

SYNTHESIS, STRUCTURAL AND BIOLOGICAL EVALUATION OF GRAMICIDIN S ANALOGUES

PROEFSCHRIFT

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Gijsbert Marnix Grotenbreg

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Promotor	:	Prof. dr. H. S. Overkleef
Co-promotores	:	Dr. G. A. van der Marel Dr. M. Overhand
Referent:	:	Prof. dr. J. C. M. van Hest (RU)
Overige leden	:	Prof. dr. H. E. Schoemaker (UvA) Prof. dr. A. van der Gen Prof. dr. J. Lugtenburg Prof. dr. J. Reedijk

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List of Abbreviations

Δ Ala	2,3-dehydroalanine	CCDC	cambridge crystallographic data centre
Δ Phe	2,3-dehydrophenylalanine	CFU	colony forming units
4Br-Phe	4-bromophenylalanine	Cha	cyclohexylalanine
4F-Phe	4-fluorophenylalanine	COSY	correlation spectroscopy
Ac	acetyl	CV	column volume
ACN	acetonitrile	d	doublet
AcOH	acetic acid	d	downfield
Ala	alanine	Dap	diaminopropionic acid
Ala	alanine	DCM	dichloromethane
Amp	4-aminoproline	dd	double doublet
Amy	2-aminomyristic acid	ddd	double doublet of doublets
aq	aqueous	DIC	<i>N,N'</i> -diisopropylcarbodiimide
ar	aromatic	DiPEA	<i>N,N'</i> -diisopropylethylamine
Arg	arginine	DMAP	4-dimethylaminopyridine
Asn	asparagine	DMF	<i>N,N'</i> -dimethylformamide
Asp	aspartic acid	DMSO	dimethylsulfoxide
ATCC	american type culture collection	DPhPC	diphytanoylphosphatidylcholin
ATR	attenuated total reflectance	DPPA	diphenylphosphoryl azide
ax	axial	EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
Azp	4-azidoproline	EDTA	ethylenediamine- <i>N,N,N',N'</i> -tetraacetic acid
BAIB	(bisacetoxiodo)benzene	eq	equitorial
Biph	biphenyl	equiv	molar equivalent
Bn	benzyl	ESI	electrospray ionization
Boc	<i>tert</i> -butyloxycarbonyl	Et	ethyl
BOP	benzotriazol-1-yl	Fmoc	9-fluorenylmethyloxycarbonyl
	oxytri(dimethylamino)phosphonium hexafluorophosphate	G ⁻	Gram-negative
Bu	butyl	G ⁺	Gram-positive
calcd	calculated	GA	gramicidin A
CAP	cationic antimicrobial peptide		

Gln	glutamine	pcp	peptidyl carrier protein domain
Glu	glutamic acid	PE	petroleum ether
Gly	glycine	PEG	polyethylene glycol
GS	gramicidin S	Pfp	pentafluorophenol
h	hour	Ph	phenyl
Hfv	hexafluorovaline	Phe	phenylalanine
His	histidine	Phth	phthaloyl
HMPB	4-(4-hydroxymethyl-3-methoxyphenoxy)butanoic acid	Piv	pivaloyl
HOBt	<i>N</i> -hydroxybenzotriazole	ppm	parts per million
HONSu	<i>N</i> -hydroxysuccinimide	Pro	proline
HPLC	high performance liquid chromatography	Pya	1-pyrenylalanine
HRMS	high-resolution mass spectrometry	PyBOP	benzotriazol-1-yloxytri-pyrrolidinophosphonium hexafluorophosphate
Hyp	4-hydroxyproline	q	quartet
Hz	hertz	quant	quantitative
<i>i</i> Pr	isopropylidene	ROESY	rotating frame nuclear Overhauser effect spectroscopy
IR	infrared spectroscopy	RP	reversed phase
<i>J</i>	coupling constant	R _t	retention time
Lac	lactic acid	rt	room temperature
LC/MS	liquid chromatography / mass spectrometry	s	singlet
Leu	leucine	SAA	sugar amino acid
Lys	lysine	sat.	saturated
M	molar	Ser	serine
m	multiplet	SNAC	<i>N</i> -acetylcysteamine thioester
<i>m/z</i>	mass to charge ratio	SPPS	solid phase peptide synthesis
MAA	morpholine amino acid	<i>t</i>	tertiary
MBHA	4-methylbenzhydramine	t	triplet
Me	methyl	TA	tyrocidine A
MIC	minimal inhibitory concentration	TE	thioesterase domain
min	minute	TEA	triethylamine
MS	mass spectrometry	TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxyl
Ms	methylsulfonyl	TFA	trifluoroacetic acid
MT	microtiter	THF	tetrahydrofuran
Naph	naphtyl	TLC	thin layer chromatography
NMP	<i>N</i> -methylpyrrolidinone	TOCSY	total correlation spectroscopy
NMR	nuclear magnetic resonance	Tos	<i>p</i> -toluenesulfonyl
NOE	nuclear Overhauser effect	Tr	triphenylmethyl
NOESY	nuclear Overhauser effect spectroscopy	Trp	tryptophan
Np	<i>p</i> -nitrophenyl	Tyr	tyrosine
NRPS	nonribosomal peptide synthetase	Tyr	tyrosine
Orn	ornithine	u	upfield
<i>p</i>	para	Val	valine
PAM	4-hydroxymethylphenyl-acetamidomethyl	Z	benzyloxycarbonyl

Chapter 1

General Introduction

1.1 Antibiotics

Antibiotics are substances that have the capacity to kill or inhibit the growth of microorganisms. The potential to exploit natural antibiotics as therapeutic agents was first put forward by Louis Pasteur, who found that anthrax bacilli (*Bacillus anthracis*) cultivated outside the body were destroyed when brought into contact with *Escherichia coli*.¹ Based on this observation, he speculated that the antagonism occurring between microorganisms could eventually be used for the treatment of human bacterial diseases. A subsequent milestone is the serendipitous discovery, by Alexander Fleming, that a contamination of a culture plate of staphylococci colonies by the mould *Penicillium notatum* resulted in the killing of the bacteria.² At the time, it was common knowledge that microorganisms possessed the means to interfere with the proliferation of one another in their competition for living space and sustenance. However, Fleming was the first to isolate an antibacterial substance which he named penicillin. Elaborating on the work of Fleming, Florey and Chain were able to obtain penicillin in its crystalline form and studied its chemical composition and structure.³ The elucidation of its structure and ensuing synthetic studies towards penicillin paved the way for its mass production. The antibiotic properties of penicillin combined with its low toxicity towards eukaryotes have been and still are of immense value in the battle against infection and bacterial disease.

