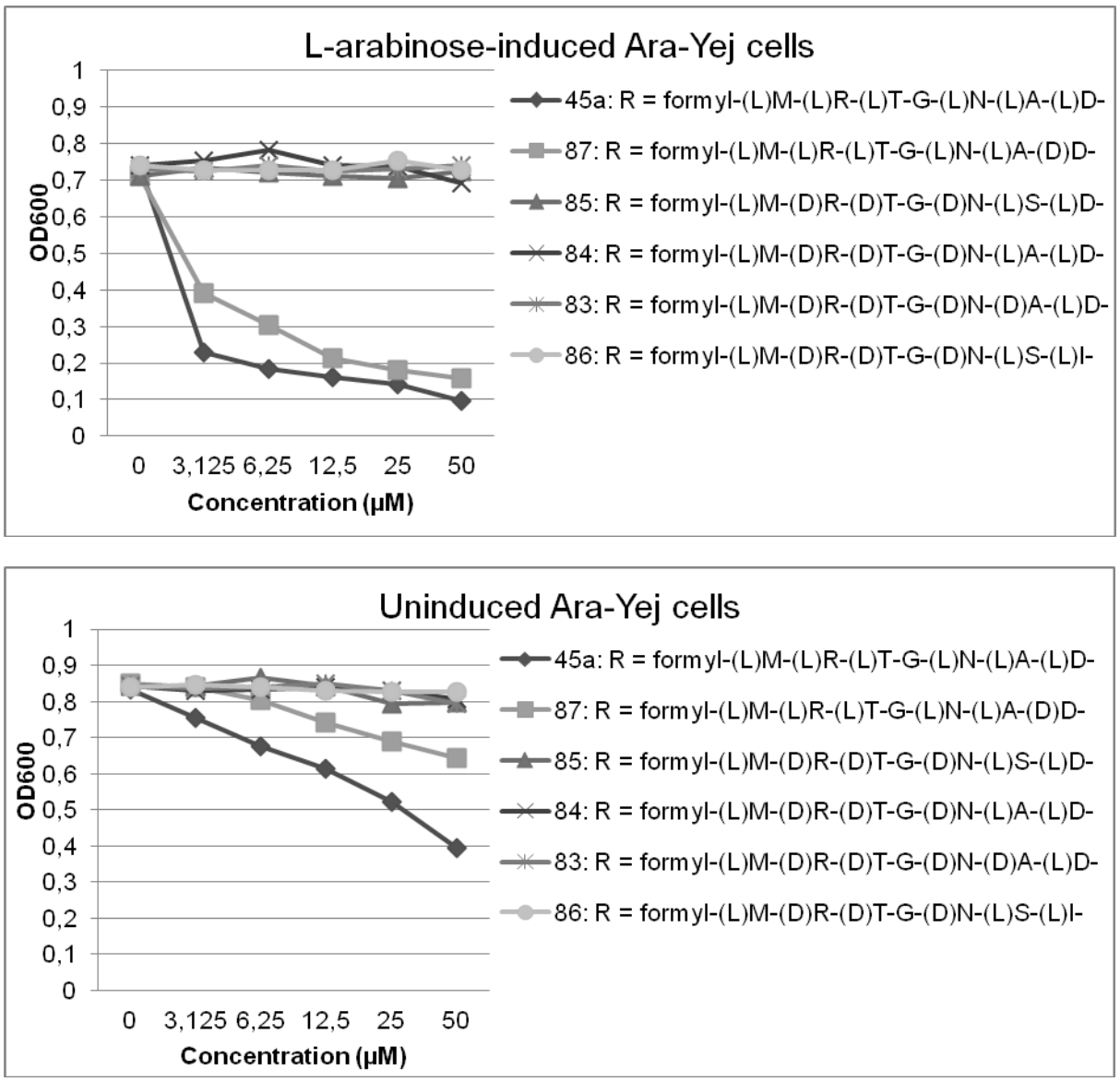



**Figure 4.2.** Inhibition profiles of the different McC analogues against L-arabinose-induced Ara-Yej cells (upper panel) and against uninduced Ara-Yej cells. OD600 refers to the optical densitiy of the cell culture measured at 600 nm.

## 4.3 Discussion

From the *in vitro* aminoacylation experiments with compounds **75** and **76** it can be seen that these compounds lack inhibitory activity against their hypothesized targets. Although with proline, ProRS allows for a secondary amine within the binding site of the enzyme, Sar-SA (**75**) is not tolerated and does not show any major inhibitory binding affinity for this enzyme. In addition, GlyRS could not be targeted with this compound, showing that, although SarSA does not have a side chain in analogy with Gly-SA, this compound can likewise not establish sufficient binding within the active site of this enzyme. It thus can be concluded that the *N*-

methyl group prevents inhibitory activity against GlyRS, or alternatively provokes active removal out of the active site. Likewise, *N*-methylated Leu-SA (**76**) was found to be inactive against LeuRS. Again, it must be concluded that the *Nα*-methyl group in this compound also prevents inhibitory activity. This then suggests that the *Nα*-amine is an important recognition point inside the active site of the enzyme which cannot be modified. In addition, since SarSA was not able to inhibit the aminoacylation reaction as catalyzed by ProRS it can be concluded that having a small secondary alpha amine is not sufficient for recognition by ProRS, but that the five-membered cyclic amine or at least a larger structure than the *N*-methylated glycine is required for recognition.

Compound **83** did not show whole-cell activity either, and was also shown incapable of inhibiting AspRS *in vitro*. Since, the *N*-terminal amino acid is *N*-formyl-(L)-methionine, whereas amino acids at positions two to six are D-amino acids, it must be concluded that the peptidases are not capable of hydrolyzing D-amino acids and can therefore not release the active moiety. This is a somewhat expected result as it has frequently been shown that the introduction of D-amino acids can prevent hydrolysis by peptidases [185].

Compounds **84**-**86** were especially designed to circumvent this problem; all three compounds contain L-amino acids at the sixth and seventh position, allowing endopeptidases to cleave between these two L-amino acids, resulting in release of the active moiety. Despite this modification, no activity could be observed. Also the modification in compound **85**, inspired by albomycin whereby PepN hydrolyzes the peptide bond between a serine and a modified amino acid carrying an acidic side chain [149], could not rescue the activity. From the *in vitro* aminoacylation experiments it can also be concluded that lack of whole-cell activity is due to inability of the peptidases to metabolize peptides containing D-amino acids. Hence, this shows that the peptidases PepA, PepB and PepN, commonly known to be responsible for processing of McC and its analogues [186], only can cleave these compounds as exopeptidases (*i.e.* sequentially) and are not able to release the active moiety in an endopeptidase manner (*i.e.* convergently).

The observation that (D)D-SA (**77b**) and its McC derivative **87** can inhibit AspRS, shows that the peptidases can metabolize peptide bonds between two amino acids, whereby the *C*-terminal amino acid has the D-configuration (as in this case a D-Asp). This suggests that the peptidases involved in this reaction are only stereoselective for the *N*-terminal amino acid.

Since it was already frequently observed that D-Asp can be esterified to tRNA, the finding that (D)D-SA (**77b**) can also inhibit AspRS can be considered being an expected result. This then also shows that the absolute configuration of the amino acid is not required for recognition inside the active site of AspRS, and most probably also for other aaRSs.

Compared to compound **45a**, compound **87** was more sensitive to inactivation by a mechanism that was not solely related to RimL. This can probably be ascribed to the intrinsic

capacity of AspRS to remove (D)D-SA from the active site. It is however unlikely that the editing site of AspRS is involved in this mechanism as it has often been shown to hydrolyze only non-cognate amino acids from tRNA. The inactivation of (D)D-SA is also reflected in a lower whole-cell activity for this compound, compared to its L-analogue (**45a**) (Figure 4.2).

In conclusion, four important observations were made in this study. First, we showed that *Nα*-methylation of GlySA and LeuSA does not result in inhibition of the respective aaRS and is therefore probably not well tolerated in other aaRSs as well. This method can therefore also not be considered as a prevention measure against acetylases that usually inactivate most aaSAs. Secondly, we showed that if amino acids at positions two to six have a D-configuration, this abolishes hydrolysis by the peptidases, thus preventing release of the active moiety and inhibition of the respective aaRS. In addition, it was shown that the peptidases primarily function as exopeptidases, as we did not observe significant inhibition of AspRS or IleRS with McC analogues containing an L-amino acid at the pre-*C*-terminal (or sixth) position. Third, if the *C*-terminal aspartic acid has a D-configuration and amino acids at positions one to six are in L-configuration, the active moiety could easily be released, resulting in inhibition of AspRS. This shows that metabolism is independent of the configuration of the *C*-terminal amino acid and only depends on the configuration of the *N*-terminal amino acid. Fourth, we have shown that (D)D-SA is perfectly capable of inhibiting AspRS. However, this proved a relatively short-lasting inhibition as over time the compound is inactivated by (predominantly) RimL. A less surprising finding, and therefore of lesser importance, is that DTD is not involved in the inactivation of (D)D-SA.

## 4.4. Materials and methods

**4.4.1** Materials are as described in section 3.4.1.

### 4.4.2 Synthesis of 5'-*O*-[*N*-[L-*N*-methyl-leucyl]-sulfamoyl] adenosine (75)

Boc-Leu-OH (2.3 g, 10 mmol) and NaH (1.3 g, 30 mmol, 3 eq.) were dissolved in THF (30 mL) at 0 °C and stirred for 30 min. Subsequently, MeI (5 mL, 80 mmol, 8 eq.) was added and the solution was stirred for another 16 h at rt. Next, EtOAc (50 mL) was added and $H_2O$ (30 mL) was added dropwise. The reaction mixture was subsequently partially evaporated and washed with ether (30 mL). The water layer was acidified to pH 3 using citric acid (5%) and subsequently extracted using EtOAc. The organic layer was then washed with 5% $Na_2S_2O_3$ (2 times 50 mL) and with brine. The organic layer was dried over $Na_2SO_4$, filtered and evaporated to yield **64**.

The crude product **64** (2.1 g, 8.6 mmol) was carefully dried in vacuo and dissolved in DMF (10 mL). Next, HOSu (986 mg, 1 eq.) and EDCI·HCl (1.64 g, 1 eq.) and DIPEA (0.75 mL, 1 eq.) were added. The reaction mixture was stirred for 16 h, after which the DMF was evaporated. The residue was taken up in EtOAc (50 mL) and subsequently washed with 0.5 N HCl (two times 30 mL), saturated $NaHCO_3$ (two times 30 mL) and once with brine (30 mL). The organic layer was dried over $Na_2SO_4$, filtered and evaporated, yielding 1.9 g (97%) of the desired product **65**.

The obtained product (1.9 g, 5.6 mmol) was carefully dried and dissolved in DMF (20 mL). To this solution, 5'-*O*-sulfamoyl-2',3'-di-*O*-(*tert*-butyldimethylsilyl)-adenosine (**40**) (3.5 g, 5.7 mmol, 1.1 eq.) and DBU (0.83 mL, 5.6 mmol, 1 eq.) were added and the reaction mixture was stirred for 6 h at rt. DMF was evaporated under reduced pressure. Next, the residue was purified by flash chromatography ($CH_2Cl_2$, MeOH 2.5 to 10%). Fractions containing the desired product were evaporated giving yellow oil. Yield: 1.2 g (53%). The compound was next treated with TFA/$H_2O$ (5/2 v/v) for 5 h at rt, after which the volatiles were evaporated. Next, the product was carefully dried and dissolved in a mixture of THF (15 mL) and $Et_3N.3HF$ (1 mL). After 3 h, another 0.8 mL of $Et_3N.3HF$ was added and the reaction mixture was stirred further for 22 h. The reaction mixture was evaporated and the residue was purified by flash chromatography ($CH_2Cl_2$, 5 to 20% MeOH). Fractions containing the desired product **75** were evaporated. Yield: 715 mg (84%)

$^1$H-NMR ($D_2O$): 0.78 – 0.86 (dd, 6H, $\delta_A$ and $\delta_B$ -Leu), 1.54 – 1.69 (m, 4H, β and γ- Leu), 2.67 (s, 3H, N-methyl), 3.56 – 3.61 (t, 1H, α-Leu, J = 7.5), 4.40 – 4.52 (m, 4H, 3',4',5'), 4.70 – 4.73 (m, 1H, 2'-H), 6.07 – 6.09 (d, 1H, 1'-H, J = 5.43), 8.17 (s, 1H, 2H), 8.36 (s, 1H, 8H), 8.46 (s, 2H, NH)

$^{13}$C-NMR ($D_2O$): 20.79 and 21.46 ($\delta_A$ and $\delta_B$ Leu), 23.82 (γ-Leu), 31.29 (N-methyl), 38.95 (β-Leu), 62.72 (5'-C), 68.22 (α-Leu), 69.84 (3'-C), 73.70 (2'-C), 81.98 (4'-C), 86.86 (1'-C), 118.14 (5-C), 139.39 (8-C), 148.54 (4-C), 152.29 (2-C), 154.95 (6-C), 174.63 (Leu -C=O).
ESI-MS calcd. for $C_{17}H_{27}N_7O_7S$ ($[M+H]^+$): 474.1; found: 474.0.

### 4.4.3 Synthesis 5'-*O*-[*N*-[sarcosyl]-sulfamoyl] adenosine (76)

Sarcosine (2.8 g, 31.4 mmol), benzyloxycarbonyloxy succinimide (7.83 g, 31.4 mmol, 1 eq.), $NaHCO_3$ (5.3 g, 62.4 mmol, 2 eq.) were dissolved in $H_2O$/dioxane (1/1, 40 mL) at 0 °C, allowing the reaction mixture to reach rt in 1 h, after which it was stirred for another 16 h. Subsequently, another 20 mL of $H_2O$ was added and the product was extracted with EtOAc. The organic layer was next extracted with aqueous saturated $NaHCO_3$ (2 times 50 mL). The water layer was then acidified to pH 1 with HCl (10%) and again extracted using EtOAc. The organic layers were combined, dried over $Na_2SO_4$, filtered and evaporated, yielding 4.7 g (68%) of the desired product (**67**).
ESI-MS calcd. for $C_{11}H_{13}N_1O_4$ ($[M-H]^-$): 222.1; found: 221.6.

The obtained product in the above step (4.7 g, 21.1 mmol) was next reacted with HOSu (2.9 g, 25.1 mmol, 1.2 eq.) and EDCI·HCl (5.23 g, 26.2 mmol, 1.3 eq.) in DMF (25 mL) for 16 h at RT. The reaction mixture was evaporated and taken up in EtOAc (200 mL), washed twice with HCl (1N, 100 mL), twice with $NaHCO_{3\ (sat.)}$ (2 x 100 mL) and once with brine (100 mL). Yield: 5.8 g (86%).
Next the succinimide activated sarcosine (1.3 g, 4.06 mmol) was reacted with 5'-*O*-sulfamoyl-2',3'-di-*O*-(*tert*-butyldimethylsilyl)-adenosine (**40**) (2.33 g, 4.06 mmol, 1 eq.) and DBU (607 µl, 4.06 mmol, 1.0 eq.) in DMF (10 mL) and the reaction mixture was stirred at rt for 8h under nitrogen atmosphere. DMF was evaporated under reduced pressure. Next, the residue was purified by flash chromatography ($CH_2Cl_2$, MeOH 2.5 to 10%). Fractions containing the desired product were evaporated giving yellow oil (**70**). Yield: 1.2 g (39%).
ESI-MS calcd. for $C_{33}H_{54}N_7O_9SSi_2$ ($[M+H]^+$): 780.32; found: 779.90.

The obtained product (610 mg, 0.78 mmol) was dissolved in MeOH at 0 ºC and Pd/C (50 mg) was added. The mixture was stirred under hydrogen atmosphere for 3 h. The mixture was filtered and evaporated yielding a white powder. The product was next carefully dried and dissolved in a mixture of THF (5 mL) and $Et_3N.3HF$ (0.3 mL). After 3 h, another 0.2 mL of $Et_3N.3HF$ was added and the mixture was stirred for another 22 h. The reaction mixture was evaporated and the residue was purified by flash chromatography ($CH_2Cl_2$, 5 to 20% MeOH). Fractions containing the desired product were evaporated, yielding **76**: 270 mg (82%). The product was further purified by means of RP-HPLC for analytical purposes.

$^{1}$H-NMR ($D_2O$): 2.67 (s, 3H, N-methyl), 3.65-3.71 (dd, α-$CH_2$, J=2.82 and J=35.76), 4.33-4.39 (m, 3H, 4', 5'), 4.45-4.46 (t, 1H, 3'H, J=4.8), 4.68-4.69 (t, 1H, 2'H, J=5.16), 6.06-6.07 (d, 1H, 1', J=5.16), 8.16 (s, 1H, 2H), 8.33 (s, 1H, 8H).
$^{13}$C-NMR ($D_2O$): 32.46 (N-methyl), 51.72 (α-$CH_2$), 68.17 (5'-C), 69.97 (3'-C), 73.86 (2'-C), 82.08 (4'-C), 87.21 (1'-C), 118.46 (5-C), 139.53 (8-C), 148.77 (4-C), 152.71 (2-C), 155.40 (6-C), 171.80 (Sar- C=O).
HR-MS calcd. for $C_{13}H_{20}N_7O_7S$ ($[M+H]^+$): 418.1145; found: 418.1133.

### 4.4.4 Synthesis of 5'-*O*-[*N*-[D-aspartyl(*O*-*tert*-butyl)]-sulfamoyl]adenosine (77a)

Synthesis of compound **77a** was performed identically to compound **43a**.

ESI-MS calcd. for $C_{38}H_{60}N_7O_{11}SSi_2$ ($[M-H]^-$): 878.4; found: 878.4.

### 4.4.5 Synthesis of 5'-*O*-[*N*-[D-aspartyl)]-sulfamoyl]adenosine (77b)

Synthesis of compound **77b** was performed identically to compound **43a**.

HR-MS calcd. for $C_{14}H_{19}N_7O_9S$ ($[M+H]^+$): 462.1043; found: 462.1514.

### 4.4.6 Synthesis of formyl-(L)M-(D)R-(D)T-G-(D)N-(D)A-(L)D-SA (83)

Synthesis of compound **83** was performed identically to compound **45a**.

HR-MS calcd. for $C_{39}H_{62}N_{17}O_{18}S_2$ ($[M+H]^+$): 1120.3900; found: 1120.3895.

### 4.4.7 Synthesis of formyl-(L)M-(D)R-(D)T-G-(D)N-(L)A-(L)D-SA (84)

Synthesis of compound **84** was performed identically to compound **45a**.

HR-MS calcd. for $C_{39}H_{60}N_{17}O_{18}S_2$ ($[M-H]^+$): 1118.3744; found: 1118.3727.

### 4.4.8 Synthesis of formyl-(L)M-(L)R-(L)T-G-(L)N-(L)S-(L)D-SA (85)

Synthesis of compound **85** was performed identically to compound **45a**.

HR-MS calcd. for $C_{39}H_{60}N_{17}O_{18}S_2$ ($[M-2H]^{2-}/2$): 566.6808; found: 566.6808.

### 4.4.9 Synthesis of formyl-(L)M-(D)R-(D)T-G-(D)N-(L)S-(L)I-SA (86)

Synthesis of compound **86** was performed identically to compound **45a**.

HR-MS calcd. for $C_{41}H_{68}N_{17}O_{17}S_2$ ($[M+H]^+$): 1134.4264; found: 1134.4231.

### 4.4.10 Synthesis of formyl-(L)M-(L)R-(L)T-G-(L)N-(L)A-(D)D-SA (87)

Synthesis of compound **87** was performed identically to compound **45a**.

HR-MS calcd. for $C_{39}H_{60}N_{17}O_{18}S_2$ ($[M-H]^-$): 1118.3744; found: 1118.3722.

### 4.4.11 Whole-cell activity determinations

Whole-cell activity screenings were perfomed as described in section 2.4.16.

Bacterial strains used for the evaluations: *E. coli* Ara-Yej (BW39758), expressing the *yejABEF* transporter upon L-arabinose induction; *E. coli* K-12 (BW28357), used as the wild type control; *E. coli ΔyejA*, lacking subunit A of the YejABEF transporter; and *E. coli ΔpepABN*, lacking all three peptidases PepA, PepB and PepN; *E. coli Δdtd*, lacking D-aminoacyl-tRNA deacylase.

### 4.4.12 Aminoacylation experiments

*In vitro* aminoacylation experiments were performed as described in section 3.4.15.2.

**Research Contributions:**

Prof. Dr. Jef Rozenski recorded the mass spectra.

# Chapter 5

## Microcin C and albomycin analogues with aryl-tetrazole substituents as nucleobase isosters are selective inhibitors of bacterial aminoacyl tRNA synthetases but lack efficient uptake

**Abstract:** In 1998 Cubist Pharmaceuticals patented a series of aminoacyl tRNA synthetase (aaRS) inhibitors, based on aaSAs where the adenine was substituted by aryltetrazole moieties linked to the ribose fragment *via* a two-carbon spacer. Although being strong inhibitors of IleRS, these compounds proved not succesful *in vivo*, due to low cell permeability and binding to serum albumin. In this work we attempted to improve these compounds by converting them into microcin C or albomycin analogues (*i.e*. Siderophore Drug Conjugates (SDCs)). Although all compounds, both in McC as in SDC form, showed nice potential when evaluated in *in vitro* aminoacylation experiments against *E. coli* cell extracts, the whole-cell activity however was virtually abolished. Only the whole-cell activity of the originally published compound (CB432) against *S. aureus* could be confirmed. It was shown that lack of activity in whole-cell assays was due to inability of these compounds to pass the cell membrane. Nevertheless, it was likewise shown that the synthesized SDCs could readily be metabolized by the peptidases PepA, PepB and PepN, a feature not observed for most other published synthetic SDCs and which could be of future use.

## 5.1 Introduction

A US patent by Cubist Pharmaceuticals® [187] reported on the synthesis and evaluation of a new type of aaRS inhibitors. Although still based upon aminoacyl-sulfamoyl-adenosine, these analogues contained an aryl-tetrazole moiety, which substituted the adenine part, and was connected through a two-carbon linker to ribose [188]. The tetrazole moiety is linked to one or two five- or six-membered heterocycles. The advantage of these compounds is the high selectivity they display against bacterial aaRSs relative to the mammalian homologues, an aspect which is not covered by the conventional aaSAs. This company synthesized many derivatives, all having the two-carbon linked aryl-tetrazole moiety in common. The most important compounds are listed in Table 5.1. These analogues exhibit good activity against *E. coli* IleRS and poor activity against the human homologue, and especially with CB168 a profound difference is seen. However CB432 was the only compound that showed moderate activity against a broad range of bacteria in whole-cell experiments against *S. aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus pyogenes, Enterococcus faecium, Enterococcus faecalis, Bacillus subtilis, Haemophilus influenzae, Klebsiella pneumoniae, Moraxella catarrhalis* and *Streptococcus pneumoniae* with MIC values ranging between 2 and 100 µg/mL) [189]. In addition, this compound showed activity in the treatment of *Streptococcus pyogenes* infected mice [189].

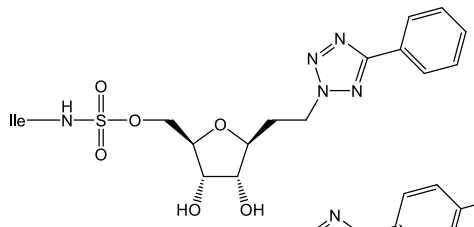
However, not long after the release of this patent the authors published that these compounds could not be further pursued as potential antibiotics, due to high affinity to serum albumin [189]. To the best of our knowledge, Cubist Pharmaceuticals® ceased all research efforts concerning this particular project following this finding.

In line with the results of Cubist Pharmaceuticals® we chose two different lead compounds, that would be modified to obtain analogues of these compounds that either contained the McC (or an analoguous) uptake-promoting peptide or the hydroxamate-based siderophore moiety as found in albomycin. CB168 was chosen for its great potential as selective inhibitor, due to its exhibited low inhibition of human IleRS. CB432 was further modified because of its broad range specificity and low inhibition of human IleRS. Furthermore, CB432 also showed moderate *in vivo* efficacy. Hereby, we aimed at developing a model compound that would retain activity or preferably would show increased activity, enhanced selectivity and decreased albumin binding.

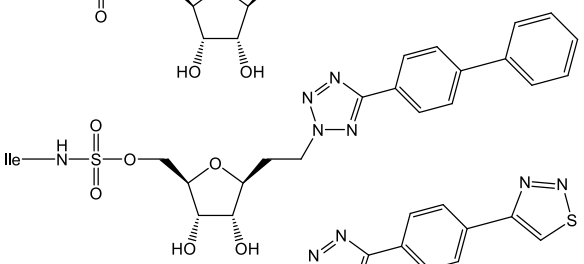
**Table 5.1 $IC_{50}$ values (in nM) of Cubist Pharmaceutical's compounds on IleRSs isolated from different organisms**

| Entry | *S. aureus* | *E. faecalis* | *E. coli* | Human |
|---|---|---|---|---|
| Ile-SA | 2.9 | 2 | 2 | 20 |
| CB 432 (**7**) | 0.5 | 9 | 3.2 | 450 |
| CB 168 (**88**) | 5 | 20 | 1.3 | 3000 |
| CB 286 (**89**) | 4 | 10 | 1.3 | 524 |
| CB 360 (**90**) | 4 | 4.4 | 12 | 190 |
| CB 447 (**91**) | 0.5 | 8.6 | 1 | 570 |
| CB 628 (**92**) | 3 | 6.3 | 27 | 455 |

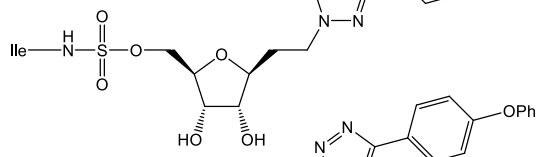
**CB168 (88)**



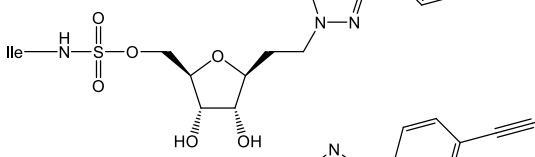
**CB286 (89)**



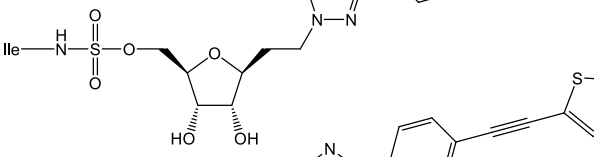
**CB360 (90)**



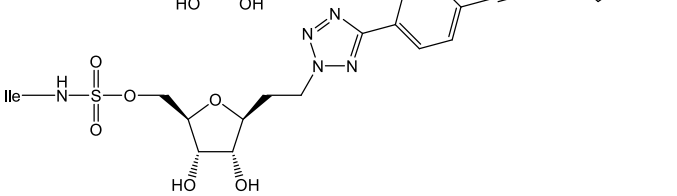
**CB432 (7)**



**CB447 (91)**



**CB628 (92)**



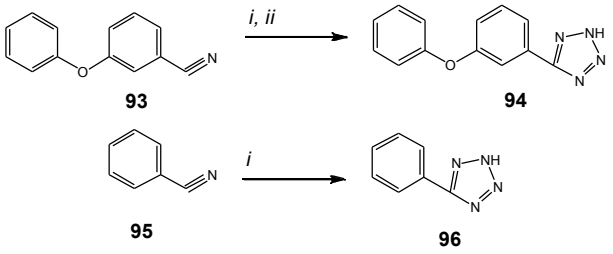
## 5.2 Results

### 5.2.1 Design and synthesis of McC and albomycin analogues carrying a 2-carbon linked aryl-tetrazole as adenine substitution

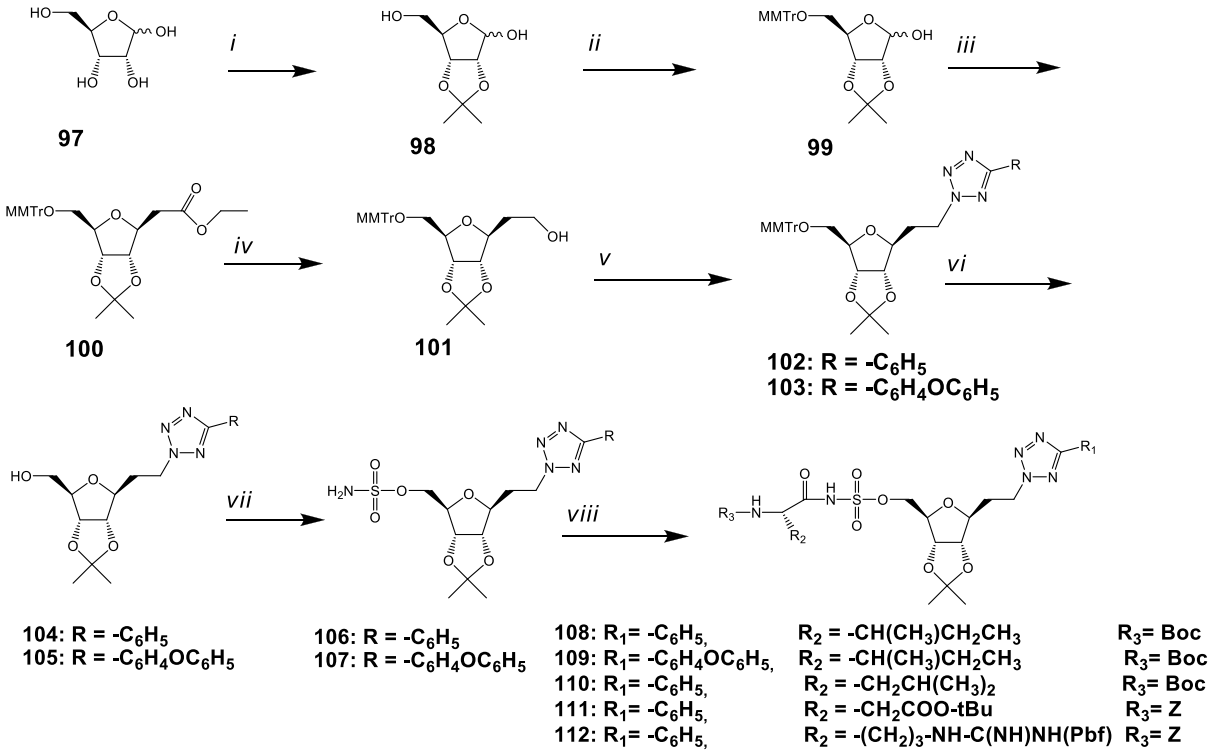
As mentioned before, two of the compounds as developed by Cubist Pharmaceuticals® were used as lead compounds (**88** and **7**). Compound **88** was further modified in several directions; either by converting this compound into a McC analogue (yielding compound **122**) and by replacing isoleucine by leucine (**123**), aspartic acid (**124**), or arginine (**125**) (see Scheme 5.2). As we initially did not find any whole-cell activity for compound **122**, we hypothesized that by replacing isoleucine by another amino acid the Trojan horse mode of action might be rescued. In addition, compound **88** was also converted into a SDC, whereby a hydroxamate-based siderophore was coupled to compound **88**, affording compound **130**. Compound **7** was likewise converted into a McC analogue, by coupling of the McC peptide, whereby the *C*-terminal amino acid remained an isoleucine (**126**). As we initially could not observe any whole-cell activity against wt *E. coli* cells for compound **126**, we hypothesized that this may be due to a failure in the metabolism of this analogue. Therefore, compound **127** was created, in which the *N*-terminal formyl group was omitted and the alanine at postion six was substituted by a serine. Thus the requirement for peptide deformylase was omitted and a possible endopeptidase cleavage site was offered to further enhance metabolism. In addition, compound **7** was coupled with a hydroxamate-based siderophore as in compound **130**, affording compound **131**.

The 5-phenoxyphenyl tetrazole (**94**) was synthesized as described by Hill *et al.*[187] The 5-phenyltetrazole (**96**) was synthesized following a method described by Herbst and Wilson [190]. (Scheme 5.1). Following the example of Cubist Pharmaceuticals® the design of the starting materials was kept identical. The synthetic procedure however was modified, and was largely based on work by Lee *et al.*[191], Mandal *et al.*[192] and most importantly Ohrui *et al.*[193] (Scheme 5.2). Starting from ribose, the 2'- and 3'-hydroxyl groups were protected with an isopropylidene moiety and the 5'-hydroxyl with an MMTrt protecting group. Following the method described by Ohrui *et al.*[193] a Wittig-Moffat reaction was carried out, providing compound **100**. Reduction of the ester, followed by a Mitsunobu reaction yielded compounds **102** and **103**, which were next selectively deprotected to liberate the 5'-hydroxyl group. Subsequent steps to obtain compounds **122**-**127** were carried out as described for the conventional McC analogues. The siderophore peptide, needed for the synthesis of the SDCs (**130** and **131**) was synthesized according to a method previously described by Miller *et al.*[194]. Compounds **130** and **131** were then synthesized by coupling of compound **7** or **88** to [$N^2$-(benzyloxycarbonyl)-$N^5$-acetyl-$N^5$-*O*-acetyl-L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-acetyl-L-

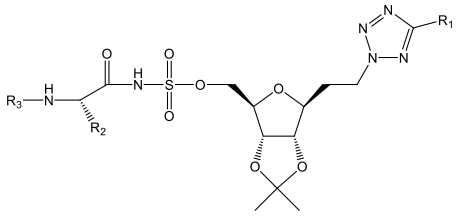
ornithinyl]-$N^5$-acetyl-$N^5$-*O*-acetyl-L-ornithine (**132**) using HBTU. Subsequent removal of the three *O*-acetyl groups with DIPEA, and removal of the Cbz group by hydrogenation yielded compounds **130** and **131** (Scheme 5.3).



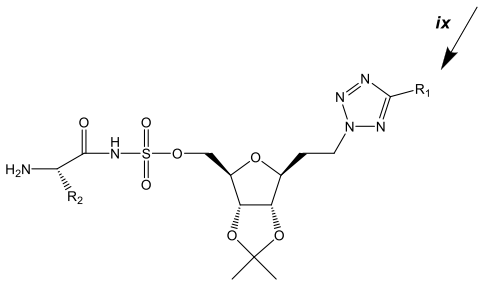

**Scheme 5.1.** Synthesis of 5-aryl tetrazoles.
Upper part: Synthesis of 5-phenoxyphenyl tetrazole: *i*) 4-phenoxybenzonitrile, dibutyltin oxide, trimethylsilylazide in toluene, 5 h, 95 °C. *ii*) work-up with NaOH (1.6 N).
Lower part: Synthesis of 5-phenyl tetrazole: *i*) benzonitrile, $NaN_3$, HOAc in *n*-butanol, 72 h, 125 °C.



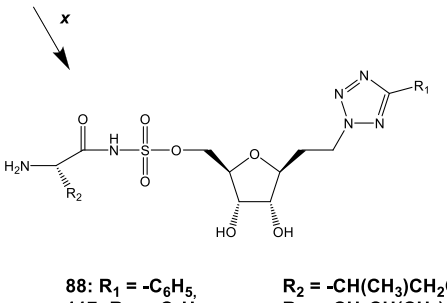

**Scheme 5.2.** Synthesis of the aryl-tetrazole derivatives. Reagents and conditions: *i*) D (-) Ribose, $H_2SO_4$, acetone, 30 min, rt. *ii*) **98**, monomethoxytrityl-Cl, pyridine, 16 h, rt. *iii*) **99**, carbethoxymethylenetriphenylphosphorane, $CH_3CN$, 8 h, 95 °C. *iv*) **100**, $LiAlH_4$, THF, 4 h, rt. *v*) **101**, $Ph_3P$, DEAD, 5-phenyltetrazole, THF, 0 °C, 76%. *vi*) **102** or **103**, ether/HOAc, 10 h, 55 °C. *vii*) **104** or **105**, sulfamoylchloride, DMAc, 0 °C. *viii*) **106** or **107**, *N*-α-Cbz-L-aminoacyl-(tBu or Boc)-succinimide, DBU in DMF, 6 h, rt.