1.2 Major targets for antibiotic action

Over the years, many different compounds that target specific bacteria have been developed, both from natural sources and through synthetic efforts.⁴ These compounds can be categorized in different ways. Some compounds lead to bacterial cell death and are called bactericidals, whereas others merely arrest bacterial cell division and are called bacteriostatics. Obviously different compound classes can be distinguished based on the origin of the bacteria they target. Often antibiotics are subdivided into those that act against Gram-positive bacteria exclusively, those that target only Gram-negative bacteria and those that act against both. Perhaps the most comprehensive subdivision is the one that takes into account the molecular mechanism that is at the basis of the antibacterial action of antibiotics. Such a categorization provides insight not only in the mechanism of action but also in how the targeted bacterial strains find their way around the antibiotic action and gain resistance. Antibacterial compounds constitute a broad class of structurally different molecules. The structural diversity is directly related to the many (sub)cellular targets they act on, ranging from DNA regulation and replication to protein synthesis, metabolic pathways and compounds that target the integrity of the cell surface. The different cellular targets and their corresponding antibiotics will be discussed here briefly.

1.2.1 *The cell wall*

The bacterial cell wall is responsible for maintaining high local concentrations of components and protects the bacteria from adverse environmental influences, such as the effects of osmotic pressure. Classification of bacteria on the basis of the complexity of their cell wall structure can be done by the ability of the cell wall to retain a crystal violet dye during Gram-staining. Both Gram-positive (G^+) and Gram-negative (G^-) bacteria are surrounded by a cytoplasmic membrane that is covered with a peptidoglycan layer. The peptidoglycan is composed of a cross-linked sugar-peptide heteropolymer that provides structural support to the cell (Figure 1). Whereas G^+ bacteria have a thick peptidoglycan layer, G^- bacteria have a relatively thin peptidoglycan coat, that is surrounded with a second membrane: the outer membrane. The surface of both classes of bacteria is decorated with a wide variety of proteins and oligosaccharides. Inhibition of bacterial cell wall biosynthesis has proven to be a very effective antibiotic strategy. For example, β -lactam antibiotics such as the penicillins and the cephalosporins (see Table 1) inhibit transpeptidases that are responsible for the cross-linking of the peptidoglycan layer, thereby disrupting the structural integrity of the cell wall. The binding of vancomycin, a glycopeptide, to the muramyl pentapeptide prevents its access to transpeptidase activity, leading to the inhibition of the cross-linking of the peptidoglycan layer in an alternative fashion. The end result of the action of β -lactams and vancomycin derivatives is the same: bacterial lysis and cell death.

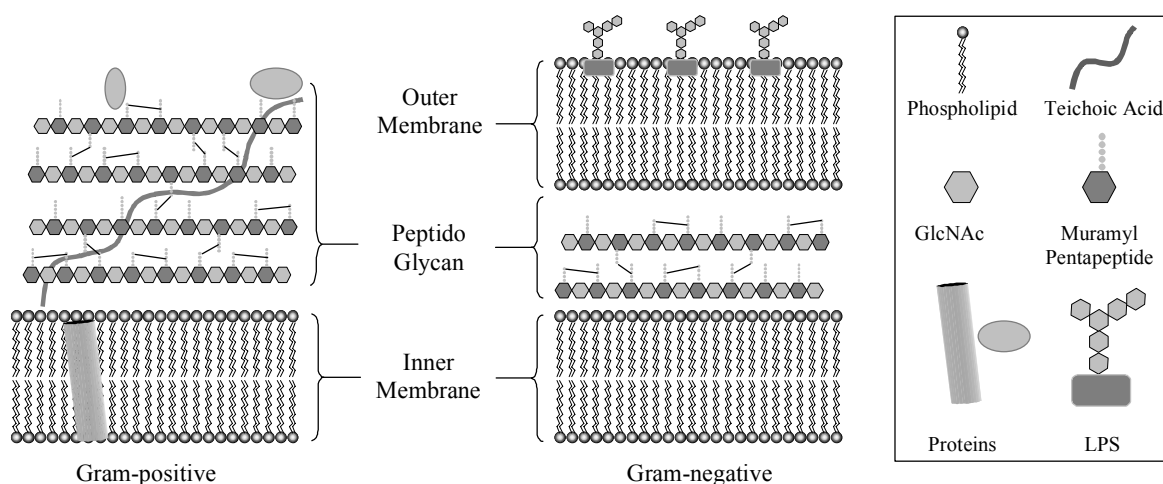


Figure 1: Cell wall composition of Gram-positive and Gram-negative bacteria.

1.2.2 Protein synthesis

The translation of genetic material into a polypeptide chain involves a great number of individual components and steps. Some representative classes of antibiotics that selectively inhibit the function of bacterial ribosomes, the primary sites of protein synthesis, are the aminoglycosides, tetracyclines and macrolides. Aminoglycosides bind to the ribosome and induce a conformational change that increases the chance of misreading of the messenger RNA information. Macrolide antibiotics inhibit protein synthesis by binding to rRNA of the bacterial ribosome in such a fashion that it blocks the exit of the growing peptide chain.

1.2.3 DNA and RNA synthesis

Topoisomerases are responsible for breaking and rejoining double-stranded DNA, thereby influencing the degree of supercoiling in DNA. Various topoisomerases relax the supercoiling of DNA, thereby enabling replication or transcription of the DNA. Conversely, gyrases return the DNA to the supercoiled state after transcription or replication has taken place. Interfering with these enzymatic pathways constitutes an entry towards arresting the multiplication of pathogens. For example, quinolone and coumarin antibiotics affect the cleavage / religation equilibrium such that the cleaved complex accumulates and the DNA cannot return to its proper topology.

1.2.4 Folic acid metabolism

Folic acid is an important co-factor in one-carbon transfer reactions involved in the biosynthesis of amino acids and nucleotides. Whereas bacteria are reliant on their own folate synthesis, eukaryotes obtain folic acid from dietary sources, making bacterial folic acid biosynthesis a valid antibiotic target. For instance, sulfamethoxazole, a member of the so-called sulfa drugs, is a structural analogue of *p*-aminobenzoic acid (PABA), one of the intermediates in the folic acid biosynthesis. As such, sulfamethoxazole acts as a competitive

inhibitor of the enzyme dihydropteroate synthetase. Sulfa drugs are the first fully synthetic antibiotics that found application in the clinic.