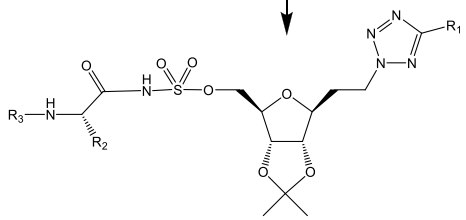
**108:** $R_1$= -$C_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$ $R_3$= Boc
**109:** $R_1$= -$C_6H_4OC_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$ $R_3$= Boc
**110:** $R_1$= -$C_6H_5$, $R_2$ = -$CH_2CH(CH_3)_2$ $R_3$= Boc
**111:** $R_1$= -$C_6H_5$, $R_2$ = -$CH_2COO$-tBu $R_3$= Z
**112:** $R_1$= -$C_6H_5$, $R_2$ = -$(CH_2)_3$-NH-C(NH)NH(Pbf) $R_3$= Z



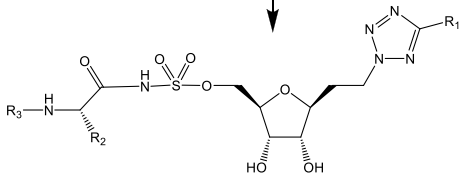
**113:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH_2COO$-tBu
**114:** $R_1$ = -$C_6H_5$, $R_2$ = -$(CH_2)_3$-NH-C(NH)NH(Pbf)



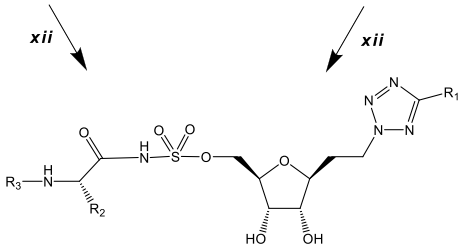
**88:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$
**117:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH_2CH(CH_3)_2$
**7:** $R_1$ = -$C_6H_4OC_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$



**115 and 116:** $R_3$ = fM(Pbf)T(tBu)GN(Trt)A-
**115:** $R_1$ = -$C_6H_5$, $R_2$= -$CH_2COOH$,
**116:** $R_1$ = -$C_6H_5$, $R_2$= -$(CH_2)_3$-NH-C(NH)$NH_2$

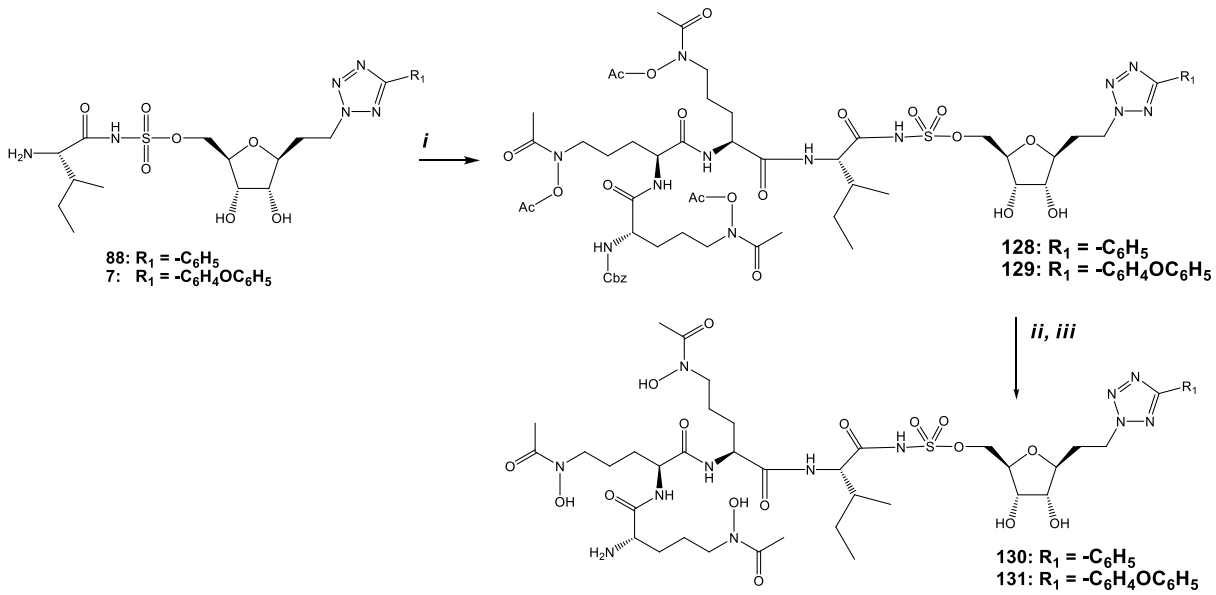


**118, 119 and 120:** $R_3$ = fM(Pbf)T(tBu)GN(Trt)A-
**118:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$
**119:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH_2CH(CH_3)_2$
**120:** $R_1$ = -$C_6H_4OC_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$
**121:** $R_1$ = -$C_6H_4OC_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$, $R_3$ = M(Pbf)T(tBu)GN(Trt)S(tBu)-



**122-126:** $R_3$ = fMRTGNA-
**122:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$
**123:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH_2CH(CH_3)_2$
**124:** $R_1$ = -$C_6H_5$, $R_2$ = -$CH_2COOH$
**125:** $R_1$ = -$C_6H_5$, $R_2$ = -$(CH_2)_3$-NH-C(NH)$NH_2$
**126:** $R_1$= -$C_6H_4OC_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$
**127:** $R_1$= -$C_6H_4OC_6H_5$, $R_2$ = -$CH(CH_3)CH_2CH_3$ $R_3$= MRTGNS-

**Scheme 5.2 continued**. General procedure for the preparation of McC analogues **122-127** *ix*) for $R_2$ = Z-group, $H_2$, Pd/C in MeOH, 3 h, rt. *x*) for $R_3$ = Boc-group TFA/$H_2O$ (5:2), 4 h, 0 °C to rt. *xi*) protected peptide, HOBt, DIC, DIPEA, DMF, 16 h, rt. *xii*) TFA/thioanisole/$H_2O$ (90/2.5/7.5), 2 h, rt.




**Scheme 5.3.** General procedure for the preparation of hydroxamate-based siderophore-IleRS-inhibitors, carrying either a phenyl tetrazole (**130**) or a phenoxyphenyl tetrazole (**131**) moiety. *i*) protected peptide, HBTU, $Et_3N$ in DMF, 16 h, rt. *ii*) DIPEA (6%) in MeOH, 12 h, rt. *iii*) $H_2$, Pd/C in MeOH, 3 h, rt.

### 5.2.2 Antibacterial activity of McC analogues.

The growth inhibitory properties on McC-sensitive *E. coli* were determined for all new compounds by measuring the optical density reached by identical cell cultures in wells of microtiter plates in the presence of various concentrations of the respective inhibitors as described earlier in section 2.2.2.

In addition, the antibacterial activities of all compounds were determined by monitoring the optical density of cellsuspensions of the following strains: *S. aureus* ATCC 6538*, Staphylococcus epidermidis* RP62A (ATCC 35984)*, Pseudomonas aeruginosa* PAO1*, Sarcina lutea* ATCC 9341*, Candida albicans* CO11.

Unfortunately, none of the tested McC analogues showed activity against any of the tested strains. As was found earlier by Cubist Pharmaceuticals®, compound **7** was active against *S. aureus* and also showed low activity against *C. albicans*. However, only compound **131** showed very low activity against *S. aureus* and *C. albicans* (Figure 5.1). This unexpected result was further evaluated by means of *in vitro* aminoacylation experiments for all final compounds targeting IleRS (compounds **7**, **88**, **122**, **124**, **125**, **128**, **129**). These compounds form one cohort and were tested in parallel in two different cell extracts. The results are grouped in Figure 5.2. All of these compounds showed nice activity against IleRS in *E. coli ΔrimL* extracts, lacking only RimL and were devoid of activity in extracts lacking functional peptidases, with the exception of compounds **7** and **88**. In general this is an expected result

since compounds **7** and **88** do not contain any cleavable peptide or siderophore chain, which needs to be removed in order for the prodrug to reach its active state.

A similar result was obtained for compounds **117** and **123** (Figure 5.3). These LeuRS inhibitors showed activity against LeuRS in wt *E. coli* extracts. In extracts lacking all three peptidases, only compound **117** showed activity, whereas compound **123** was largely inactive against LeuRS. Again, compound **123** requires processing of the prodrug in order to inhibit LeuRS, whereas compound **117** does not as it is devoid of an uptake-promoting signal. Compound **124** however, did not even show activity against AspRS in wt *E. coli* extracts (data not shown), in contrast to all other analogues with degradable uptake-promoting signals.

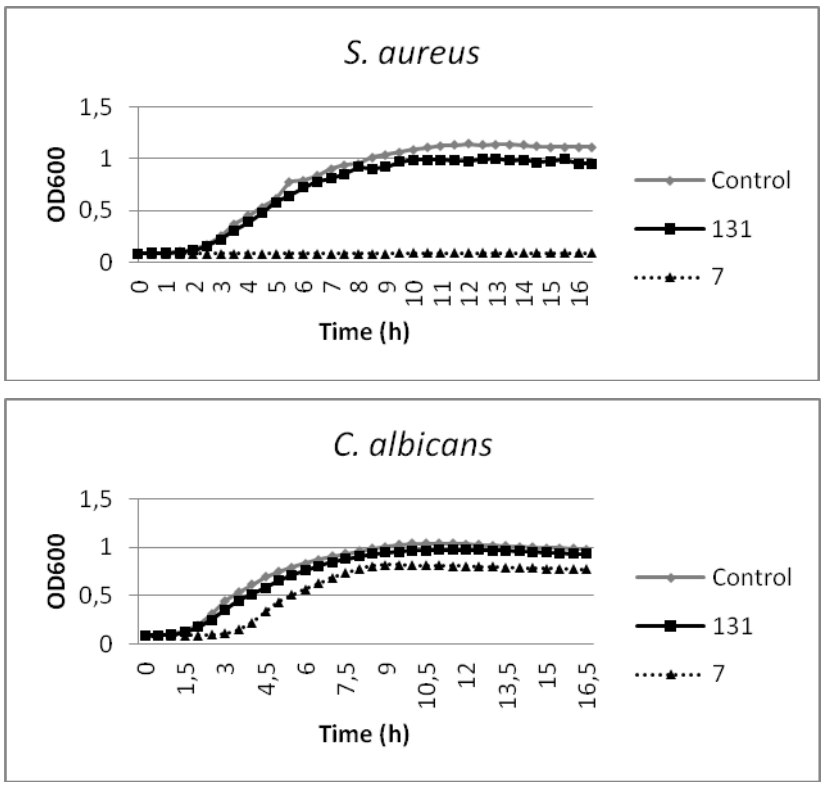
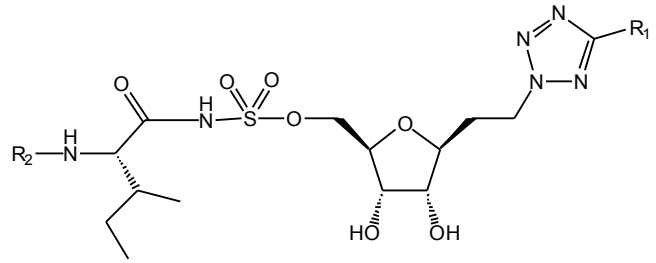



**Figure 5.1.** Inhibitory activity of compounds **7** and **131** against *S. aureus* and *C. albicans* over time in LB medium. OD600 refers to the optical densitiy of the cell culture measured at 600 nm.



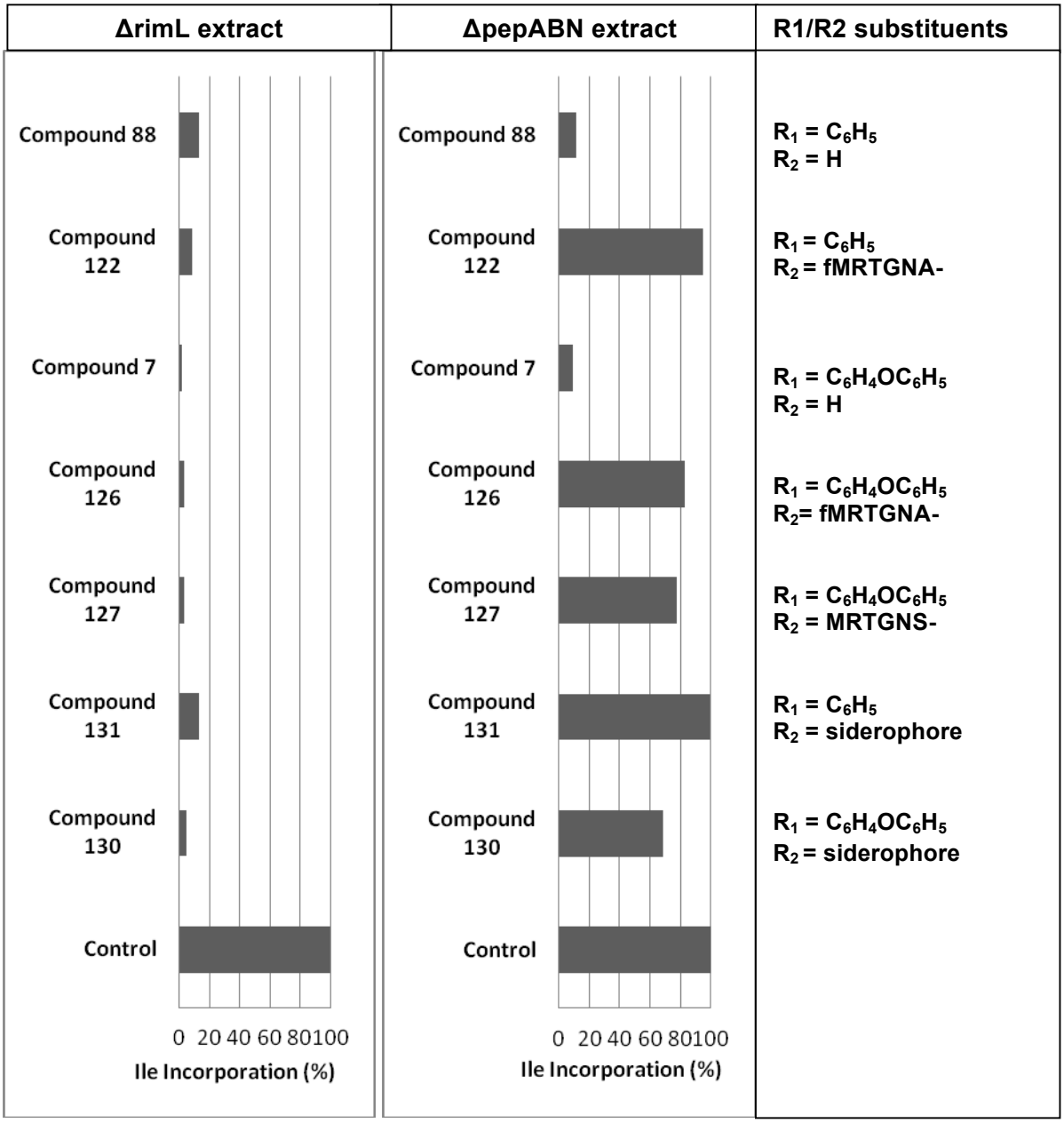



**Figure 5.2** Inhibition of the aminoacylation reaction.
Left panel: IleRS inhibition in S30 extracts of McC-sensitive *E. coli* cells. Middle panel: IleRS inhibition in S30 extracts of McC-resistant *E. coli* cells lacking peptidases A, B and N.
In all tests, the different extracts were incubated with the respective IleRS inhibitors (25 µM), taking samples after 15 min for evaluation of the aminoacylation reaction. The amounts of aminoacylated $tRNA^{Ile}$ were measured *via* liquid scintillation counting.

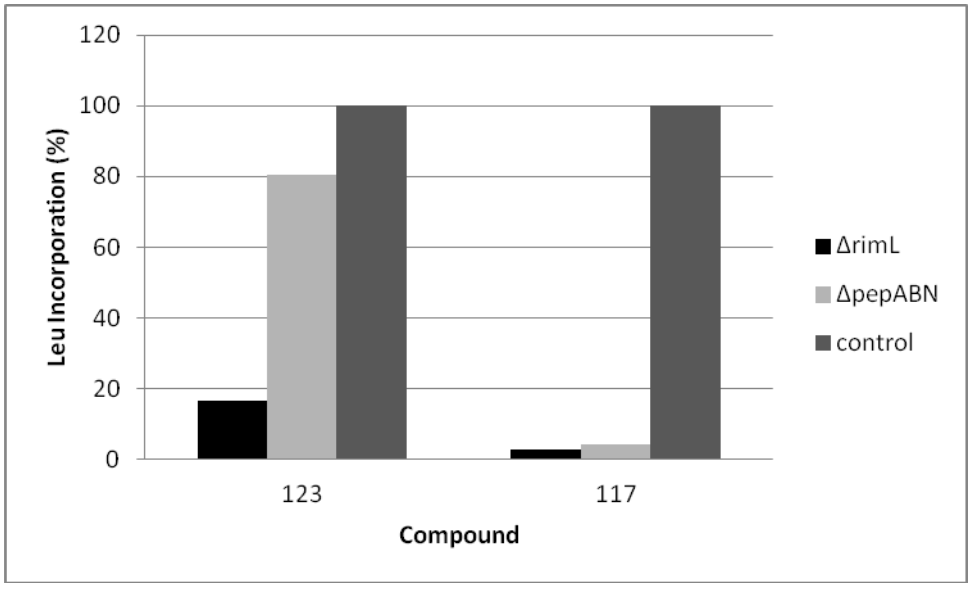



**Figure 5.3** Inhibition of LeuRS by compounds **123** and **117**. In all tests, the different extracts were incubated with the respective IleRS inhibitors (25 µM), taking samples after 15 min. for evaluation of the aminoacylation reaction. The amounts of aminoacylated $tRNA^{Ile}$ were measured *via* liquid scintillation counting.

## 5.3 Discussion

In spite of all our attempts to promote uptake of well-known inhibitors, none of the newly synthesized compounds showed whole-cell activity, while *in vitro*, all compounds (compound **124** excluded) proved potent inhibitors of either LeuRS or IleRS. However, since compound **124** did not inhibit AspRS, it can be concluded that not all modifications in the amino acid constituency are allowed. Since we have shown that all McC analogues with L-amino acids can be efficiently metabolized, it is unlikely that this observation results from problems during the metabolism. This suggests that LeuRS and IleRS can accommodate and effectively bind the aryl-tetrazole moiety, substituting for adenine, while AspRS does not allow for such modification.

Also the siderophore-containing analogues (**130** and **131**) proved unable to effectively inhibit bacterial growth of the different strains tested for sensitivity. Only compound **131** showed activity against *S. aureus*, although compared to compound **7** this activity was insignificant.

Three important conclusions can be drawn from these results. First, the major premise of this work is that although no activity was observed in a whole-cell screening, these compounds showed very nice activity in an *in vitro* setting using only cell extracts. Hence, it must be concluded that all compounds can be efficiently metabolized, thereby liberating the active moiety that can inhibit the aminoacylation reaction of IleRS and LeuRS, whereas release of the active moiety of synthetic SDC prodrugs has often been marked as the main bottleneck [114]. Secondly, it must be concluded that, in case of the McC analogues, absence of whole-

cell activity is attributable to failure in the uptake of these compounds. In the previous three chapters it was clearly shown that the YejABEF transporter tolerates modifications in the peptide moiety of all tested McC analogues relatively well. In this case, the only modification is the replacement of the adenine base by two-carbon-linked aryl tetrazoles. Therefore, the YejABEF transporter may be a selective transporter recognizing only peptidyl-adenylates or very closely resembling derivatives. However, it is not clear if the lack of activity is attributable to the YejABEF transporter. Other factors may affect efficient uptake as well. McC predominantly targets Gram-negative bacteria, for which it has to pass two membranes. It was observed by Severinov *et al*. (unpublished data) that McC diffuses through the OmpF pore, after which it can actively be transported accros the inner membrane by YejABEF. Hence, the McC analogues presented here may be unable to pass the OmpF pore or are impaired otherwise. The same may be true for the SDCs that would rely on FhuA and the TonB complex for efficient uptake. Further research is required to resolve this issue. It is clearly shown that substitution of the base results in absence of ability to pass the cellmembrane. It would therefore be highly desirable to investigate if the uptake by one or both transport systems can be improved by modifying the two-carbon-linked aryl-tetrazole moiety, allowing for efficient uptake.

## 5.4 Materials and Methods

**5.4.1** Materials are as described in section 3.4.1

### 5.4.2 Synthesis of 5-phenoxyphenyltetrazole (94)

4-Phenoxybenzonitrile (200 mg, 1.02 mmol, 1.0 eq.), dibutyltinoxide (79 mg, 3.16 mmol, 3.0 eq), and trimethylsilylazide (679 µL, 5.17 mmol, 5.0 eq) were dissolved in toluene (5 mL) and the mixture was refluxed for 5 h, after which the reaction mixture was cooled down to rt. To this mixture another 100 mL of toluene was added and the organic layer was extracted twice with 50 mL of 1.6 N NaOH. The aqueous layer was subsequently washed twice with 50 mL diethyl ether, after which the aqueous layer was acidified to pH 6 with concentrated HCl. The aqueous layer was then extracted with EtOAc. The organic layer was dried over $Na_2SO_4$, filtered and evaporated yielding 215 mg (87%) of the desired compound.
$^{13}$C-NMR (DMSO-$d_6$): 119.49, 120.24, 120.52, 125.36, 129.90, 131.23, 156.41, 160.16. HR-MS: calcd. for $C_{13}H_{10}N_4O_1$ ($[M+H]^+$): 239.0933; found: 239.0918.

### 5.4.3 Synthesis of 5-phenyltetrazole (96)

Benzonitrile (15.5 mL), sodium azide (13 g) and acetic acid (11.4 mL) were dissolved in *n*-butanol (60 mL) and refluxed. After three days another portion of sodium azide (2.6 g), acetic acid (3.8 mL) and *n*-butanol (20 mL) were added and the mixture was stirred for another 24 h. The mixture was cooled down till 0 °C and a white precipitate was formed. Water (250 mL) was added and the mixture was concentrated to 100 mL. The resulting solution was neutralized with 1 N NaOH and was next washed with toluene (2 x 100 mL). The water layer was acidified with 1 N HCl until a white precipitate was formed. This precipitate was washed with cold water (0 °C) and recrystallized from 20% isopropanol in water. The white crystals were filtered yielding 11.3 g (77.4 mmol, 39%).

$^{13}$C-NMR (DMSO-$d_6$): 124.23 (*i*-aryl), 127.08 (*o*-aryl), 129.52 (*m*-aryl), 131.36 (*p*-aryl), 155.38 (C-tetrazole).

ESI-MS calcd. for $C_7H_7N_4$ ($[M+H]^+$): 147.07; found: 146.90.

### 5.4.4 Synthesis of 5'-*O*-*p*-anisyldiphenylmethyl-2',3'-*O*-isopropylidene-β-D-ribofuranose (99)

A solution of D(-)-ribose (6.5 g, 43.16 mmol, 1 eq.), acetone (80 mL) and $H_2SO_4$ (0.2 mL) was stirred for 30 min at rt under Ar. $NaHCO_3$ (430 mg, 5.14 mmol, 0.12 eq.) was added, the mixture was stirred for 30 min, filtered and the volatiles were evaporated, yielding 2',3'-*O*-isopropylidene-β-D-ribofuranose (**98**). The crude product was dissolved in pyridine (30 mL). Next, a solution of monomethoxytrityl-chloride (MMTr-Cl) (10 g, 32.38 mmol, 0.67 eq.) in pyridine was added. This mixture was stirred overnight at rt under Ar. The volatiles were evaporated and the residue was purified by flash-chromatography (20% EtOAc in hexane with 0.1% $Et_3N$). The fractions containing the desired product, as determined by TLC analysis, were evaporated yielding product **99** (2.8 g, 6.054 mmol, 43% calculated over two steps).

$^{13}$C-NMR ($CDCl_3$): 24.98, 25.31, 26.36 and 26.75 (2x isopropylidene $CH_3$); 55.39 (O-$CH_3$); 65.19 and 65.56 (C5'); 79.68 and 82.27(C1'); 80.32 and 86.23 (C3'); 82.41 and 87.20 (C2'); 87.44 and 88.04 (C4'); 98.22, 103.66, 112.41, 113.33, 113.48 (trityl); 113.56 (C-isopropylidene); 127.51-159.04 (aromatic signals).

ESI-MS calcd. for $C_{28}H_{30}O_6Na$ ($[M+Na]^+$): 485.19; found: 484.80.

### 5.4.5 Synthesis of ethyl-3,6-anhydro-7-*O*-*p*-anisyldiphenylmethyl-2-deoxy-4,5-*O*-isopropylidene-D-allo-heptanoate (100)

Product **99** (11 g, 23.8 mmol, 1 eq.) and carbethoxymethylene-triphenylphosphorane (12.5 g, 35.7 mmol, 1.5 eq.) were dissolved in $CH_3CN$ (100 mL). The solution was refluxed for 8 h. The solution was evaporated yielding a pale yellow oil.

The product was purified by flash-chromatography (22% EtOAc in hexane). The fractions containing the desired product were evaporated affording product **100** (12.5 g, 23.49 mmol, 99%).

$^{13}$C-NMR ($CDCl_3$): 14.44 ($CH_3$ ethyl); 25.89 and 27.77 ($CH_3$ isopropylidene); 39.00 (C2); 55.38 (C1); 60.85 ($CH_2$ ethyl); 64.44 (C7); 81.17 (C3); 82.57 (C5); 83.77 (C4); 84.54 (C6); 86.61 (MMTr-C($Ph_3$)); 113.34 (trityl); 114.39 (C-isopropylidene); 127.10-158.81 (aromatic signals), 170.75 (C=O).

ESI-MS calcd. for $C_{32}H_{36}O_7Na$ ($[M+Na]^+$): 555.24; found: 554.83.

### 5.4.6 Synthesis of 3,6-anhydro-7-*O*-*p*-anisyldiphenylmethyl-2-deoxy-4,5-*O*-isopropylidene-D-allo-heptitol (101)

Compound **100** (12.5 g, 23.48 mmol, 1 eq.) was dried overnight *in vacuo*. THF (10 mL) was added and to the solution, $LiAlH_4$ (1.3 g, 35.23 mmol, 1.5 eq.) was carefully added. After 30 min, the reaction was quenched by adding 2-propanol (50 mL). The volatiles were evaporated and the product was purified by flash-chromatography (40% EtOAc in hexane). The fractions containing the desired product **101** were collected and evaporated. Yield: 9.5 g (19.38 mmol, 83%).

$^{13}$C-NMR (DMSO-$d_6$): 25.95 and 27.88 ($CH_3$ isopropylidene), 36.09 (C2), 55.55 ($CH_3$ para-anisyl), 61.21 (C1), 64.40 (C7), 82.48 (C3), 83.65 (C5), 84.35 (C4), 85.10 (C6), 86.71 (C-trityl), 113.48 (C-isopropylidene), 114.82-158.94 (aromatic signals).

ESI-MS calcd. for $C_{30}H_{34}O_6$ ($[M+H]^+$): 513.22; found: 513.00.

### 5.4.7 Synthesis of 3,6-anhydro-7-*O*-*p*-anisyldiphenylmethyl-1,2-dideoxy-4,5-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol (102)

Product **101** (9.4 g, 19.1 mmol, 1.0 eq.) and triphenylphosphine ($Ph_3P$, 10 g, 38.2 mmol, 1.0 eq.) were combined and dried *in vacuo*. THF (140 mL) was added and the mixture was cooled to 0 °C. Diethyl 1,2-diazenedicarboxylate (DEAD) was slowly added to the cooled mixture over 1 h, after which **96** (3.6 g, 24.8 mmol, 0.33 eq.) was added. The mixture was stirred for 3 h at rt under Ar. The product was purified using flash-chromatography (10% MeOH in chloroform). All fractions containing the desired product were collected and evaporated yielding **102** (9.0 g, 14.6 mmol, 76%).

$^{13}$C-NMR ($CDCl_3$): 25.91 and 27.81 ($CH_3$ isopropylidene); 31.91 (C2); 50.46 (C1); 55.54 (O-$CH_3$); 64.56 (C7); 81.66 (C3); 82.71 (C5); 83.82 (C4); 85.01 (C6); 114.78 (C-isopropylidene); 113.49-165.45 (aromatic signals).

ESI-MS calcd. for $C_{37}H_{39}N_4O_5$ ($[M+H]^+$): 641.27; found: 640.79.

### 5.4.8 Synthesis of 3,6-anhydro-2-deoxy-7-hydroxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol (104)

Product **102** (5.6 g, 9.1 mmol, 1.0 eq.) was dissolved in diethylether/HOAc (20 mL, 1 v/v) and refluxed for 10 h. The mixture was evaporated and neutralized with $NaHCO_{3(sat.)}$. The mixture was extracted with ether (2 x 20 mL) and washed with brine (20 mL). The organic layer was dried ($Na_2SO_4$), filtered and evaporated. The product could easily be purified by flash-chromatography (60% EtOAc in hexane). Yield: 1.2 g (3.4 mmol, 38%).

$^1$H-NMR (DMSO-$d_6$): 1.25 (s, 3H, $CH_3$), 1.40 (s, 3H, $CH_3$), 2.17-2.33 (m, 2H, C1) 3.44-3.46 (*t*AB, 2H, C7, $J_{AB}$=11.60/$J_{A,OH}$≈$J_{A,6}$≈5.4/$J_{B,OH}$≈$J_{B,6}$≈5.3), 3.83-3.87 (m, 2H, C6, C3), 4.45-4.47 (dd, 1H, C4, J=4.2/6.5), 4.59-4.61 (dd, 1H, C5, J=3.3/6.5), 4.79-4.84 (m, 2H, C2), 4.87-4.88 (t, 1H, OH, J=5.5), 7.54-7.58 (m, 1H, *m/p*-aryl), 8.05-8.05 (d, 1H, *o*-aryl, J=1.5), 8.07-8.07 (d, 1H, *o*-aryl, J=1.8).

$^{13}$C-NMR (DMSO-$d_6$): 25.34 and 27.23 ($CH_3$ isopropylidene), 32.88 (C2), 49.95 (C1), 61.88 (C7), 80.94 (C3), 81.90 (C5), 83.99 (C4), 84.84 (C6), 113.00 (C-isopropylidene), 126.39 (C-aryl), 127.05 (*i*-aryl), 129.33 (C-aryl), 130.59 (*p*-aryl),164.11 (tetrazole).

ESI-MS calcd. for $C_{17}H_{23}N_4O_4$ ($[M+H]^+$): 347.17; found: 346.74.

### 5.4.9 Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene- 1-[5-phenyltetrazole]-D-allo-heptitol-7-sulfamate (106)

Chlorosulfonyl isocyanate (2.3 mmol, 198 µL) and formic acid (2.2 mmol, 85 µL) were combined at 0 °C. A white foam was formed and the mixture was allowed to come to rt. After 15 min, the mixture was cooled to 0 °C again and acetonitrile (1 mL) was added. This mixture was stirred for 15 min. The obtained product was used as such for the next step.

An amount of **106** (450 mg, 1.3 mmol) was dissolved in dimethylacetamide (DMAc, 2.3 mL) at rt. This solution was cooled to 0 °C and the cooled chlorosulfonamide was added. This mixture was stirred for 50 min at 0 °C to rt. After 50 min the mixture was cooled to 0 °C again and $Et_3N$ (460 µL) was added. The mixture was stirred for 15 min. Then MeOH (2.3 mL) was added and the mixture was stirred for another 15 min. The solvents were evaporated. The desired product was taken up in EtOAc and washed twice with $NaHCO_3$ (50 mL) and once with brine (50 mL). The organic layer was dried over $Na_2SO_4$, filtered and evaporated. Yield of **106**: 410 mg, 0.96 mmol (74%).

$^1$H-NMR (DMSO-$d_6$): 1.32 (s, 3H, $CH_3$), 1.48 (s, 3H, $CH_3$), 2.31-2.48 (m, 2H, C1), 3.21-3.33 (m, 2H, C7), 3.99-4.02 (m, 2H, C6, C3), 4.48-4.52 (dd, 1H, C4, J=4.6/6.5), 4.71-4.74 (dd, 1H, 5C, J=2.8/6.5), 4.79-4.84 (m, 2H, C2), 7.51-7.53 (m, 3H, *m/p*-aryl), 8.09-8.13 (m, 2H, *o*-aryl, J=1.5)

$^{13}$C-NMR (DMSO-$d_6$): 26.44 and 28.46 ($CH_3$ isopropylidene), 34.99 (C2), 52.03 (C1), 71.08 (C7), 83.89 (C3), 84.03 (C5), 84.29 (C4), 86.68 (C6), 116.52 (C-isopropylidene), 128.59 (C-aryl), 129.43 (i aryl), 130.93 (C-aryl), 132.41 (p C-aryl),167.05 (tetrazole).
ESI-MS calcd. for $C_{17}H_{26}N_5O_6S_1$ ($[M+H]^+$): 426.14; found: 426.15.

### 5.4.10 Synthesis of 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-(Boc-Leu)]-sulfamate (110)

Product **106** (95 mg, 0.22 mmol, 1.0 eq.) was dried together with *N-tert*-butoxycarbonyl-leucine-*O*-succinimide (110 mg, 0.33 mmol, 1.5 eq.) *in vacuo*. Diazobicyclo[5,4,0]undecene, (DBU, 50 µL, 1.5 eq.) was added. This mixture was stirred for 8 h. The volatiles were evaporated and the product was purified using flash-chromatography (40% hexane in EtOAc). The fractions containing the product were collected and evaporated yielding product **108** (20 mg, 0.03 mmol, 14%).
$^{13}$C-NMR ($CDCl_3$): 22.18 (Leu-$\delta_B$-$CH_3$), 23.41 (Leu-$\delta_A$-$CH_3$), 25.13 (Leu-γ-CH) 25.74 and 27.60 ($CH_3$ isopropylidene), 28.68 (*t*Bu Boc), 33.35 (C2), 41.27 (Leu-β-$CH_3$), 50.18 (C1), 56.51 (Leu-α), 69.71 (C7), 80.74 (C3), 81.88 (C5), 82.28 (C4), 84.83 (C6), 115.26 (C-isopropylidene), 127.21 (C-aryl), 127.66 (*i*-C-aryl), 129.22 (C-aryl), 130.67 (*p*-C-aryl),156.69 (Boc C=O), 165.48 (tetrazole).
ESI-MS calcd. for $C_{28}H_{43}N_6O_9S$ ($[M+H]^+$): 639.28; found: 639.20.

### 5.4.11 Synthesis of 2-deoxy-4,5-dihydroxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-*N*-(Leu)-sulfamate (117)

To **110** (20 mg, 0.03 mmol, 1 eq.) was added a mixture of TFA/water (5:2). This mixture was reacted for 2 h. The volatiles were evaporated and co-evaporated with toluene. The desired product was purified using HPLC (10% MeOH). Yield **117**: 4.5 mg, 0.01 mmol, 30%.
$^1$H-NMR (MeOD): 0.96-0.97 (d, 3H, Leu-$\delta_B$-$CH_3$, J=5.9 ), 0.98-0.99 (d, 3H, Leu-$\delta_A$-$CH_3$, J=6.1) 1.56-1.62 and 1.76-1.80 (m, 2H, C1), 1.76-1.80 (m, 1H, Leu-γ-CH), 2.20-2.26 (m, 1H, Leu-$\beta_A$-$CH_2$), 2.41-2.43 (m, 1H, Leu-$\beta_B$-$CH_2$), 3.57-3.60 (dd, 1H, Leu-α, J=5.2/8.5), 3.77-3.80 (m, 1H, C4), 3.83-3.85 (t, 1H, C5, J=4.8), 4.00-4.02 (dd, 1H, C6, J=3.7/7.5), 4.11-4.19 (m, 1H, C3 and C7), 4.90 (m, 2H, C2), 7.50-7.51 (m, 1H, *m/p*-aryl), 8.10-8.11 (dd, 1H, *o*-aryl, J=1.5/ 7.26).
$^{13}$C-NMR (MeOD): 22.13 (Leu-$\delta_B$-$CH_3$), 23.23 (Leu-$\delta_A$-$CH_3$), 25.86 (Leu-γ-CH), 34.14 (C2), 42.33 (Leu-β-$CH_2$), 51.36 (C1), 55.55 (Leu-α), 69.75 (C7), 73.01 (C3), 76.02 (C5), 80.59 (C4), 83.53 (C6), 127.74 (C-aryl), 128.71 (*i*-C-aryl), 130.09 (C-aryl), 131.54 (*p*-C-aryl), 166.20 (tetrazole), 176.47 (C=O Leu).
HR-MS calcd. for $C_{20}H_{29}N_6O_7S$ ($[M-H]^-$): 497.1819; found: 497.1822.

### 5.4.12 Synthesis of peptide coupled 2-deoxy-4,5-dihydroxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-*N*-(Leu)-sulfamate (123)

f-MR(Pbf)T(*t*Bu)GN(Trt)A-OH (7 mg, 6 µmol, 2 eq.) was combined with HOBt (1.5 mg, 0.01 mmol, 3 eq.) and dried *in vacuo*. DMF (0.5 mL) and DIC (1.6 µL, 0.01 mmol, 3 eq.) were added and the solution was stirred for 1 h. The previously obtained product **117** (1.5 mg, 0.003 mmol, 1 eq.) and DIPEA (1 µL, 6 µmol, 2 eq.) were added. This solution was stirred overnight. The evaporated desired product was purified on a Porapak®-column (25% to 100% $CH_3CN$ in water).

The protecting groups were removed by addition of a mixture of TFA/water/thioanisol (92.5/7.5/2.5, v/v/v). This solution was stirred for 2 h. The mixture was coevaporated with toluene. Next, the mixture was purified on a PLRP-S 100Å HPLC column (2% $CH_3CN$ in water).

HRMS calcd for $C_{45}H_{71}N1_6O_{16}S_2$ ($[M-H]^-$): 1155.4676; found: 1155.4683.

### 5.4.13 Synthesis of 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-(Boc-Ile)-sulfamate] (108)

Further synthesis was performed analogously to the preparation of **110**, affording 550 mg (82% total yield) of the title compound.

$^{13}$C-NMR ($CDCl_3$): 11.58 (Ile-δ-$CH_3$), 16.04 (Ile-γ-$CH_3$), 25.21 (Ile-γ'-$CH_3$), 25.73 and 27.59 ($CH_3$ isopropylidene), 28.71 (t-Bu-Boc), 33.33 (C2), 37.45 (Ile-β-$CH_2$), 50.17 (C1), 63.16 (Ile-α-$CH_2$), 69.11 (C7), 80.32 (C3), 81.80 (C5), 82.33 (C4), 84.82 (C6), 115.17 (C-isopropylidene), 127.18 (C-aryl), 127.67 (*i*-C-aryl), 129.19 (C-aryl), 130.63 (*p*-C-aryl), 157.23 (C=O Boc), 165.44 (tetrazole), 182.3 (C=O Ile).

ESI-MS calcd. for $[M-H]^-$: $C_{28}H_{43}N_6O_9S$; 637.27; found: 637.11.

### 5.4.14 Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-(Ile)-sulfamate] (88)

Further synthesis was performed analogously to the preparation of **117**, affording 460 mg (89% total yield) of the title compound.

$^1$H-NMR (MeOD): 0.95-0.98 (t, 3H, Ile-δ-$CH_3$, J=7.4), 1.05-1.06 (d, 3H, Ile-γ'-$CH_3$, J=7.0), 1.23-1.31 (m, 1H, Ile-$\gamma_B$-$CH_2$), 1.58-1.66 (m, 1H, Ile-$\gamma_A$-$CH_2$), 1.99 (m, 1H, Ile-β-CH), 2.19-2.26 and 2.41-2.47 (m, 2H, C2), 3.566-3.574 (d, 1H, Ile-α-CH, J=4.0), 3.78-3.81 (m, 1H, C4), 3.83-3.85 (t, 1H, C5, J=5.5), 4.00-4.03 (dd, 1H, C6, J=3.7/ 7.7), 4.11-4.13 (dd, 1H, C3, J=4.5/9.6 ), 4.17-4.24 (dAB, 1H, C7, $J_{A,4'}$=3.63/$J_{A,B}$=10.81), 4.90 (m, 2H, C1), 7.50-7.51 (m, 3H, *m/p*-phenyl), 8.09-8.11 (m, 2H, *o*-phenyl).