1.2.5 Cellular membrane

Over the years, a number of bactericidal peptides have been identified that interfere in one way or another with the integrity of the bacterial cell membrane. Some of these have found therapeutic application as systemic antibiotic but more frequently as topical agent, such as gramicidin S and polymyxin. These cationic antimicrobial peptides will be discussed in detail in the section 2 of this chapter.

Table 1: Common antibiotics in clinical use

Class	Target	Examples
Penicillins	Peptidoglycan biosynthesis	Penicillin G, Amoxicillin
Cephalosporins	Peptidoglycan biosynthesis	Cephazolin, Cefuroxim
Glycopeptides	Peptidoglycan biosynthesis	Vancomycin, Teicoplanin
Aminoglycosides	Protein biosynthesis	Kanamycin, Neomycin
Tetracyclins	Protein biosynthesis	Tetracyclin, Chlortetracyclin
Macrolides	Protein biosynthesis	Erythromycin, Telithromycin
Oxazolidinones	Protein biosynthesis	Linezolid, Eperezolid
Quinolones	DNA replication	Ciprofloxacin, Gatifloxacin
Coumarins	DNA replication	Novobiocin
Sulpha drugs	Folate biosynthesis	Sulphamethoxazole
Peptide antibiotics	Cell membrane	Polymyxin, Daptomycin

1.3 Resistance towards antibiotics

From the onset of the therapeutic application of antibiotics, it was evident that certain species of bacteria were not sensitive to the drugs.⁵ Moreover, the effectivity of antibiotic agents is often compromised after prolonged use, due to the development of drug-resistant bacterial strains.⁶ The emergence of antibiotic-resistant strains can be viewed as an evolutionary selection in which bacteria with an acquired mutation that confers resistance to the antibiotic have a selective survival advantage over those that do not have the mutation. Upon encountering an antibiotic, the resistant bacteria flourish due to an increase in nutrients which their nonresistant counterparts would have competed for. The spread of antibiotic resistance can be accelerated through gene exchange between different bacterial species.⁷

1.3.1 Antibiotic efflux

An important mechanism by which bacteria counter the effects of antibiotics is to transport the antibiotics out of the cell. This efflux of antibiotics is mediated by transmembrane pumps that promote the unidirectional export from cytoplasmic compartments. Several of these transporter protein complexes act upon a narrow range of structurally related substrates. However, export systems that bacteria previously used for the uptake and excretion of metabolic products have evolved into multidrug efflux pumps and can handle a variety of structurally dissimilar compounds.⁸ Multidrug efflux pumps can be subdivided into a number of distinct families with varying molecular architecture, mechanisms of action and energy requirements.⁹

1.3.2 Antibiotic modification

Bacteria can resist the action of antibiotics by the enzymatic destruction or modification of the antibiotic. For example, the hydrolytic activity of β -lactamases is responsible for degradation of penicillins and cephalosporins.¹⁰ The hydrolysis of the β -lactam ring disables the acylating activity of the antibiotic. Aminoglycoside antibiotics are also sensitive to deactivation by the covalent modification of specific amino- or hydroxyl functionalities. The binding affinity of aminoglycosides for the bacterial ribosome can be severely impaired through *N*-acetylation, *O*-phosphorylation or *O*-adenylation at susceptible positions.¹¹

1.3.3 Target modification

The action of an antibiotic can be nullified by the replacement or modification of cellular targets such as the cell wall constituents, proteins or genetic material, into insensitive forms. A striking example of target modification is found in the emergence of resistance towards the glycopeptide antibiotic vancomycin. The binding of vancomycin to the ^DAla-^DAla terminus of the muramyl pentapeptide, being the substrate of transpeptidases, prohibits the cross-linking of the peptidoglycan. Through a series of genetic modifications, vancomycin resistant pathogens have been able to modify their ^DAla-^DAla terminus into the ^DAla-^DLac depsipeptide that confers a considerable loss of affinity for the antibiotic.¹²

2.1 Cationic antimicrobial peptides

Cellular membranes are crucial for the viability of bacterial cells because they separate the intracellular from the extracellular world. The membrane architecture, primarily a lipid bilayer composed of phospholipids, is targeted by cationic antimicrobial peptides (CAPs). The disruption of the membrane integrity by CAPs causes a loss in barrier function.¹³ Prokaryotic and eukaryotic organisms employ a plethora of structurally and functionally diverse CAPs in

their nonadaptive immune defense systems.¹⁴ These nonspecific effectors display their cell-lytic activity against a variety of microorganisms such as G^+ and G^- bacteria. In this paragraph, general structural characteristics found in CAPs as well as several models describing their mode of action will be discussed.

2.2 Structural characteristics of CAPs

A plethora of primary structures of CAPs have been identified over the past decades, as is documented in several reviews.^{13,14} What becomes evident from the various primary structures is the prevalence of lipophilic and cationic amino acid residues. Furthermore, CAPs are often found to adopt specific secondary structures resulting in the distribution of hydrophobic and hydrophilic residues onto separate surfaces. Finally, CAPs regularly contain nonproteinogenic residues. To highlight the extensive differences in the number of residues, primary sequences, positioning of charged residues, secondary structures and their origin, some examples (peptides **1-8**) are given in Table 2.

Table 2: Cationic antimicrobial peptides.

	Peptide	Sequence	Structure	Origen
1	gramicidin A	VGA ^D LA ^D VV ^D VW ^D LW ^D LW ^D LW-NHCH ₂ CH ₂ OH	α -helix	<i>B. Brevis</i>
2	mellitin	GIGAVLKVTLTGLPALISWIKRKRQ	α -helix	Bee venom
3	maigainin 2	GIGKFLHSAKKFGKAFVGEIMNS	α -helix	Frog
4	cathelicidin LL37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	α -helix	Human
5	gramicidin S	<i>cyclo</i> -(^D FPVOL ^D FPVOL)	β -sheet	<i>B. Brevis</i>
6	tachyplesin I	KWC ₁ FRVC ₂ YRGIC ₂ YRRC ₁ R	β -sheet	Horseshoe crab
7	bactenecin	RLCRIVVIRVCR	β -sheet	Cow
8	θ -defensin	<i>cyclo</i> -(GFC ₁ RC ₂ LC ₃ RRGVC ₃ RC ₂ IC ₁ TR)	β -sheet	Monkey

2.2.1 Lipophilic and cationic amino acid residues

CAPs are generally comprised of cationic residues (that is Lys, Arg, Orn) with an overall net charge of +2 or more. The overall positive charge is believed to facilitate the initial interactions with negatively charged membrane phospholipids. The preferential binding to negatively charged bacterial membranes confers some specificity to the CAPs, because CAPs are less attracted by zwitterionic mammalian plasma membranes. After arrival of the CAP at the membrane surface, the intrinsic hydrophobicity stemming from the lipophilic amino acid residues (for instance Val, Leu and Ala) allows the CAP to partition into the lipid bilayer.