$^{13}$C-NMR (MeOD): 12.18 (Ile-δ-$CH_3$), 15.56 (Ile-γ-$CH_3$), 25.60 (Ile-γ'-$CH_2$), 34.19 (C2), 38.15 (Ile-β-CH), 51.40 (C1), 61.22 (Ile-α-$CH_2$), 70.25 (C7), 73.00 (C3), 76.06 (C5), 80.70 (C6),

83.43 (C4), 127.77 (*i*-C-aryl), 128.72 (*o*-C-aryl), 130.13 (*m*-C-aryl), 131.58 (*p*-C-aryl), 166.22 (tetrazole), 173.99 (C=O Ile).
HR-MS calcd. for $C_{20}H_{29}N_6O_7S$ ([M-H]$^-$): 497.1819; found: 497.1822.

### 5.4.15 Synthesis of peptide coupled 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-(Ile)-sulfamate] (122)

Further synthesis was performed analogously to the preparation of **123**, affording 1.8 mg (4% total yield) of the title compound.
HR-MS calcd. for $C_{45}H_{71}N1_6O_{16}S_2$ ([M-H]$^-$): 1155.4676; found: 1155.4664.

### 5.4.16 Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[[$N^2$-(benzyloxycarbonyl)-$N^5$-acetyl-$N^5$-*O*-acetyl- L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-acetyl-L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-acetyl-L-ornithinyl]-L-Ile)-sulfamate] (128)

Compound **88** (25 mg, 0.05 mmol) was dissolved in DMF (0.5 mL), to which $Et_3N$ (13 µL, 0.1 mmol, 2 eq.) was added. This mixture was next combined with a mixture of **132** (32.4 mg, 0.05 mmol, 1 eq.) and HBTU (23 mg, 0.06 mmol, 1.2 eq.) in DMF (0.5 mL). The reaction mixture was stirred overnight at rt under Ar. The following day, the DMF was evaporated and the residue was taken up in EtOAc. The organic layer was washed two times with water. Subsequently, the EtOAc was evaporated and the product was dissolved in $H_2O/CH_3CN$, whereby the volume of $CH_3CN$ was kept to a minimum. This mixture was then purified over a Porapak™ column (0 to 100 % $CH_3CN$ in $H_2O$). The fractions containing the product were dried, affording the desired product **128**. Yield: 21 mg (34%).
ESI-MS calcd. for $C_{55}H_{77}N_{12}O_{21}S$ ([M-H]$^-$): 1273.5; found: 1273.1.

### 5.4.17 Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[[$N^5$-acetyl-$N^5$-*O*-hydroxyl-L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-hydroxyl-L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*- hydroxyl-L-ornithinyl]-L-Ile)-sulfamate] (130)

Product **128** (11 mg, 8.2 µmol) was dissolved in MeOH, containing 6% DIPEA, and the mixture stirred for 24 h. The product was dried and purified on a PLRP-S 100Å RP-HPLC column (5 to 80 % $CH_3CN$ in $H_2O$), yielding 1.8 mg (5%) of the desired compound.
$^{13}$C-NMR (MeOH): 12.08 ($CH_3$ DIPEA), 13.20 (Ile-δ-$CH_3$), 16.37 (Ile-γ-$CH_3$), 17.38 (DIPEA), 19.41 (Orn- γ-$CH_2$), 20.27 (N-acetyl-$CH_3$), 25.79 (Ile-γ'-$CH_2$), 30.29 (Orn-β-$CH_2$), 34.15 (C2), 38.59 (Ile-β-CH), 43.79 (DIPEA-$CH_2$), 51.43 (C1), 54.21 (Orn-α-CH), 55.83 (DIPEA-CH), 61.62 (Ile-α-$CH_2$), 67.76 (Cbz-$CH_2$), 69.81 (C7), 73.08 (C3), 76.10 (C5), 80.45 (C6), 83.73 (C4), 127.77-138.46 (aromatic signals), 158.77 (C=O Cbz), 166.15 (tetrazole), 173.84 (C=O Ile).
HR-MS calcd. for $C_{49}H_{71}N_{12}O_{18}S$ ([M-H]$^-$): 1147.4730; found: 1147.4774.

Compound **128** was dissolved in 5% aqueous DMF (100 µL), to which 10 µL 0.5 M $FeCl_3$ was added. Hereto, 10 mg of 10% Pd-C was added and the reaction mixture was stirred for 5 h under hydrogen atmosphere. Subsequently, the RM was filtred and evaporated, yielding 1.2 mg (98%) of the desired compound.
HR-MS calcd. for $C_{41}H_{65}N_{12}O_{16}S$ ($[M-H]^-$): 1013.4360; found: 1013.4360.

### 5.4.18 Synthesis of 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[*N*-benzyloxycarbonyl-L-aspartyl(*O*-*t*Bu)]-sulfamate] (111)

A solution of *Z*-aspartyl(*O*-*tert*-butyl)-*O*-Su (460 mg, 0.15 mmol, 2.0 eq.), 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-sulfamate (**106**) (320 mg, 0.75 mmol, 1 eq.) and DBU (171 µl, 0.9 mmol, 1.2 eq.) in DMF (5 mL) was stirred at rt for 8 h. DMF was evaporated under reduced pressure. Next, the residue was purified by flash chromatography ($CH_2Cl_2$, 10% MeOH). Fractions containing the desired product were evaporated affording **111** as colourless oil. Yield: 420 mg (81%).
$^{13}$C-NMR ($CDCl_3$): 25.57 and 27.47 (isopropylidene $CH_3$), 28.35 (*t*-Bu-Boc), 29.84 (C2), 38.45 (Asp-$CH_2$) 50.04 (C1), 53.59 (Cα), 67.46 ($CH_2$-Cbz), 69.83 (C7), 81.78 (C3), 81.96 (C-*t*Bu), 82.09 (C5), 82.46 (C4), 84.76 (C6), 115.47 (C-isopropylidene), 127.18, 165.49 (aromatic signals), 172.42 (C=O Asp).
ESI-MS calcd. for $C_{38}H_{62}N_7O_{11}SSi_2$ ($[M+H]^+$): 880.4; found: 880.1.

### 5.4.19 Synthesis of 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[L-aspartyl(*O*-*tert*-butyl)]-sulfamate] (113)

Compound **111** (420 mg) was dissolved in a mixture of MeOH with 4.4% HCOOH (15 mL) while Pd/C (100 mg) was added. The mixture was stirred for 0.5 h, after which it was filtered and evaporated yielding a white foam (**113**). Yield: 250 mg (76%)
$^{13}$C-NMR ($CDCl_3$): 25.79 and 27.63 (isopropylidene $CH_3$), 28.39 (*t*Bu-Boc), 33.42 (C2), 35.63 (Asp-$CH_2$) 50.27 (C1), 53.05 (Asp-Cα), 69.53 (C7), 81.91 (3C), 82.02 (C-*t*Bu), 82.44 (C5), 83.11 (C4), 84.84 (C6), 115.02 (C-isopropylidene), 127.18 165.39 (aromatic signals), 171.49 and 174.10 (C=O Asp).
ESI-MS calcd. for $C_{32}H_{31}N_6O_9S$ ($[M-H]^-$): 555.2; found: 554.9.

### 5.4.20 Synthesis of peptide coupled 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[L-aspartyl(*O*-tert-butyl)]-sulfamate] (124)

Further synthesis was performed analogously to the preparation of **122**, affording 1.7 mg (15% total yield) of the title compound.
HR-MS calcd. for $C_{42}H_{67}N_{16}O_{17}S_2$ ($[M+H]^+$): 1131.4311; found: 1131.4850.

### 5.4.21 Synthesis of 3,6-anhydro-7-*O*-p-anisyldiphenylmethyl-1,2-dideoxy-4,5-isopropylidene-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol (103)

Further synthesis was performed analogously to the preparation of **102**, affording 410 mg (71% total yield) of the title compound.

$^{13}$C-NMR ($CDCl_3$): 25.93 and 27.83 ($CH_3$ isopropylidene), 33.96 (C2), 50.45 (C1), 55.56 (O-$CH_3$), 64.57 (C7), 81.68 (C3), 82.72 (C5), 83.83 (C4), 85.02 (C6), 86.79 (C-trityl), 114.79 (C-isopropylidene), 113.49-159.64 (aromatic signals)

ESI-MS calcd. for $C_{43}H_{42}N_4O_6Na$ ($[M+Na]^+$): 733.30; found: 733.24.

### 5.4.22 Synthesis of 3,6-anhydro-2-deoxy-7-hydroxy-4,5-*O*-isopropylidene-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol (105)

Further synthesis was performed analogous to the preparation of **104**, affording 248 mg (98% total yield) of the title compound.

$^{1}$H-NMR ($CDCl_3$): 1.34 (s, 3H, $CH_3$), 1.53 (s, 3H, $CH_3$), 2.26-2.56 (m, 2H, 1C) 3.67-3.46 (ABX, 2H, 7C, $J_{AB}$=12.60/$J_{AX}$≈3.0/$J_{BX}$≈3.75), 3.95-4.05 (m, 2H, C6, C3), 4.34-4.38 (m, 1H, 4C), 4.64-4.89 (m, 3H, C5, C2), 7.07-7.19 and 7.36-7.41 and 8.09-8.12 (aromatic signals).

$^{13}$C-NMR ($CDCl_3$): 25.71 and 27.65 ($CH_3$ isopropylidene), 33.18 (C2), 50.23 (C1), 62.90 (C7), 81.75 (C3), 81.86 (C5), 84.79 (C4), 84.83 (C6), 115.17 (C-isopropylidene), 118.87-165.03 (aromatic signals).

ESI-MS calcd. for $C_{23}H_{26}N_4O_5Na$ ($[M+Na]^+$): 461.18; found: 461.25.

### 5.4.23 Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol-7-sulfamate (107)

Further synthesis was performed analogous to the preparation of **106**, affording 170 mg (84% total yield) of the title compound.

$^{13}$C-NMR ($CDCl_3$): 25.69 and 27.59 ($CH_3$ isopropylidene), 33.74 (C2), 50.28 (C1), 70.57 (C7), 81.59 (C3), 82.35 (C5), 82.74 (C4), 85.05 (C6), 118.93-130.31 (aromatic signals).

HR-MS calcd. for $C_{23}H_{28}N_5O_7S$ ($[M+H]^+$): 518.1709; found: 518.1708.

### 5.4.24 Synthesis of 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol-7-[*N*-(Boc-L-Ile)-sulfamate] (109)

Further synthesis was performed analogous to the preparation of **110**, affording 210 mg (76% total yield) of the title compound.

$^{13}$C-NMR ($CDCl_3$): 11.85 (Ile-δ-$CH_3$), 15.90 (Ile-γ-$CH_3$), 25.22 (Ile-γ'-$CH_3$), 25.69 and 27.56 (isopropylidene $CH_3$), 28.67 (*t*Bu-Boc), 33.33 (C2), 37.47 (Ile-β-$CH_2$), 50.13 (C1), 69.00 (C7), 79.95 (C3), 81.78 (C5), 82.38 (C4), 84.78 (C6), 115.01 (C-isopropylidene), 118.85-159.64 (aromatic signals), 156.51 (C=O Boc), 164.98 (tetrazole).

ESI-MS calcd. for $C_{34}H_{45}N_6O_{10}S$ ([M-H]$^-$): 729.29; found: 729.18.

### 5.4.25 Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol-7-[*N*-(L-Ile)-sulfamate] (7)

Further synthesis was performed analogous to the preparation of **88**, affording 180 mg (80% total yield) of the title compound.

$^1$H-NMR (MeOD): 0.88-0.90 (t, 3H, Ile-δ-$CH_3$, J=7.4), 0.98-0.99 (d, 3H, Ile-γ'-$CH_3$, J=7.0), 1.23-1.37 (m, 2H, Ile-$\gamma_B$-$CH_2$), 1.53-1.57 (m, 1H, Ile-$\gamma_A$-$CH_2$), 1.86-1.93 (m, 1H, Ile-β-CH), 2.15-2.19 and 2.36 (m, 2H, C2), 3.48-3.49 (d, 1H, Ile-α-CH, J=4.0), 3.73-3.81 (m, 1H, C4), 3.95-3.96 (m, 1H, C5), 4.05-4.11 (m, 3H, C6, C3 and C7), 6.99-7.14 and 7.32-7.37 aromatic signals).

$^{13}$C-NMR ($CDCl_3$): 13.03 (Ile-δ-$CH_3$), 16.38 (Ile-γ-$CH_3$), 26.56 (Ile-γ'-$CH_3$), 34.99 (C2), 39.06 (Ile-β-$CH_2$), 52.18 (C1), 62.27 (Ile-α-CH), 70.66 (C7), 73.86 (C3), 76.86 (C5), 81.59 (C6), 83.34 (C4), 120.36-131.97 (aromatic signals).

HR-MS calcd. for $C_{26}H_{33}N_4O_8S$ ([M-H]$^-$): 589.2081; found: 589.2090.

### 5.4.26 Synthesis of peptide coupled 3,6-anhydro-2-deoxy-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol-7-[*N*-(L-Ile)-sulfamate] (126)

Further synthesis was performed analogous to the preparation of **123**, affording 1.0 mg (18% total yield) of the title compound.

HR-MS calcd. for $C_{51}H_{75}N_{16}O_{17}S_2$ ([M-H]$^-$): 1247.4938; found: 1247.4900.

### 5.4.27 Synthesis of peptide coupled 3,6-anhydro-2-deoxy-1-[5-phenoxyphenyl tetrazole]-D-allo-heptitol-7-[*N*-(L-Ile)-sulfamate] (127)

Further synthesis was performed analogous to the preparation of **123**, affording 1.0 mg (18% total yield) of the title compound.

HR-MS calcd. for $C_{51}H_{76}N_{16}O_{18}S_2$ ([M-H]$^-$): 1263.4887; found: 1263.4774.

### 5.4.28 Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenoxyphenyltetrazole]-D-allo-heptitol-7-[*N*-[[$N^2$-(benzyloxycarbonyl)-$N^5$-acetyl-$N^5$-*O*-acetyl- L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-acetyl-L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-acetyl-L-ornithinyl]-L-Ile)-sulfamate] (129)

Further synthesis was performed analogous to the preparation of **128**, affording 17 mg (28% total yield) of the title compound.

ESI-MS calcd. for $C_{61}H_{82}N_{12}O_{22}SNa$ ([M+Na]$^+$): 1389.53; found: 1389.21.

### 5.4.29 Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenoxtphenyltetrazole]-D-allo-heptitol-7-[*N*-[[$N^5$-acetyl-$N^5$-*O*-hydroxyl- L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-hydroxyl -L-ornithinyl]-[$N^5$-acetyl-$N^5$-*O*-hydroxyl-L-ornithinyl]-L-Ile)-sulfamate] (131)

Further synthesis was performed analogous to the preparation of **130**, affording 1.3 mg (20% total yield) of the title compound.

HR-MS calcd. for $C_{55}H_{75}N_{12}O_{19}S$ ([M-H]$^-$): 1239.4992; found: 1239.4967.

### 5.4.30 Synthesis of 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[*N*-benzyloxycarbonyl-L-arginyl(Pbf)]-sulfamate] (112)

Further synthesis was performed analogous to the preparation of **111**, affording 50 mg (78% total yield) of the title compound.

ESI-MS calcd. for $C_{49}H_{56}N_9O_{12}S_2$ [M-H]$^-$: 966.36; found: 966.17.

### 5.4.31 Synthesis of 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[L-arginyl(Pbf)]-sulfamate] (114)

Further synthesis was performed analogous to the preparation of **113**, affording 27 mg (58% total yield) of the title compound.

ESI-MS calcd. for $C_{36}HN_9O_{10}S_2$ [M-H]$^-$: 832.3; found: 831.9.

### 5.4.32 Synthesis of peptide coupled 3,6-anhydro-2-deoxy-4,5-ispropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[L-arginyl]-sulfamate] (125)

Further synthesis was performed analogously to the preparation of **122**, affording 1.7 mg (15% total yield) of the title compound.

ESI-MS calcd. for $C_{45}H_{73}N_{19}O_{16}S_2$ ([M-H]$^-$): 1198.4; found: 1198.0.

### 5.4.33 Biological activity

#### 5.4.33.1 Whole-cell activity determinations

Evaluations of all compounds were performed as described in secion 2.4.15.

Bacterial strains used for the evaluations: *E. coli* Ara-Yej (BW39758), expressing the *yejABEF* transporter upon L-arabinose induction; *E. coli* K-12 (BW28357), used as the wild-type control. The antibacterial activities of all compounds were determined by monitoring the optical density of suspensions of cell-cultures of the following strains: *S. aureus* ATCC 6538, *S. epidermidis* RP62A (ATCC 35984), *P. aeruginosa* PAO1, *S. lutea* ATCC 9341, *C. albicans* CO11.

#### 5.4.33.2 Aminoacylation experiments

*In vitro* aminoacylation experiments were performed as described in section 3.4.15.2.

**Research Contributions**

Sophie Geboers assisted in the synthesis of some of the compounds. Bharat Gadakh synthesized the siderophore peptide, compound **132**. Prof. Dr. Busson confirmed the presence and configuration of compound **100**. Prof. Dr. Jef Rozenski recorded the mass spectra.

# Chapter 6

# General Discussion

## 6.1 Introduction

The basic premise of this work is that it forms a solid foundation for further development of bacterial aaSA inhibitors. In Chapters 2 and 5 we firmly established requirements for efficient uptake of McC analogues. Chapter 2 shows that uptake of McC analogues is most efficient with peptide-chain lengths of more than 6 amino acids. Chapter 5 shows that alteration of the nucleoside, by replacing the adenine base moiety with aryl-tetrazoles linked with a 2-carbon spacer, abolishes whole-cell activity. Chapters 2-5 show that intracellular metabolism is only hampered by the incorporation of D-amino acids, while all other modifactions (use of different amino acids) are well tolerated. It was also clearly shown in Chapter 4 that the use of D-amino acids at the *C*-terminal position are well tolerated, although these compounds may be less effective aaRS inhibitors. However, upon methylation of the alpha amine of the aaSA, the inhibitory properties for the respective aaRSs are lost.
In this general discussion focus is given to further ideas for future work and to reflections on the obtained data, described in the previous Chapters.
In general, aaRSs are structurally quite conserved in prokaryotes and eukaryotes. As a consequence, all aaRSs use the same reaction intermediates in the aminoacylation of tRNA. However, some divergence has occurred throughout evolution, making it possible to develop selective aaRS inhibitors. Many natural compounds have been found by means of high-throughput screening, although few have reached the level of clinical trials. Mupirocin is the only drug that is used in the clinic, although high-level resistance to this drug has restricted its use. Hereto, new compounds that limit resistance should be developed.

## 6.2 McC as lead compound for future antibiotics development

AaSAs for a long time have been evaluated as high-potential antibiotics. However, these compounds lack bioavailability and selectivity, and therefore cannot be used as such. The most important finding however is that the Trojan horse concept, as presented by McC and its newly developed analogues, could offer some solutions to address the selectivity and bioavailability issues, opening the doors for a new plethora of antibacterial agents. However, insufficient research so far has been carried out on the possible cytotoxic effects on mammalian cells. A recent report by Messmer *et al.*[73] confirmed Asp-SA to be an efficient inhibitor of many different bacterial AspRSs, but for the first time likewise a strong inhibition was found on the human mitochondrial synthetase. However, it is not clear yet whether such possible side effects on the host mitochondrial enzymes will be translated to an *in vivo* setting. To the best of our knowledge, it is not known if the signaling peptide of McC is

specific for prokaryotes. Indeed, further studies are needed to corroborate these assumptions and to evaluate the toxicity and resistance to such compounds. The Trojan horse concept may be further developed, by creating peptides that are selectively recognized by either prokaryotes or eukaryotes. To address the resistance issue, a multisynthetase inhibitor may become the new standard, such as an inhibitor which would target at the same time ValRS, IleRS and LeuRS, as these use isosteric amino acids. Similarly, a single inhibitor for AspRS and GluRS, and AsnRS and GlnRS may be envisaged. Whether this is a realistic perspective needs to be borne out. The Trojan horse concept, as presented by McC, might not be limited to antibiotic treatment by means of an aaRS inhibitor. The feature of a drug coupled to a signal peptide that is actively pumped into cells, and is subsequently cleaved off, is extraordinary. This concept in theory might be applied to other potent, but poorly bioavailable drugs, as well. Thus, new McC-based drugs could also open doors for other applications.

In this work we showed that such compounds are attainable, holding on to the Trojan horse concept. Also we showed that by replacing the *C*-terminal amino acid in McC analogues, different aaRSs could be targeted. With the proposed synthetic protocol it was shown that it is possible to synthesize almost any McC analogue (except fXP-SA) and thus to target virtually any aaRS. Most alterations at this position had little effect on the uptake of these compounds by the YejABEF transporter. It is not clear yet whether all modifications at other positions allow for efficient uptake by this transporter.

An obvious drawback of McC and its analogues is its relatively large size. With a molecular weight of ~1.1 kDa, 17 hydrogen bond donors, and 35 H-bond acceptors this compound exceeds three out of four rules as stated by Lipinski *et al.*[195], limiting its drug likeness. In shortening the peptide tail, as found in McC, we evaluated the possibility of small McC analogues that retained activity, but would do so under more Lipinski-favorable conditions. In addition we hoped to find other transporters that would allow for active uptake of these compounds. Although only low activity of these shortened compounds was found, no alternative transport mechanism was found that could replace the effectiveness of the YejABEF transporter. However, it was shown that the threshold for efficient uptake by this transporter is at a peptide chain-length of at least five amino acids. In addition, it was found that the combination of a methionyl-arginyl moiety is important for efficient uptake and thus this combination may serve as an identity element that is used by the YejABEF transporter.

We showed that replacing the adenine moiety by two-carbon linked aryl-tetrazole moieties has dramatic consequences on the uptake of McC analogues carrying such moieties. It was shown that this modification abolishes uptake completely. It was also shown that these compounds work perfectly in cell extracts and depend on the peptidases PepA, PepB, and PepN for liberating the active moiety. The most plausible explanation is that the peptide

transporter YejABEF does not support the transport of peptides smaller than 5 amino acids. In Chapter 3 it was shown that peptides can compete for uptake with McC or its analogues. This shows that YejABEF is a peptide transporter that is not designed specifically for uptake of nucleoside-coupled peptides. Alternatively, the aryl-tetrazole moiety may interact with membrane-associated structures abolishing effective uptake and thus antibacterial activity. Since compound **7** is active against *S. aureus*, this presumption would only be valid for *E. coli*. Since none of the other McC analogues shows activity against *S. aureus*, this suggests that *S. aureus* does not have a YejABEF transporter homologue, and thus it cannot be ruled out that the YejABEF transporter is the (only) bottleneck in the uptake of aryl-tetrazole-containing compounds. Thus far there is no molecular basis for this finding.

## 6.3 Siderophore drug conjugates as Trojan horse – aaRS inhibitors

Since the discovery of the first sideromycins half a century ago, valuable information has been obtained on this interesting class of antibiotics. Despite tireless efforts in unraveling siderophore uptake mechanisms, on siderophore biosynthesis as well as in research on the natural and synthetic sideromycins, none of the compounds investigated reached clinical phase trials.

Thus far, the most promising potential antibiotic is represented by the albomycins because of their proven broad-spectrum antibacterial activity and *in vivo* efficacy. However, to date no total synthesis of these compounds have been achieved and only low, insufficient, amounts can be obtained by the producing strain.

Although large numbers of SDCs have been chemically synthesized, most of these have shown decreased activity compared to the non-conjugated drug. As mentioned before, this is largely due to the fact that there is insufficient intracellular metabolism, so that the drug moiety is not released after uptake. In the case of salmycins, good antibacterial activities were found, but due to instability issues only low *in vivo* activity was observed. Thus, it can be concluded that the main bottleneck in this field is attributable to the linker between siderophore and drug. Ester linkages are too labile, while most other linkages cannot be cleaved so that the intracellular target is not reached. Therefore it is recommended that the relatively simple albomycin linker is inserted between siderophore and drug, so that a serine protease can be utilized as a drug-releasing enzyme, a concept that has shown its value in this study.

Alternatively, targets that do not require processing in order to release the active moiety from the SDC, probably display the highest potential. As mentioned before, SDCs targeting PBP show the greatest potency as they do not require activation by metabolism and do not need

to be transported across the inner membrane as they can inhibit the PBP within the periplasmic space. Recently Möllmann *et a*l. [97] synthesized a number of β-lactam-containing antimicrobial compounds that were linked to a siderophore where the catecholate moieties were acylated to form acetoxy groups. Furthermore this group showed that the conjugates displayed activity against the difficult-to-treat *P. aerigunosa* strains that were even resistant against carbapenems. Less activity was observed against MRSA strains.
However, for antibacterial drug development as such, this may not be the right course to take, as these compounds will inevitably meet with resistance since they operate using the same mechanism of action and hence will suffer from the same resistance mechanism as all other β-lactam antibiotics. Therefore, it would be more desirable to continue the development of SDCs targeting other processes.

## 6.4 Suggestions for further development of McC/albomycin analogues as antibiotics

Several directions, related to this field, would be interesting to explore. Along the same line as the aryl-tetrazole-containing compounds presented in this work, it would be desirable to also conjugate the other aryl-tetrazole compounds that were developed by Cubist Pharmaceuticals®. These would then need to be evaluated for their whole-cell activity to establish the potency of such compounds.
Another interesting project would be the synthesis and evaluation of McC analogues having 3-deaza adenine moieties. As briefly mentioned in this work, aaSAs suffer from degradation due to attack of the $N^3$ of adenine on the 5' postition of the ribose moiety, with the sulfamoyl-conjugated peptide as a leaving group [170]. Also from a biological perspective this would be an interesting course to take. It would show the influence of $N^3$ on the binding capacity inside the active site of the aaRS. Secondly, a comparative study could show the influence of this modification on the selectivity for either bacterial or human aaRSs. This can easily be established by *in vitro* aminoacylation experiments as described in the previous chapters.
As mentioned before, the use of D-amino acids in the peptide tail prevents efficient processing of the McC analogues, except if the *C*-terminal amino acid has a D-configuration. Although we attempted to circumvent this problem by incorporating L-amino acids at the sixth position, hoping for an endopeptidase that could release the drug, this could not rescue activity. Along the same line it would be worthwhile to investigate whether other modifications in the peptide chain could rescue activity. This would be interesting for two reasons. First, the use of D-amino acids in prodrugs aids their stability against peptidases, and therefore enhances their plasma half life [182]. Hence, this modification could improve the druggability of

such compounds. Secondly, the evaluation of McC analogues containing D-amino acids will give valuable information about the requirements for efficient metabolism by the different peptidases involved in the processing of these prodrugs.

PepN is the major aminopeptidase, forming about 1% of the total protein concentration inside the bacterial cell, and showing broad specificity. Chandu *et al*. showed that PepN has a preference for amino acids being either small (Ala, Gly) or having a basic side chain (Arg, Lys) [196]. Therefore McC analogues that contain either a combination of these four amino acids at positions six and seven could be interesting. This work is currently ongoing in our laboratories.

The major premise of the SDCs, as synthesized in this PhD work, is the proof of the earlier stated hypothesis that when hydroxamate-based siderophores as in albomycin are coupled to an antibacterial moiety via another amino acid as linker moiety, the compound is readily cleavable. To the best of our knowledge this is the first observation of synthetic SDCs that can reach full activity through intracellular activation, while SDCs synthesized by others suffered from low activity due to the inability of peptidases to metabolize these compounds. Unfortunately, both SDCs presented in this work did not show whole-cell activity. This again may be due to lack of efficient uptake, this time by the FhuA transporter or the TonB complex. Also the possible interference of other membrane components cannot be ruled out.

Since both the McC compounds as well as the SDCs containing an aryl-tetrazole moiety, lack whole-cell activity, this suggests that another problem different from active uptake by either transporter prevents transport across the bacterial cell membrane(s). Possible binding to membrane associated proteins might explain this observation. One way to investigate this presumption, would be to synthesize one or several SDCs containing a simple aaSA. The evaluation of such a compound would either confirm or rule out the involvement of the aryl-tetrazole moiety in the absence of whole-cell activity.

However, several attempts were already undertaken to obtain such a compound, without success. It seems that these compounds are relatively unstable, again due to $N^3$-5'-O cyclization. As already suggested in the previous section, the use of 3-deaza aaSAs, or perhaps even 1,3-dideaza aaSAs, could circumvent this problem, and could possibly solve the problem which we encountered as described in Chapter 5. In addition this could lead to a very potent antibiotic that could make it to the market.

## Summary

This dissertation developed from an initial interest in extending the McC concept to a series of potent antimicrobial compounds that could target a variety of aaRSs (Ch.2). Along the way, several new side projects were investigated that evolved in response to further optimize drug-likeness (Ch. 3) and selectivity for the bacterial enzyme (Ch. 5). We also undertook several attempts to prevent possible resistance mechanisms to potential antibiotics that might result from this work (Ch. 4).

Increasing resistance to antibiotics is a major problem worldwide and provides the stimulus for development of new bacterial inhibitors with preferably different modes of action. In search for new leads, several new bacterial targets are being exploited beside the use of traditional screening methods. Hereto, inhibition of bacterial protein synthesis is a long-standing validated target. Aminoacyl-tRNA synthetases (aaRSs) play an indispensable role in protein synthesis and their structures proved quite conserved in prokaryotes and eukaryotes. However, some divergence has occurred allowing the development of selective aaRS inhibitors. Following an outline on the action mechanism of aaRSs, an overview has been given of already existing aaRS inhibitors, which are largely based on mimics of the aminoacyl-adenylates, the natural reaction intermediates. This is followed by a discussion on more recent developments in the field and the bioavailability problem and the so-called Trojan horse inhibitors, as based on microcin C (McC) and the sideromycins.

Microcin C (**21**) is a potent antibacterial compound produced by some *E. coli* strains. McC functions through a Trojan horse mechanism: it is actively taken up inside a sensitive cell through the function of the YejABEF transporter and then processed by cellular aminopeptidases. Processed McC (**22**) is a non-hydrolyzable aspartyl-adenylate analogue that inhibits aspartyl-tRNA synthetase (AspRS). A new synthesis is described that allows for the production of a wide variety of McC analogues in acceptable amounts. Using this synthesis a number of diverse compounds was synthesized with altered target specificity. It was shown that these compounds exhibit potent whole-cell activity. In addition, further characteristics of the YejABEF transporter were determined using these compounds, showing a preference for the more polar aminoacyl residues at the *C*-terminal position.

McC analogues with variable length of the peptide moiety were synthesized and evaluated in order to characterize substrate preferences of the YejABEF transporter. It was shown that a minimal peptide-chain length of six amino acids and the presence for *N*-terminal formyl-methionyl-arginyl sequence are required for transport.

*N*-methylated aaSAs were synthesized to investigate their potential as aaRS inhibitors and to establish if these would escape acetylation of the alpha amine (with concomitant inactivation of the inhibitor), which is a self-protective mechanism in the bacterial cell. It was shown however that these compounds are not able anymore to effectively inhibit their respective aaRSs. In addition we showed that (D)D-SA (*i.e.* Asp-SA with a D-configuration of the Asp), is a potent inhibitor of AspRS. However, we also showed that the inhibitory effect of (D)D-SA is relatively short-lasting. This was shown to be attributable to a large extent to acetylation by RimL, although it was also shown that other unknown factors also play a role in the inactivation of this compound.

McC analogues with D-amino acids at positions two to six were shown to be resistant against metabolization by the different peptidases and could therefore not liberate the active moiety. This observation could not be reversed by the incorporation of L-amino acids at position six, showing that none of the peptidases could exhibit endopeptidase activity.

The substitution of the adenine by aryl-tetrazole moieties linked *via* a two-carbon spacer in aaSA proved unsuccessful. Although all synthesized compounds, both in McC as in SDC form, showed nice potential when evaluated in *in vitro* aminoacylation experiments, the whole-cell activity was virtually abolished. Only the activity of compound **7** (CB432) against *S. aureus* could be confirmed. It was shown that lack of activity in whole-cell assays was due to an inability of these compounds to pass the cell membrane. Nevertheless, it was also shown that the synthesized SDCs could readily be metabolized by the peptidases PepA, PepB and PepN.

## Samenvatting

Dit doctoraal proefschrift ontstond oorspronkelijk uit een poging het microcin C (McC) concept te kopiëren en verder uit te breiden tot een serie antibiotica, die op de aminoacyl-tRNA-synthetasen aangrijpen. Gedurende de thesisperiode werd het project verder uitgebreid om zodoende het medicijnpotentieel (Hfdst. 3) en selectiviteit voor bacteriële enzymen te verbeteren (Hfdst. 5). Zo werden ook verschillende pogingen om mogelijke resistentie te voorkomen, ondernomen (Hfdst 4).

Toenemende resistentie tegen de bestaande antibiotica vormt een wereldwijd probleem en duidt op de noodzaak nieuwe antibiotica te blijven ontwikkelen, die bij voorkeur op nog niet eerder beproefde vitale celprocessen aangrijpen. In de zoektocht naar nieuwe antibiotica, worden verschillende nieuwe bacteriële targets onderzocht in uitgebreide screenings. De bacteriële proteïnesynthese is al gedurende enkele decennia een gevalideerd aangrijpingspunt. De aminoacyl-tRNA-synthetasen (aaRSs) spelen een noodzakelijke rol in de proteïne synthese en hun structuren zijn relatief geconserveerd in prokaryoten en eukaryoten. Nochtans is er gedurende de evolutie voldoende divergentie opgetreden, die ruimte geeft voor de ontwikkeling van selectieve aaRS inhibitoren.

McC is een anbtibacteriële verbinding die door verschillende *E. coli*-stammen wordt geproduceerd. McC werkt *via* een Trojan horse mechanisme: het wordt actief opgenomen door de cellen in het bezit van een YejABEF-transporter en vervolgens gemetaboliseerd door cellulaire aminopeptidasen. Deze metabolisatie resulteert in een niet-hydrolyseerbaar aspartyl adenylaat-analoog dat aspartyl-tRNA-synthetase (AspRS) kan inhiberen. Een nieuwe chemische synthese die het mogelijk maakt een grote variëteit aan McC-analogen aan te maken met acceptabele opbrengsten werd ontwikkeld. Gebruikmakende van deze synthese werden verschillende verbindingen aangemaakt, waarvan werd aangetoond dat zij elk hun respectievelijk enzym konden inhiberen en *via* het Trojan horse-mechanisme functioneerden. Verder werd met deze verbindingen de YejABEF-transporter verder gekarakteriseerd, waarbij werd aangetoond dat er een lichte voorkeur is voor de meer polaire amino zuren op de zevende positie.

McC-analogen met variabele peptidelengten werden gesynthetiseerd en geëvalueerd, om zodoende verdere voorkeuren van de YejABEF-transporter te karakteriseren. Wij toonden aan dat er een minimale peptidelengte van zes aminozuren vereist is om efficiënte opname te bewerkstelligen. Daarnaast werd ondervonden dat de combinatie van *N*-terminaal formyl-methionyl-arginyl noodzakelijk is voor efficiënt transport over de bacteriële membraan.

*N*-Gemethyleerde aaSAs werden gesynthetiseerd om te achterhalen of het mogelijk is op deze wijze deze verbindingen te beschermen tegen acetylering van de alfa amine, wat normaliter leidt tot inactivatie van zulke verbindingen. Echter, het blijkt dat *Nα*-methylering resulteert in niet-functionele verbindingen die dus de aminoacylatie reactie niet inhiberen. Verder werd aangetoond dat het gebruik van (D)D-SA (*i.e.* Asp-SA met een D-configuratie van het Asp-residue), een krachtige inhibitor van AspRS oplevert. Anderzijds werd echter ook gevonden dat het inhiberende effect van (D)D-SA van relatief kortere duur is dan van het L-analoog. Dit kon in overwegende mate worden toegeschreven aan acetylering door RimL, alhoewel dit niet de enige inactiverende factor is. Tot dusver is onbekend waardoor inactivatie van (D)D-SA versneld wordt.

McC-analogen met D-aminozuren op posities twee tot zes bleken resistent tegen metabolisering door de verschillende peptidasen. Hierdoor kan het inhiberende deel van deze prodrugs niet afgesplitst worden, waardoor deze verbindingen inactief bleken. Deze observatie hebben we getracht te pareren door op positie zes van de peptideketen een aminozuur met een L-configuratie in te bouwen, waardoor in theorie de mogelijkheid werd geboden aan endopeptidasen om de peptidebinding tussen de L-aminozuren op posities zes en zeven te hydrolyseren. Deze ingreep bleek echter niet effectief, waardoor geconcludeerd moet worden dat alle bij de metabolisatie van McC-analogen betrokken peptidasen als exopeptidasen fungeren.

De substitutie van adenine door twee-koolstof-gekoppelde aryl tetrazolen in aaSAs, bleek niet succesvol. Hoewel van alle verbindingen, zowel in McC- als in SDC-vorm, werd aangetoond dat deze goede activiteit vertonen in bacteriële celextracten, kon geen activiteit tegen bacteriën cellen worden aangetoond. Enkel van verbinding **7** (CB432) werd de activiteit tegen *S. aureus* bevestigd. Er moet worden aangenomen dat het uitblijven van activiteit van de hier beschreven verbindingen, te wijten is aan het onvermogen van deze verbindingen om actief te worden opgenomen door de YejABEF- of FhuA-TonB transporters.