2.2.2 Secondary structure and amphiphilicity

CAPs frequently assume a specific three-dimensional conformation, aided by secondary structure elements, that segregates the hydrophobic and cationic amino acid residues. This results in the nonpolar amino acid side-chains making up a hydrophobic face and the positively charged polar residues making up a hydrophilic face. Such an arrangement is referred to as either amphipathic or amphiphilic. The adoption of secondary structure allows the crude classification of CAPs into two groups, namely the α -helical and β -sheet peptides (see Table 2).

The structural determinants influencing the permeabilizing properties as well as antimicrobial and hemolytic activity of α -helical CAPs have been extensively studied and charted.^{13a-e} However, it remains difficult to discern guiding principles in the biological activity of α -helical CAPs, for changes in primary structure directly influences the hydrophobicity, hydrophilicity, helicity and consequently the polar and hydrophobic domains. In a characteristic example of α -helical CAPs, maigainin 2 (**3**) is depicted in a helical wheel presentation (Figure 2A). The peptide is viewed along the helical axis which clearly demonstrates the positively charged and lipophilic amino acid residue distribution. The β -sheet CAPs are comprised of a variable number of β -strands that are arranged in parallel or antiparallel fashion. Disulfide bridges and/or a cyclic backbone further stabilize an extended conformation. The β -sheet structure of these CAPs enables the positioning of the amino acid side chains in amphiphilic arrangements. Interestingly, the resulting conformations are not always perfectly amphiphilic, as can be gauged from the example of tachyplesin I (**6**) in Figure 2B.

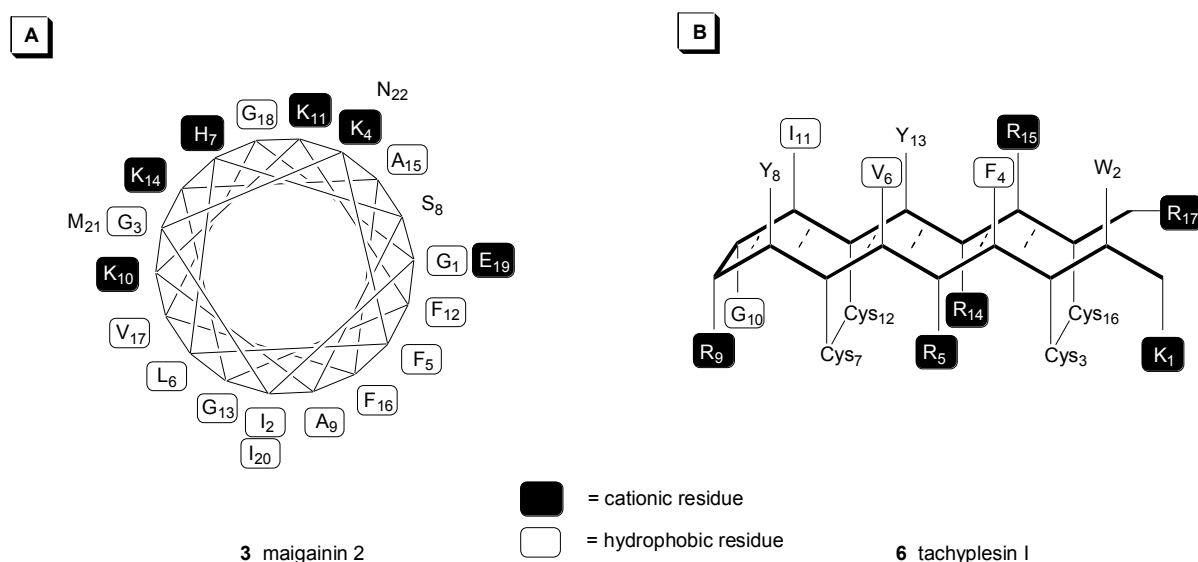


Figure 2: Schematic distribution of amino acid side chains in α -helical and β -sheet CAPs. (A) Helical wheel presentation of maigainin 2 (B) Side view cartoon of a tachyplesin I.

2.2.3 Nonribosomal peptide synthesis and nonproteinogenic residues

The ribosomally produced peptide antibiotics form a major component of the natural immune defense in all species of life. In addition, the biosynthesis of bacterial CAPs is often accomplished by multidomain enzymes known as nonribosomal peptide synthetases (NRPS).¹⁵ These large multimodular enzymes form an assembly-line in which multiple domains are responsible for the activation and incorporation of a specific amino acid, as well as the optional modification of the separate amino acids, as will be discussed in more detail for gramicidin S in section 3 of this chapter. The number and order of this modular architecture usually corresponds to the number of amino acids and the sequence in which the peptide is being constructed, respectively. Several domains embedded within the modules of the enzymatic assembly line are able to introduce modifications to the amino acids that are incorporated. For example, racemases provide the requisite D-amino acids from the L-amino acid pool, N-methylation domains are able to methylate the α -amino group of amino acids, and serine, threonine or cysteine residues can be heterocyclized. Next to the incorporation of these nonproteinogenic amino acids, postsynthetic modifications such as oxidative cross-linking, glycosylation, C-terminal amidation and halogenation are amongst those associated with the peptides assembled by NRPS production lines, thereby making these secondary metabolites extraordinarily diverse.

2.3 Mechanism of action of CAPs^{16,17}

The initial CAP interactions with the target cell surface occurs through electrostatic attraction between the cationic peptide and the negatively charged phospholipid membranes of bacteria. Other common constituents of bacterial membranes such as lipopolysaccharides (LPS) and teichoic acid in Gram-negative and Gram-positive bacteria, respectively, also donate to the overall negative charge of the target cell surface, thereby increasing the electrostatic interaction. Having arrived at the cell surface, the peptidoglycan (for G^+ bacteria) and LPS-containing outer membrane (in the case of G^- bacteria) needs to be traversed by the CAP, before reaching the inner membrane (see Figure 1). In this respect, Hancock and coworkers have postulated the self-promoted uptake in which the positively charged CAPs take the place of divalent cations on surface LPS.¹⁸ By binding to anionic sites of the LPS, barrier function of the outer membrane dissipates which supports the further uptake of antibiotics. This sensibilization of Gram-negative bacteria is used clinically to enhance the uptake of other antibiotics.

Upon arrival of the CAP on the inner membrane, insertion of the lipophilic side-chains of the peptide into the hydrophobic environment of the lipid bilayer takes place. When the α -helical CAP interacts with a lipid surface, a conformational phase transition can precede lytic activity. The α -helical CAPs first exist as disordered structures in aqueous solution but fold

into their α -helical amphiphilic arrangement upon interaction with the lipid surfaces. In contrast, the structural constraints (such as disulfide bridges or cyclic structures) already present in β -sheet CAPs preserve the secondary structure. Therefore β -sheet CAPs adopt the same conformation both in aqueous media and in lipid environments. Accumulation of either α -helical or β -sheet CAPs in the lipid bilayer ultimately results in a threshold concentration of CAPs, after which both nonspecific membrane disruption or self-association and the assembly of quarternary structures with ensuing pore formation will take place. The mechanism by which these peptides induce permeability and traverse the microbial membranes is likely to differ for various CAPs and the membrane environments in which they are studied. Several models have been postulated to describe the *modus operandi* of CAPs (see Figure 3) is discussed below.

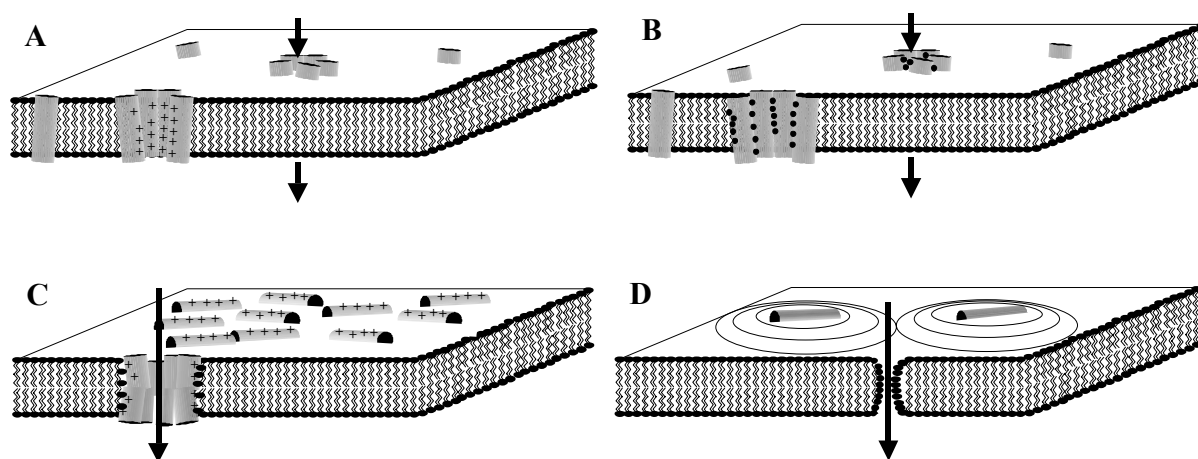


Figure 3: Transmembrane helical bundle model (A), wormhole model (B), carpet model (C), In-plane diffusion model (D).

2.3.1 The transmembrane helical bundle model¹⁹

The oldest model for the formation of pores across lipid bilayers that are induced by membrane associated peptides is the “barrel-stave” or “transmembrane helical bundle” model (Figure 3A). In this model, the individual peptides traverse the membrane and are bundled together around an aqueous pore. The hydrophobic amino acid residues face towards the acyl chains of the phospholipids whilst the hydrophilic inner surface of the barrel is lined with the cationic moieties stemming from the CAPs. The self-aggregation towards distinct quarternary structures helps to explain the reproducible stepwise increases of conductivity observed in some biophysical studies.

2.3.2 The wormhole model²⁰

The “toroid” or “wormhole” model, as depicted in Figure 3B, is an adaptation of the helical bundle model. In the helical bundle model, a large amount of positive charge is confined to a

small space. The negatively charged headgroups of lipids separate this charge in the wormhole model, thus forming a transient supramolecular membrane-spanning complex with the interior surface composed of polar peptide side-chains and phospholipid head groups.

2.3.3 *The carpet model*²¹

The above described two models do not give a satisfactory explanation for the fact that most active peptides are actually too small to completely traverse the lipid bilayer. Moreover, biophysical studies indicate that lytic peptides are often orientated parallel to the membrane surface. Subsequently, a model was proposed in which the peptides are initially adsorbed onto the membrane and cover the surface in a carpet-like manner (see Figure 3C). At a high local density of peptide, the structural organization of the membrane will become perturbed which causes a change in membrane fluidity and reduces the membranes barrier function. This type of peptide-induced membrane instability occurs in a disperse manner without requiring the insertion of CAPs into the hydrocarbon chain section of the membrane or adoption of a given secondary or macromolecular structure.

2.3.4 *The in-plane diffusion model*²²

Even in the presence of negatively charged phospholipids, aggregation of cationic peptides in the membrane surface is an entropically and electrostatically disfavoured process. To further take into consideration that CAPs can induce their lytic effects at comparatively low peptide-to-lipid ratios, the in-plane diffusion model (Figure 3D) was conceived. In this model, membrane-associated peptides disturb the lipid packing over a large surface area. By diffusion of the CAPs these disturbances can overlap resulting in the collapse of lipid packing and inducing temporary openings in the membrane.

Finally, the effect CAPs have on lipid bilayers by acting as detergent-like substances should also be taken into account. By inserting the hydrophobic residues of the antimicrobial peptides in the acyl portion of lipid bilayer, the polar head groups of the lipids are displaced and interact with the cationic residues of the CAPs. The ensuing membrane dissolution introduces strain and thinning of the surface which in turn leads to permeabilization and depolarization.

3.1 Isolation and structural identification of gramicidin S

In 1939, several crude CAPs were isolated by Dubos from the sporulating bacteria *Bacillus Brevis*. Partial fractionation provided three crystalline products that were named graminic acid, gramidinic acid and gramicidin.²³ The latter substance could be further fractionated into two individual crystalline substances, with a neutral fraction comprised of linear polypeptides

(gramicidin A-D) and an acidic fraction comprised of cyclic polypeptides (tyrocidine A-C). The mixture of gramicidins and tyrocidines was later renamed to tyrothricin.²⁴ After these pioneering investigations, Gause and Brazhnikova reported the isolation of a tyrothricin-like substance from cultures of *Bacillus Brevis* found in russian garden soil.²⁵ Extracts of this new *B. Brevis* strain consisted almost entirely of a single substance that could be readily obtained in crystalline form, and which was designated gramicidin S (GS, gramicidin Soviet). Clinical application demonstrated that GS (**5**) could effectively be used to combat G^+ and certain G^- bacterial infections.²⁶