# References


[1] G. H. Vondenhoff, A. Van Aerschot, *Nucleosides Nucleotides Nucleic Acids* **2011**, *30*, 465.
[2] G. H. Vondenhoff, A. Van Aerschot, *Eur J Med Chem* **2011**, *46*, 5227.
[3] D. M. Livermore, *J Antimicrob Chemother* **2009**, *64 Suppl 1*, i29.
[4] S. Rachakonda, L. Cartee, *Current Medicinal Chemistry* **2004**, *11*, 775.
[5] K. Bockstael, A. Van Aerschot, *Central European Journal of Medicine* **2009**, *4*, 141.
[6] R. C. Moellering, Jr., *International Journal of Antimicrobial Agents* **2011**, *37*, 2.
[7] J. J. Barker, *Drug Discovery Today* **2006**, *11*, 391.
[8] J. Pohlmann, *Drugs of the future* **2004**, *29(3)*, 243.
[9] J. A. Krzycki, *Current Opinion in Microbiology* **2005**, *8*, 706.
[10] P. O'Donoghue, Z. Luthey-Schulten, *Microbiology and molecular biology reviews : MMBR* **2003**, *67*, 550.
[11] C. R. Woese, G. J. Olsen, M. Ibba, D. Soll, *Microbiology and molecular biology reviews : MMBR* **2000**, *64*, 202.
[12] P. Schimmel, J. Tao, J. Hill, *FASEB J.* **1998**, *12*, 1599.
[13] L. Moulinier, S. Eiler, G. Eriani, J. Gangloff, J. C. Thierry, K. Gabriel, W. H. McClain, D. Moras, *The EMBO journal* **2001**, *20*, 5290.
[14] F. L. Rock, W. Mao, A. Yaremchuk, M. Tukalo, T. Crepin, H. Zhou, Y. K. Zhang, V. Hernandez, T. Akama, S. J. Baker, J. J. Plattner, L. Shapiro, S. A. Martinis, S. J. Benkovic, S. Cusack, M. R. Alley, *Science* **2007**, *316*, 1759.
[15] H. Ueda, Y. Shoku, N. Hayashi, J. Mitsunaga, Y. In, M. Doi, M. Inoue, T. Ishida, *Biochimica et Biophysica Acta* **1991**, *1080*, 126.
[16] M. J. Brown, L. M. Mensah, M. L. Doyle, N. J. Broom, N. Osbourne, A. K. Forrest, C. M. Richardson, P. J. O'Hanlon, A. J. Pope, *Biochemistry* **2000**, *39*, 6003.
[17] D. Heacock, C. J. Forsyth, K. Shiba, K. MusierForsyth, *Bioorganic Chemistry* **1996**, *24*, 273.
[18] B. Ruan, H. Nakano, M. Tanaka, J. A. Mills, J. A. DeVito, B. Min, K. B. Low, J. R. Battista, D. Soll, *Journal of Bacteriology* **2004**, *186*, 8.
[19] S. F. Ataide, M. Ibba, *ACS chemical biology* **2006**, *1*, 285.
[20] M. Mirande, *FEBS Letters* **2010**, *584*, 443.
[21] S. T. Rao, M. G. Rossmann, *Journal of Molecular Biology* **1973**, *76*, 241.
[22] U. A. Ochsner, X. Sun, T. Jarvis, I. Critchley, N. Janjic, *Expert opinion on investigational drugs* **2007**, *16*, 573.
[23] J. G. Hurdle, A. J. O'Neill, I. Chopra, *Antimicrob. Agents Chemother.* **2005**, *49*, 4821.
[24] S. Kim, S. W. Lee, E. C. Choi, S. Y. Choi, *Applied Microbiology and Biotechnology* **2003**, *61*, 278.
[25] J. Levengood, S. F. Ataide, H. Roy, M. Ibba, *The Journal of biological chemistry* **2004**, *279*, 17707.
[26] R. Fukunaga, S. Yokoyama, *Journal of Molecular Biology* **2006**, *359*, 901.
[27] B. e. a. Alberts, *Molecular biology of the cell*, fourth ed., Garland Science, New York, **2002**.
[28] S. A. Martinis, M. T. Boniecki, *FEBS Letters* **2010**, *584*, 455.
[29] K. Sheppard, P. M. Akochy, J. C. Salazar, D. Soll, *The Journal of biological chemistry* **2007**, *282*, 11866.
[30] J. L. Huot, C. Balg, D. Jahn, J. Moser, A. Emond, S. P. Blais, R. Chenevert, J. Lapointe, *Biochemistry* **2007**, *46*, 13190.
[31] C. Balg, M. De Mieri, J. L. Huot, S. P. Blais, J. Lapointe, R. Chenevert, *Bioorganic & medicinal chemistry* **2010**, *18*, 7868.
[32] J. S. Cisar, J. A. Ferreras, R. K. Soni, L. E. Quadri, D. S. Tan, *Journal of the American Chemical Society* **2007**, *129*, 7752.

[33] R. Finking, A. Neumuller, J. Solsbacher, D. Konz, G. Kretzschmar, M. Schweitzer, T. Krumm, M. A. Marahiel, *Chembiochem : a European journal of chemical biology* **2003**, *4*, 903.
[34] S. Bernier, D. Y. Dubois, M. Therrien, J. Lapointe, R. Chenevert, *Bioorganic & Medicinal Chemistry Letters* **2000**, *10*, 2441.
[35] C. M. Thomas, J. Hothersall, C. L. Willis, T. J. Simpson, *Nature reviews. Microbiology* **2010**, *8*, 281.
[36] R. G. Werner, L. F. Thorpe, W. Reuter, K. H. Nierhaus, *European journal of biochemistry / FEBS* **1976**, *68*, 1.
[37] J. G. Hurdle, A. J. O'Neill, I. Chopra, *The Journal of antimicrobial chemotherapy* **2004**, *54*, 549.
[38] T. Kanamaru, Y. Nakano, Y. Toyoda, K. I. Miyagawa, M. Tada, T. Kaisho, M. Nakao, *Antimicrobial Agents and Chemotherapy* **2001**, *45*, 2455.
[39] R. L. Jarvest, J. M. Berge, V. Berry, H. F. Boyd, M. J. Brown, J. S. Elder, A. K. Forrest, A. P. Fosberry, D. R. Gentry, M. J. Hibbs, D. D. Jaworski, P. J. O'Hanlon, A. J. Pope, S. Rittenhouse, R. J. Sheppard, C. Slater-Radosti, A. Worby, *Journal of Medicinal Chemistry* **2002**, *45*, 1959.
[40] I. A. Critchley, C. L. Young, K. C. Stone, U. A. Ochsner, J. Guiles, T. Tarasow, N. Janjic, *Antimicrobial Agents and Chemotherapy* **2005**, *49*, 4247.
[41] A. S. Faqi, S. J. Bell, S. Gill, D. B. Colagiovanni, *Regulatory toxicology and pharmacology : RTP* **2009**, *55*, 28.
[42] B. Nare, S. Wring, C. Bacchi, B. Beaudet, T. Bowling, R. Brun, D. Chen, C. Ding, Y. Freund, E. Gaukel, A. Hussain, K. Jarnagin, M. Jenks, M. Kaiser, L. Mercer, E. Mejia, A. Noe, M. Orr, R. Parham, J. Plattner, R. Randolph, D. Rattendi, C. Rewerts, J. Sligar, N. Yarlett, R. Don, R. Jacobs, *Antimicrob Agents Chemother*, *54*, 4379.
[43] K. Ziegelbauer, P. Babczinski, W. Schonfeld, *Antimicrobial Agents and Chemotherapy* **1998**, *42*, 2197.
[44] J. Mittendorf, F. Kunisch, M. Matzke, H. C. Militzer, A. Schmidt, W. Schonfeld, *Bioorganic & Medicinal Chemistry Letters* **2003**, *13*, 433.
[45] B. Ruan, M. L. Bovee, M. Sacher, C. Stathopoulos, K. Poralla, C. S. Francklyn, D. Soll, *The Journal of biological chemistry* **2005**, *280*, 571.
[46] D. V. R. N. Bhikshapathi, Y. S. Kumar, Y. M. Rao, V. Kishan, *Indian Journal of Biotechnology* **2010**, *9*, 18.
[47] D. V. R. N. Bhikshapathi, D. R. Krishna, V. Kishan, *Indian Journal of Biotechnology* **2010**, *9*, 265.
[48] J. S. Reader, P. T. Ordoukhanian, J. G. Kim, V. de Crecy-Lagard, I. Hwang, S. Farrand, P. Schimmel, *Science* **2005**, *309*, 1533.
[49] J. G. Kim, B. K. Park, S. U. Kim, D. Choi, B. H. Nahm, J. S. Moon, J. S. Reader, S. K. Farrand, I. Hwang, *Proc Natl Acad Sci U S A* **2006**, *103*, 8846.
[50] T. Moriguchi, N. Asai, T. Wada, M. Sekine, *Nucleic Acids Symposium Series* **1999**, 15.
[51] M. Ubukata, H. Osada, K. Isono, *Nucleic Acids Symposium Series* **1985**, 81.
[52] H. Osada, K. Isono, *Antimicrobial Agents and Chemotherapy* **1985**, *27*, 230.
[53] M. Uramoto, C. J. Kim, K. Shin-Ya, H. Kusakabe, K. Isono, D. R. Phillips, J. A. McCloskey, *The Journal of antibiotics* **1991**, *44*, 375.
[54] M. Sekine, K. Okada, K. Seio, H. Kakeya, H. Osada, T. Obata, T. Sasaki, *The Journal of organic chemistry* **2004**, *69*, 314.
[55] A. Z. Metlitskaya, G. S. Katrukha, A. S. Shashkov, D. A. Zaitsev, T. A. Egorov, I. A. Khmel, *FEBS Lett.* **1995**, *357*, 235.
[56] J. I. Guijarro, J. E. Gonzalez-Pastor, F. Baleux, J. L. San Millan, M. A. Castilla, M. Rico, F. Moreno, M. Delepierre, *J. Biol. Chem.* **1995**, *270*, 23520.
[57] D. E. Fomenko, A. Z. Metlitskaya, J. Peduzzi, C. Goulard, G. S. Katrukha, L. V. Gening, S. Rebuffat, I. A. Khmel, *Antimicrob. Agents Chemother.* **2003**, *47*, 2868.
[58] J. E. Gonzalez-Pastor, J. L. San Millan, F. Moreno, *Nature* **1994**, *369*, 281.
[59] R. F. Roush, E. M. Nolan, F. Lohr, C. T. Walsh, *J. Am. Chem. Soc.* **2008**, *130*, 3603.

[60] A. Metlitskaya, T. Kazakov, G. H. Vondenhoff, M. Novikova, A. Shashkov, T. Zatsepin, E. Semenova, N. Zaitseva, V. Ramensky, A. Van Aerschot, K. Severinov, *J. Bacteriol.* **2009**, *191*, 2380.

[61] M. Novikova, T. Kazakov, G. H. Vondenhoff, E. Semenova, J. Rozenski, A. Metlytskaya, I. Zukher, A. Tikhonov, A. Van Aerschot, K. Severinov, *J. Biol. Chem.* **2010**, *285*, 12662.

[62] A. Tikhonov, T. Kazakov, E. Semenova, M. Serebryakova, G. Vondenhoff, A. Van Aerschot, J. S. Reader, V. M. Govorun, K. Severinov, *The Journal of biological chemistry* **2010**, *285*, 37944.

[63] K. Beautement, Chrystal, E.J.T., Howard, J., Ridley, S.M., in *Proc. International Meeting on Biodiversity: A Source of New Leads for the Pharmaceutical and Agrochemical Industries.* (Ed.: S. K. Wrigley, Hayes, M.A.,Thomas, R. Chrystal, E.J.T., Nicholson, N.), The Royal Society of Chemistry, University St. Andrews, St. Andrews, Scotland, **1999**, pp. 288.

[64] M. Novikova, A. Metlitskaya, K. Datsenko, T. Kazakov, A. Kazakov, B. Wanner, K. Severinov, *Journal of Bacteriology* **2007**, *189*, 8361.

[65] T. Kazakov, G. H. Vondenhoff, K. A. Datsenko, M. Novikova, A. Metlitskaya, B. L. Wanner, K. Severinov, *J. Bacteriol.* **2008**, *190*, 2607.

[66] A. Metlitskaya, T. Kazakov, A. Kommer, O. Pavlova, M. Praetorius-Ibba, M. Ibba, I. Krasheninnikov, V. Kolb, I. Khmel, K. Severinov, *J. Biol. Chem.* **2006**, *281*, 18033.

[67] C. W. Carter, *Annu. Rev. Biochem.* **1993**, *62*, 715.

[68] S. Bernier, P. M. Akochy, J. Lapointe, R. Chenevert, *Bioorg. Med. Chem.* **2005**, *13*, 69.

[69] X. Y. Yu, J. M. Hill, G. Yu, W. Wang, A. F. Kluge, P. Wendler, P. Gallant, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 375.

[70] J. Lee, S. E. Kim, J. Y. Lee, S. Y. Kim, S. U. Kang, S. H. Seo, M. W. Chun, T. Kang, S. Y. Choi, H. O. Kim, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1087.

[71] T. Kazakov, A. Metlitskaya, K. Severinov, *J. Bacteriol.* **2007**, *189*, 2114.

[72] P. Van de Vijver, G. H. Vondenhoff, T. S. Kazakov, E. Semenova, K. Kuznedelov, A. Metlitskaya, A. Van Aerschot, K. Severinov, *J Bacteriol* **2009**, *191*, 6273.

[73] M. Messmer, S. P. Blais, C. Balg, R. Chenevert, L. Grenier, P. Lague, C. Sauter, M. Sissler, R. Giege, J. Lapointe, C. Florentz, *Biochimie* **2009**, *91*, 596.

[74] A. K. Forrest, R. L. Jarvest, L. M. Mensah, P. J. O'Hanlon, A. J. Pope, R. J. Sheppard, *Bioorganic & Medicinal Chemistry Letters* **2000**, *10*, 1871.

[75] P. Brown, C. M. Richardson, L. M. Mensah, P. J. O'Hanlon, N. F. Osborne, A. J. Pope, G. Walker, *Bioorganic & medicinal chemistry* **1999**, *7*, 2473.

[76] H. Belrhali, A. Yaremchuk, M. Tukalo, K. Larsen, C. Berthet-Colominas, R. Leberman, B. Beijer, B. Sproat, J. Als-Nielsen, G. Grubel, et al., *Science* **1994**, *263*, 1432.

[77] J. Lee, S. U. Kang, M. K. Kang, M. W. Chun, Y. J. Jo, J. H. Kwak, S. Kim, *Bioorganic & Medicinal Chemistry Letters* **1999**, *9*, 1365.

[78] M. Ora, M. Murtola, S. Aho, M. Oivanen, *Organic & Biomolecular Chemistry* **2004**, *2*, 593.

[79] D. R. Phillips, *Journal of Organic chemistry* **1993**, *58*, 854.

[80] S. Bernier, P. M. Akochy, J. Lapointe, R. Chenevert, *Bioorg Med Chem* **2005**, *13*, 69.

[81] X. Y. Yu, J. M. Hill, G. Yu, W. Wang, A. F. Kluge, P. Wendler, P. Gallant, *Bioorganic & Medicinal Chemistry Letters* **1999**, *9*, 375.

[82] J. Y. Winum, A. Scozzafava, J. L. Montero, C. T. Supuran, *Medicinal Research Reviews* **2005**, *25*, 186.

[83] P. Van de Vijver, G. H. Vondenhoff, S. Denivelle, J. Rozenski, J. Verhaegen, A. Van Aerschot, P. Herdewijn, *Bioorganic & medicinal chemistry* **2009**, *17*, 260.

[84] M. Mattiuzzo, A. Bandiera, R. Gennaro, M. Benincasa, S. Pacor, N. Antcheva, M. Scocchi, *Mol Microbiol* **2007**, *66*, 151.

[85] J. W. Payne, B. M. Grail, S. Gupta, J. E. Ladbury, N. J. Marshall, R. O'Brien, G. M. Payne, *Arch. Biochem. Biophys.* **2000**, *384*, 9.

[86] D. Weitz, D. Harder, F. Casagrande, D. Fotiadis, P. Obrdlik, B. Kelety, H. Daniel, *J. Biol. Chem.* **2007**, *282*, 2832.

[87] Farhanullah, T. Kang, E. J. Yoon, E. C. Choi, S. Kim, J. Lee, *European journal of medicinal chemistry* **2009**, *44*, 239.
[88] Z. P. Xiao, X. B. He, Z. Y. Peng, T. J. Xiong, J. Peng, L. H. Chen, H. L. Zhu, *Bioorganic & medicinal chemistry* **2011**, *19*, 1571.
[89] D. Ding, Q. Meng, G. Gao, Y. Zhao, Q. Wang, B. Nare, R. Jacobs, F. Rock, M. R. Alley, J. J. Plattner, G. Chen, D. Li, H. Zhou, *Journal of Medicinal Chemistry* **2011**, *54*, 1276.
[90] H. Eum, Y. Lee, S. Kim, A. Baek, M. Son, K. W. Lee, S. W. Ko, S. Kim, S. Y. Yun, W. K. Lee, H. J. Ha, *Bulletin of the Korean Chemical Society* **2010**, *31*, 611.
[91] M. Hoffmann, M. Torchala, *Journal of Molecular Modeling* **2009**, *15*, 665.
[92] Y. Wu, K. Yu, B. Xu, L. Chen, X. Chen, J. Mao, A. Danchin, X. Shen, D. Qu, H. Jiang, *The Journal of antimicrobial chemotherapy* **2007**, *60*, 502.
[93] V. Braun, *Drug Resist Updat* **1999**, *2*, 363.
[94] M. J. Miller, H. Zhu, Y. Xu, C. Wu, A. J. Walz, A. Vergne, J. M. Roosenberg, G. Moraski, A. A. Minnick, J. McKee-Dolence, J. Hu, K. Fennell, E. Kurt Dolence, L. Dong, S. Franzblau, F. Malouin, U. Mollmann, *Biometals* **2009**, *22*, 61.
[95] X. P. Jiang, R. L. Elliott, J. F. Head, *Anticancer Res*, *30*, 759.
[96] K. K. Neilands J. B., Schwyn B., Coy M., Francis R. T., Paw B. H., Bagg A., *VCH Press, Weinheim* **1987**, pp. 3.
[97] U. Mollmann, L. Heinisch, A. Bauernfeind, T. Kohler, D. Ankel-Fuchs, *Biometals* **2009**, *22*, 615.
[98] V. Braun, H. Killmann, *Trends in Biochemical Sciences* **1999**, *24*, 104.
[99] B. Halliwell, J. M. Gutteridge, *Biochem J* **1984**, *219*, 1.
[100] H. Boukhalfa, A. L. Crumbliss, *Biometals* **2002**, *15*, 325.
[101] K. D. Krewulak, H. J. Vogel, *Biochim Biophys Acta* **2008**, *1778*, 1781.
[102] W. R. Harris, C. J. Carrano, S. R. Cooper, S. R. Sofen, A. E. Avdeef, J. V. McArdle, K. N. Raymond, *Journal of the American Chemical Society* **1979**, *101*, 6097.
[103] K. Konopka, J. B. Neilands, *Biochemistry* **1984**, *23*, 2122.
[104] D. G. Moore, C. F. Earhart, *Infect Immun* **1981**, *31*, 631.
[105] J. M. Roosenberg, 2nd, Y. M. Lin, Y. Lu, M. J. Miller, *Curr Med Chem* **2000**, *7*, 159.
[106] L. Escolar, J. Perez-Martin, V. de Lorenzo, *J Bacteriol* **1999**, *181*, 6223.
[107] J. D. Faraldo-Gomez, M. S. Sansom, *Nat Rev Mol Cell Biol* **2003**, *4*, 105.
[108] A. D. Ferguson, J. Deisenhofer, *Biochim Biophys Acta* **2002**, *1565*, 318.
[109] R. A. Larsen, M. G. Thomas, K. Postle, *Mol Microbiol* **1999**, *31*, 1809.
[110] K. Postle, R. A. Larsen, *Biometals* **2007**, *20*, 453.
[111] D. C. Scott, Z. Cao, Z. Qi, M. Bauler, J. D. Igo, S. M. Newton, P. E. Klebba, *J Biol Chem* **2001**, *276*, 13025.
[112] S. Silver, M. Walderhaug, *Microbiol Rev* **1992**, *56*, 195.
[113] M. S. Diarra, M. C. Lavoie, M. Jacques, I. Darwish, E. K. Dolence, J. A. Dolence, A. Ghosh, M. Ghosh, M. J. Miller, F. Malouin, *Antimicrob Agents Chemother* **1996**, *40*, 2610.
[114] T. A. Wencewicz, U. Mollmann, T. E. Long, M. J. Miller, *Biometals* **2009**, *22*, 633.
[115] S. Gong, S. W. Bearden, V. A. Geoffroy, J. D. Fetherston, R. D. Perry, *Infect Immun* **2001**, *69*, 2829.
[116] A. Pierce, D. Legrand, *Biochimie* **2009**, *91*, 1.
[117] D. H. Goetz, M. A. Holmes, N. Borregaard, M. E. Bluhm, K. N. Raymond, R. K. Strong, *Mol Cell* **2002**, *10*, 1033.
[118] T. H. Flo, K. D. Smith, S. Sato, D. J. Rodriguez, M. A. Holmes, R. K. Strong, S. Akira, A. Aderem, *Nature* **2004**, *432*, 917.
[119] M. A. Fischbach, H. Lin, D. R. Liu, C. T. Walsh, *Nat Chem Biol* **2006**, *2*, 132.
[120] H. R. Strange, T. A. Zola, C. N. Cornelissen, *Infect Immun*.
[121] S. W. Bearden, T. M. Staggs, R. D. Perry, *J Bacteriol* **1998**, *180*, 1135.
[122] J. D. Fetherston, V. J. Bertolino, R. D. Perry, *Mol Microbiol* **1999**, *32*, 289.
[123] J. Heesemann, K. Hantke, T. Vocke, E. Saken, A. Rakin, I. Stojiljkovic, R. Berner, *Mol Microbiol* **1993**, *8*, 397.