In the first investigations towards the chemical properties of GS, Synge found that GS consists of five distinct amino acids, namely valine, ornithine, leucine, D-phenylalanine and proline and suggested that GS is a cyclic peptide.²⁷ Subsequently, the primary sequence of GS was determined by partial hydrolysis and partition chromatography to be $^D\text{Phe-Pro-Val-Orn-Leu}$. Judging by the molecular weight it was concluded that GS is a cyclodecapeptide that contains two copies of this sequence (see Figure 4).²⁸ Thereafter, several models have been put forward that describe the secondary structure adopted by GS. The synthesis of several derivatives of GS and crystallographic studies thereof did not lead to elucidation of the structure of GS, although the information obtained was sufficient to propose a molecular model.²⁹ In the Hodgkin-Oughton model of GS, the primary sequence $\text{cyclo-}(^D\text{Phe-Pro-Val-Orn-Leu})_2$ adopts a C_2 -symmetric β -sheet structure that is stabilized by four interstrand hydrogen bonds between the Leu and Val residues. The $^D\text{Phe-Pro}$ dipeptide sequences hold the $i+1$ and $i+2$ position in two type II' β -turns that further contribute to the stabilization of the pleated sheet structure. In this conformation, the hydrophobic (*i.e.* Val, Leu) and hydrophilic (*i.e.* Orn) residues of the two antiparallel β -strands are positioned on opposite sides of the molecule.

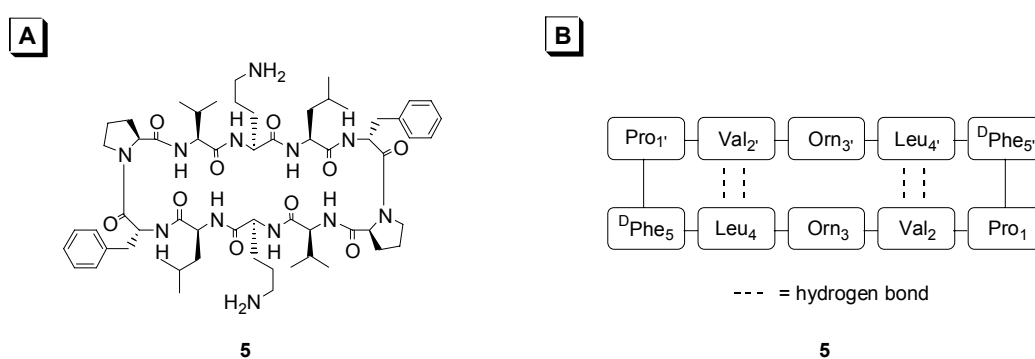


Figure 4: The primary structure (A) and the relative numbering of amino acids (B) of gramicidin S.

Final confirmation of the Hodgkin-Oughton model was provided by Dodson and coworkers, who were able to solve the single-crystal structure of a hydrated gramicidin S-urea complex to a resolution of $1\text{ \AA}$.³⁰ In the crystal structure, a slightly twisted β -sheet is observed for GS (see Figure 5) that maintains its C_2 -symmetry. Unexpectedly, the side-chains of the Orn residues take part in hydrogen bonds with the carbonyl oxygen atom of the ^DPhe -residue.

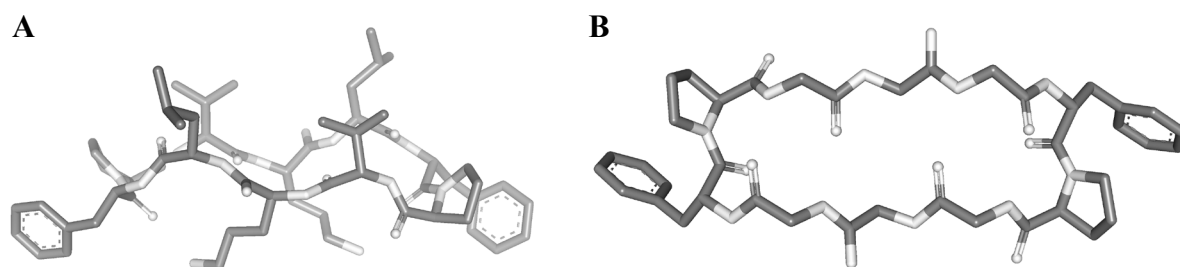


Figure 5: The crystal structure of gramicidin S (A) viewed from the side, (B) viewed from the top with selected amino acid side chains omitted for clarity.

Recently, Dodson and coworkers reported a refined structure of the hydrated gramicidin S-urea complex that appears to contain channels.³¹ As can be gauged from Figure 6, six equivalent GS molecules are assembled into a left-handed double spiral. The outside surface is comprised of the hydrophobic side-chains, whereas the inner surface of the channel is lined with the hydrophilic side-chains. Another striking feature of this crystal structure is that there was no experimental evidence for the presence of chloride-ions. These findings suggest the absence of charge on the Orn side-chains in the crystal structure although GS existed as hydrochloric acid salt in solution. While the authors speculate on the potential biological relevance of these channels, the mechanism by which GS elicits transmembrane ion-transport was not conclusively established. In additional studies, several derivatives of GS have been obtained in crystalline form and their structures were resolved. These efforts include the acylation of the Orn-residues with trichloroacetyl and *m*-bromobenzoyl-group³² and a Boc-protected GS analogue having the amide functionalities of the Orn and ^DPhe residues methylated.³³

Detailed NMR studies and ensuing distance geometry calculations have been carried out to assess the three-dimensional structure of GS in solution.³⁴ These investigation largely corroborated the Hodgkin-Oughton model of GS, displaying C_2 -symmetry with an extraordinary prevalence for intramolecular hydrogen bonding, and have shed light on the position and rotamer populations of amino acid residue side-chains.

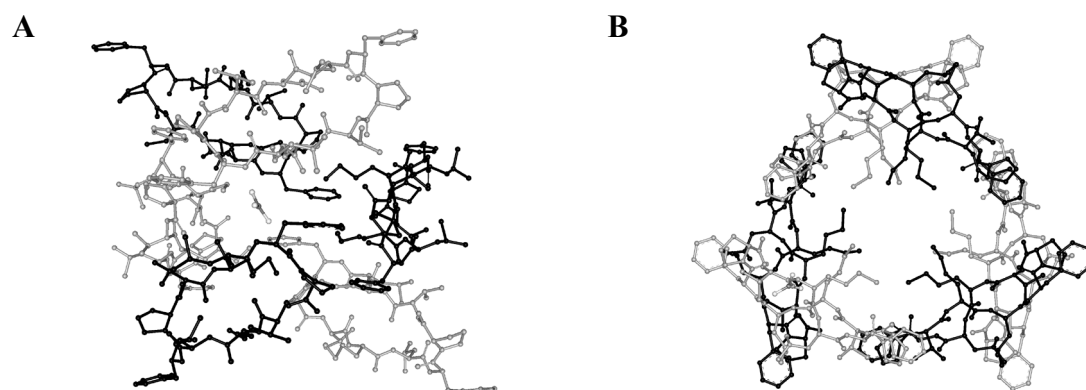


Figure 6: Channel formation observed in the crystal structure of GS (A) side-view, (B) top-view.