[124] A. J. Baumler, K. Hantke, *J Bacteriol* **1992**, *174*, 1029.
[125] R. Koebnik, K. Hantke, V. Braun, *Mol Microbiol* **1993**, *7*, 383.
[126] G. M. R. a. I. Smith, *J. Bact* **2005**, *188*, 424.
[127] V. Braun, A. Pramanik, T. Gwinner, M. Koberle, E. Bohn, *Biometals* **2009**, *22*, 3.
[128] R. Wayne, J. B. Neilands, *J Bacteriol* **1975**, *121*, 497.
[129] R. C. Hider, X. L. Kong, *Natural Product Reports* **2010**, *27*, 637.
[130] V. Braun, C. Bos, M. Braun, H. Killmann, *J Infect Dis* **2001**, *183 Suppl 1*, S12.
[131] J. H. Crosa, *Microbiol Rev* **1989**, *53*, 517.
[132] S. I. Muller, M. Valdebenito, K. Hantke, *Biometals* **2009**, *22*, 691.
[133] W. Bitter, I. S. van Leeuwen, J. de Boer, H. W. Zomer, M. C. Koster, P. J. Weisbeek, J. Tommassen, *Mol Gen Genet* **1994**, *245*, 694.
[134] P. Cornelis, *Appl Microbiol Biotechnol*, *86*, 1637.
[135] R. G. Ankenbauer, *J Bacteriol* **1992**, *174*, 4401.
[136] C. R. Dean, K. Poole, *Mol Microbiol* **1993**, *8*, 1095.
[137] C. R. Dean, K. Poole, *J Bacteriol* **1993**, *175*, 317.
[138] U. A. Ochsner, M. L. Vasil, *Proc Natl Acad Sci U S A* **1996**, *93*, 4409.
[139] B. Beall, G. N. Sanden, *Microbiology* **1995**, *141 ( Pt 12)*, 3193.
[140] T. J. Brickman, S. K. Armstrong, *J Bacteriol* **1999**, *181*, 5958.
[141] B. Beall, *Res Microbiol* **1998**, *149*, 189.
[142] C. Sauvage, T. Franza, D. Expert, *J Bacteriol* **1996**, *178*, 1227.
[143] S. Kuhn, V. Braun, W. Koster, *J Bacteriol* **1996**, *178*, 496.
[144] J. R. Butterton, J. A. Stoebner, S. M. Payne, S. B. Calderwood, *J Bacteriol* **1992**, *174*, 3729.
[145] G. F. Gause, *Br Med J* **1955**, *2*, 1177.
[146] G. e. a. Benz, *Angewandte Chemie* **1982**, 1322.
[147] G. Benz, *Liebigs Annalen Der Chemie* **1984**, 1399.
[148] G. Benz, *Liebigs Annalen Der Chemie* **1984**, 1424.
[149] G. Benz, L. Born, M. Brieden, R. Grosser, J. Kurz, H. Paulsen, V. Sinnwell, B. Weber, *Liebigs Annalen Der Chemie* **1984**, 1408.
[150] G. Benz, L. Born, J. Kurz, K. Metzger, H. Paulsen, D. Schmidt, *Abstracts of Papers of the American Chemical Society* **1984**, *188*, 111.
[151] G. Benz, D. Schmidt, *Liebigs Annalen Der Chemie* **1984**, 1434.
[152] V. Braun, K. Gunthner, K. Hantke, L. Zimmermann, *J Bacteriol* **1983**, *156*, 308.
[153] A. Pramanik, U. H. Stroeher, J. Krejci, A. J. Standish, E. Bohn, J. C. Paton, I. B. Autenrieth, V. Braun, *Int J Med Microbiol* **2007**, *297*, 459.
[154] W. Koster, B. Bohm, *Mol Gen Genet* **1992**, *232*, 399.
[155] G. Schultz-Hauser, W. Koster, H. Schwarz, V. Braun, *J Bacteriol* **1992**, *174*, 2305.
[156] H. B. Paulsen, M. Benz, G, *Liebigs Annalen Der Chemie* **1987**, 565.
[157] Y. M. Lin, M. J. Miller, *Journal of Organic Chemistry* **1999**, *64*, 7451.
[158] A. W. Vertesy L, Fehlhaber H-W, Koger H, *Helvetica Chim. Acta* **1995**, *78*, 46.
[159] J. M. Roosenberg, 2nd, M. J. Miller, *J Org Chem* **2000**, *65*, 4833.
[160] A. Pramanik, V. Braun, *J Bacteriol* **2006**, *188*, 3878.
[161] L. Dong, J. M. Roosenberg, 2nd, M. J. Miller, *J Am Chem Soc* **2002**, *124*, 15001.
[162] H. Bickel, P. Mertens, V. Prelog, J. Seibl, A. Walser, *Tetrahedron* **1966**, *S*, 171.
[163] G. H. Vondenhoff, S. Dubiley, K. Severinov, E. Lescrinier, J. Rozenski, A. Van Aerschot, *Bioorg Med Chem*, *19*, 5462.
[164] G. H. Vondenhoff, B. Blanchaert, S. Geboers, T. Kazakov, K. A. Datsenko, B. L. Wanner, J. Rozenski, K. Severinov, A. Van Aerschot, *J Bacteriol* **2011**, *193*, 3618.
[165] M. Novikova, A. Metlitskaya, K. Datsenko, T. Kazakov, A. Kazakov, B. Wanner, K. Severinov, *J Bacteriol* **2007**, *189*, 8361.
[166] C. Qiao, A. Gupte, H. I. Boshoff, D. J. Wilson, E. M. Bennett, R. V. Somu, C. E. Barry, 3rd, C. C. Aldrich, *J Med Chem* **2007**, *50*, 6080.

[167] R. V. Somu, H. Boshoff, C. Qiao, E. M. Bennett, C. E. Barry, 3rd, C. C. Aldrich, *J Med Chem* **2006**, *49*, 31.
[168] J. Vannada, E. M. Bennett, D. J. Wilson, H. I. Boshoff, C. E. Barry, 3rd, C. C. Aldrich, *Org Lett* **2006**, *8*, 4707.
[169] C. Balg, S. P. Blais, S. Bernier, J. L. Huot, M. Couture, J. Lapointe, R. Chenevert, *Bioorg Med Chem* **2007**, *15*, 295.
[170] E. M. Peterson, J. Brownell, R. Vince, *J Med Chem* **1992**, *35*, 3991.
[171] H. Ueda, Y. Shoku, N. Hayashi, J. Mitsunaga, Y. In, M. Doi, M. Inoue, T. Ishida, *Biochim Biophys Acta* **1991**, *1080*, 126.
[172] M. Antal, V. Bordeau, V. Douchin, B. Felden, *J Biol Chem* **2005**, *280*, 7901.
[173] A. Metlitskaya, T. Kazakov, G. H. Vondenhoff, M. Novikova, A. Shashkov, T. Zatsepin, E. Semenova, N. Zaitseva, V. Ramensky, A. Van Aerschot, K. Severinov, *J Bacteriol* **2009**, *191*, 2380.
[174] T. Kazakov, A. Metlitskaya, K. Severinov, *J Bacteriol* **2007**, *189*, 2114.
[175] M. Novikova, T. Kazakov, G. H. Vondenhoff, E. Semenova, J. Rozenski, A. Metlytskaya, I. Zukher, A. Tikhonov, A. Van Aerschot, K. Severinov, *J Biol Chem*, *285*, 12662.
[176] A. Tikhonov, T. Kazakov, E. Semenova, M. Serebryakova, G. Vondenhoff, A. Van Aerschot, J. S. Reader, V. M. Govorun, K. Severinov, *J Biol Chem*, *285*, 37944.
[177] S. Wydau, G. van der Rest, C. Aubard, P. Plateau, S. Blanquet, *J Biol Chem* **2009**, *284*, 14096.
[178] A. K. Malde, A. E. Mark, *J Am Chem Soc* **2009**, *131*, 3848.
[179] J. Soutourina, P. Plateau, S. Blanquet, *J Biol Chem* **2000**, *275*, 32535.
[180] G. Zheng, W. Liu, Y. Gong, H. Yang, B. Yin, J. Zhu, Y. Xie, X. Peng, B. Qiang, J. Yuan, *Biochem J* **2009**, *417*, 85.
[181] H. Yang, G. Zheng, X. Peng, B. Qiang, J. Yuan, *FEBS Lett* **2003**, *552*, 95.
[182] M. Werle, A. Bernkop-Schnurch, *Amino Acids* **2006**, *30*, 351.
[183] P. Van de Vijver, G. H. Vondenhoff, S. Denivelle, J. Rozenski, J. Verhaegen, A. Van Aerschot, P. Herdewijn, *Bioorg Med Chem* **2009**, *17*, 260.
[184] S. T. a. B. Cheung, L, *Can. J. Chem.* **1977**, *55*.
[185] R. Rink, A. Arkema-Meter, I. Baudoin, E. Post, A. Kuipers, S. A. Nelemans, M. H. Akanbi, G. N. Moll, *J Pharmacol Toxicol Methods*, *61*, 210.
[186] T. Kazakov, G. H. Vondenhoff, K. A. Datsenko, M. Novikova, A. Metlitskaya, B. L. Wanner, K. Severinov, *J Bacteriol* **2008**, *190*, 2607.
[187] J. Hill, Cubist Pharmaceuticals, Inc., United States, **1998**.
[188] J. Hill, in *37th ICCAC*, **1999**.
[189] P. Schimmel, J. Tao, J. Hill, *Faseb J* **1998**, *12*, 1599.
[190] R. M. Herbst, K. R. Wilson, *Journal of Organic Chemistry* **1957**, *22*, 1142.
[191] J. Lee, S. U. Kang, S. Y. Kim, S. E. Kim, M. K. Kang, Y. J. Jo, S. Kim, *Bioorganic & Medicinal Chemistry Letters* **2001**, *11*, 961.
[192] S. B. Mandal, B. Achari, *Synthetic Communications* **1993**, *23*, 1239.
[193] H. Ohrui, G. H. Jones, J. G. Moffatt, M. L. Maddox, A. T. Christensen, S. K. Byram, *Journal of the American Chemical Society* **1975**, *97*, 4602.
[194] Y.-M. L. a. M. J. Miller, *Journa of Organic chemistry* **1999**, *64*, 7451.
[195] C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney, *Adv Drug Deliv Rev* **2001**, *46*, 3.
[196] D. Chandu, D. Nandi, *Microbiology* **2003**, *149*, 3437.

## Curriculum vitae

Gaston Vondenhoff was born in 1983 in Meerssen, The Netherlands. He commenced his bachelor studies at Utrecht University in 2002 where he obtained two bachelors: one in chemistry, the second in biomedical sciences. At the same university he obtained his master degree (*cum laude*) in chemistry in 2008. During the same year he started his PhD studies at the Laboratory of Medicinal research, Rega Institute for Medical Research, KU Leuven under supervision of Prof. Dr. Arthur Van Aerschot.

### *Publications*


1. Kazakov, T., **Vondenhoff, G. H.**, Novikova, M., Datsenko, K., Wanner, B., and Severinov, K. E. coli peptidases A, B, or N can process translation inhibitor Microcin C. J. Bacteriol. 2008, 190, 2607-2610.
2. Van de Vijver P, **Vondenhoff GH**, Denivelle S, Rozenski J, Verhaegen J, Van Aerschot A, Herdewijn P. Antibacterial 5'-O-(N-dipeptidyl)-sulfamoyladenosines. Bioorg Med Chem. 2009, 17(1):260-9
3. Metlitskaya, A., Kazakov, T., **Vondenhoff, G. H.**, Novikova, M., Semenova, E., Shashkov, A., Zaitseva, N., Ramensky, V., Van Aerschot, A and Severinov, K. Maturation of translation inhibitor microcin C. J. Bacteriol. 2009, 191, 2380-2387
4. **Vondenhoff, G.H.M.***, Van de Vijver, P*., Kazakov, T*., Semenova, E., Kuznedelov, K., Metlitskaya, A., Van Aerschot, A., and Severinov, K. Synthetic Microcin C analogs targeting different aminoacyl-tRNA synthetases. J. Bacteriol. 2009, 20, 6273-80. *equally contributing
5. Novikova M, Kazakov T, **Vondenhoff GH**, Semenova E, Rozenski J, Metlytskaya A, Zukher I, Tikhonov A, Van Aerschot A, Severinov K. MccE provides resistance to protein synthesis inhibitor Microcin C by acetylating the processed form of the antibiotic. J Biol Chem. 2010, 285, 12662-12669
6. Tikhonov, A. Kazakov, T. Semenova, E. Serebryakova, M. **Vondenhoff, G**. Van Aerschot, A. Reader, J. S. Govorun, V. M. Severinov, K. The mechanism of microcin C resistance provided by the MccF peptidase. J. Biol.Chem, 2010, 285(49): 37944-52.
7. **Gaston H. M. Vondenhoff**, Bart Blanchaert, Sophie Geboers, Teymur Kazakov, Kirill A. Datsenko, Barry L. Wanner, Jef Rozenski, Konstantin Severinov and Arthur Van Aerschot. Characterization of peptide chain length and constituency requirements for YejABEF-mediated uptake of Microcin C analogues. J Bacteriol, 2011 193, 3618-3623
8. **Gaston H. M. Vondenhoff** and Arthur Van Aerschot. Microcin C: biosynthesis, mode of action and potential as a lead in antibiotics development. Nucleosides Nucleotides Nucleic Acids, 2011, 30, 465-474
9. **Gaston H. M. Vondenhoff**, Svetlana Dubiley, Konstantin Severinov, Eveline Lescrinier, Jef Rozenski and Arthur Van Aerschot. Extended targeting potential and

improved synthesis of Microcin C analogues as antibacterials. Bioorganic and Medicinal Chemistry. Bioorg Med Chem, 2011, 19, 5462-5467

10. **Gaston H. M. Vondenhoff** and Arthur Van Aerschot. Aminoacyl-tRNA Synthetase Inhibitors as potential antibiotics. Eur J Med Chem, 2011, 46, 5227-5236
11. Boguslaw Nocek, Anton Tikhonov, Gyorgy Babnigg, Minyi Gu, Min Zhou, Kira S. Makarova, **Gaston Vondenhoff**, Arthur Van Aerschot, Wayne Anderson, Konstantin Severinov and Andrzej Joachimiak. Structural and functional characterization of microcin C resistance peptidase MccF from *Bacillus anthracis*. Submitted to JMB
12. Teymur Kazakov, Konstantin Kuznedelov, Ekaterina Semenova, Damir Mukahmedjarov, Kirill A. Datsenko, Anastasiya Metlytskaja, **Gaston H. Vondenhoff**, Barry L. Wanner, Arthur Van Aerschot, and Konstantin Severinov The RimL Transacetylase Provides Resistance to Translation Inhibitors Microcin C and albomycin. Submitted to JBC
13. **Gaston H. Vondenhoff**, Bharat Gadakh, Konstantin Severinov, Arthur Van Aerschot. Microcin C and albomycin analogues with aryl-tetrazole substituents as nucleobase isosters are selective inhibitors of bacterial aminoacyl tRNA synthetases but lack efficient uptake. Submitted to ChemBioChem.

### *Patent*

**US Patent** (US Provisional Patent Number: 61/373,314). Patent filed for Konstantin Severinov, Teymur Kazokov, Gaston Vondenhoff, Arthur Van Aerschot, Pieter Van De Vijver, Ekaterina Semenova

### *Awards and Scholarships*

| | |
|---|---|
| 2009-present | PhD Scholarship from Agentschap voor Innovatie door Wetenschap en Technologie (IWT) |
| 21/5/2008 | USPS-award for excellent study results and stimulation of inter-University co-operation |

### *Attended Symposia, summer-schools and meetings*

- 1/8- till 17/8 2011 LEADER Summer School Aarhus University, Aarhus, Denmark
- 19/5 till 24/5 2011 American Society for Microbiology Meeting. New Orleans, USA. **Poster presentation**
- 1/2 and 2/2 2010 10$^{th}$ Flemish Youth Congress of Chemistry, Blankenberge, Belgium. **Oral Presentation**
- 9/10 2009 International Symposium on chemical protein synthesis, Maastricht, The Netherlands
- 26/6 till 4/7 2009 ULLA Summer School, Copenhagen, Denmark. **Poster presentation**
- 2008 Roche Continents, Salzburg, Austria.