3.2 The biosynthesis of gramicidin S

The biosynthesis of the decameric cyclopeptide GS by the Gause-Brazhnikova strain of *B. Brevis* is performed on a nonribosomal peptide synthetase (NRPS). This multienzyme complex acts as an assembly line that catalyzes peptide condensation in a stepwise fashion as is illustrated in Figure 7A.³⁵ The NRPS for GS consists of two distinct enzymatic subunits, GrsA and GrsB. These two subunits together consist of five modules (M1-M5) and each activates a specific amino acid residue. Therefore, the location of each module dictates the primary structure of the peptidic construct. The modules are divided into several functional domains. The A-domain catalyses the amino acid activation through adenylation, which is followed by attack of the thiol moiety of the phosphopantetheine cofactor appended from the pcg-domain (peptidyl carrier protein) to furnish an aminoacyl thioester. Subsequently, the activated peptide is transferred to the condensation domain (C) which is responsible for the peptide bond formation between two amino acids on adjacent modules. However, condensation can be preceded by an additional tailoring domain, as is the case for phenylalanine, where the E-domain facilitates its epimerisation into the nonproteinogenic D-amino acid. It is then proposed that at the end of this modular assembly line, the first linear pentapeptide is transferred to the thioesterase domain (TE) as is shown in Figure 7B. The TE-domain catalyses the acyl transfer of this pentapeptide onto a second pentapeptide that arrives at the final pcg-domain. The resulting pcg-tethered decapeptide is transferred to the TE-domain where intramolecular attack of the terminal amine ensures release of the product.

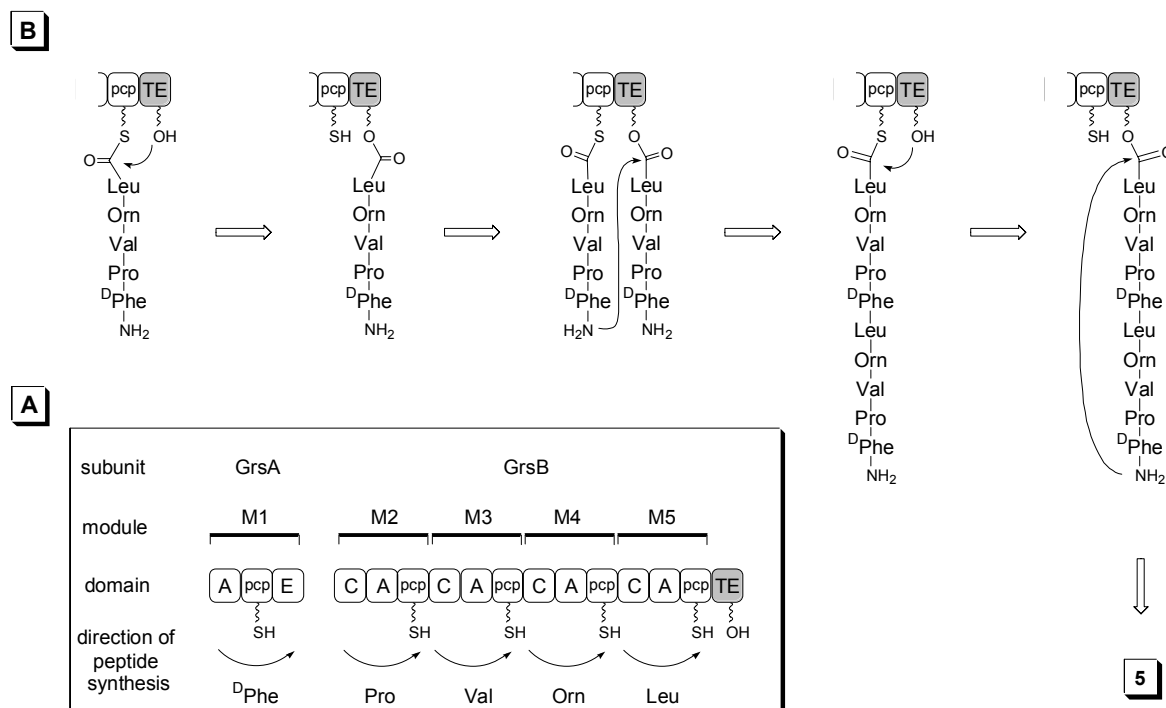


Figure 7: The nonribosomal peptide synthetase of GS (A) and the proposed dimerization-cyclization of pentapeptides on the NRPS of GS (B).

3.3 Cyclodecapeptides analogous to gramicidin S

Several microbial strains have been identified that produce cyclodecapeptides analogous to GS. For example, the Dubos-strain of *B. Brevis* produces the tyrocidines (A-E, **9-13**) that share five amino acid residues at identical positions to GS (see Figure 8).^{24,36} However, the other five amino acid residues are different from those found in GS. Within the series of tyrocidines, three positions have varying amino acid compositions. The biosynthesis of tyrocidine A (**9**) is orchestrated by a different NRPS that is composed of three subunits (TycA, TycB and TycC) that bear ten separate modules for all ten amino acid residues. The recently isolated streptocidins A-D (**14-17**) from culture broth extracts of *Streptomyces* sp. Tü 6071, obtained from Ghanaen tropical rain forest soil samples, are also structurally related to GS.³⁷ Streptocidins share a pentapeptide sequence (Val-Orn-Leu-^DPhe-Pro) that is identical to both GS and the tyrocidines. However, the streptocidins have three invariant amino acid residues that are not shared with GS and two positions that are varied within the series of these cyclodecapeptides. NMR spectroscopic studies provided conformational data which indicate a molecular topology similar to the β -sheet structure of GS. Biological assays demonstrated that the streptocidins are potent antibiotics against G^+ pathogens.³⁸

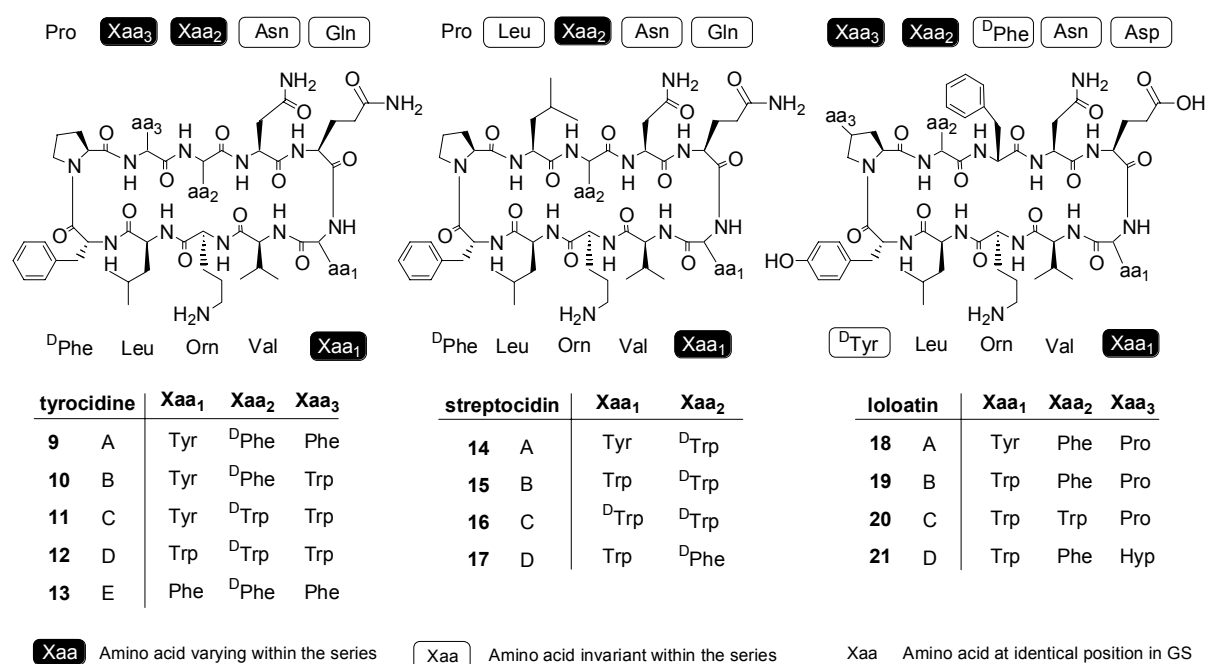


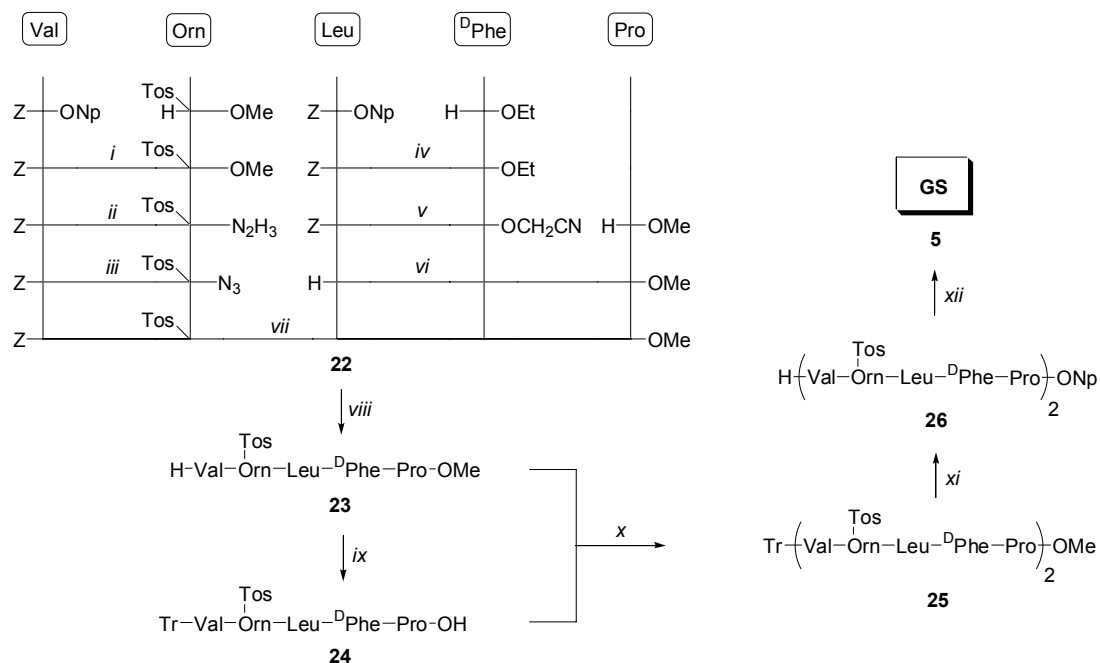
Figure 8: Decapeptide antimicrobial peptides analogous to GS.

Finally, cyclodecapeptides analogous to GS have been isolated from a tropical marine bacterium collected near the southern coast of Papua New Guinea and were named lolotatins.³⁹ The lolotatins (A-D, **18-21**) have the Val-Orn-Leu tripeptide sequence in common with GS. Furthermore, the ^DTyr-Pro or ^DTyr-Hyp dipeptide sequence bears significant resemblance to the reverse turn structure of GS. The remaining pentapeptide sequence has two variable aromatic amino acid residues within the series and three invariant residues. Lolotatins have a

higher degree of conformational freedom compared to GS and can adopt dumbbell-like conformation under specific conditions. The amphiphilic arrangement, together with the zwitterionic character of the loloatins, are believed to be at the basis of their potent antimicrobial activity.⁴⁰

4.1 Synthetic strategies towards gramicidin S

Twelve years after the discovery of GS, Schwyzer and Sieber described the synthesis of GS, and with it the first total synthesis of a naturally occurring cyclic peptide.⁴¹ Using an earlier reported solution-phase block-coupling procedure,⁴² fully protected linear pentapeptide **22** could be efficiently obtained (see Scheme 1). Hydrogenolysis of the *N*-terminal benzyloxycarbonyl (Z) protection group furnished amine **23**, of which a portion was converted into the *N*-trityl-protected (Tr) free carboxylic acid **24**. The linear pentapeptides **23** and **24** were condensed towards linear decapeptide **25**, that was subsequently transformed into the *p*-nitrophenyl (Np) ester **26**. Ensuing cyclization under dilute conditions provided the ditosyl-GS derivative in 28% yield, that was deprotected in 70% yield, to furnish GS. The synthesis of pentameric precursors in this divergent solution phase approach, followed by their linking and final cyclization of the decamer has been frequently used for the synthesis of GS and analogues thereof.

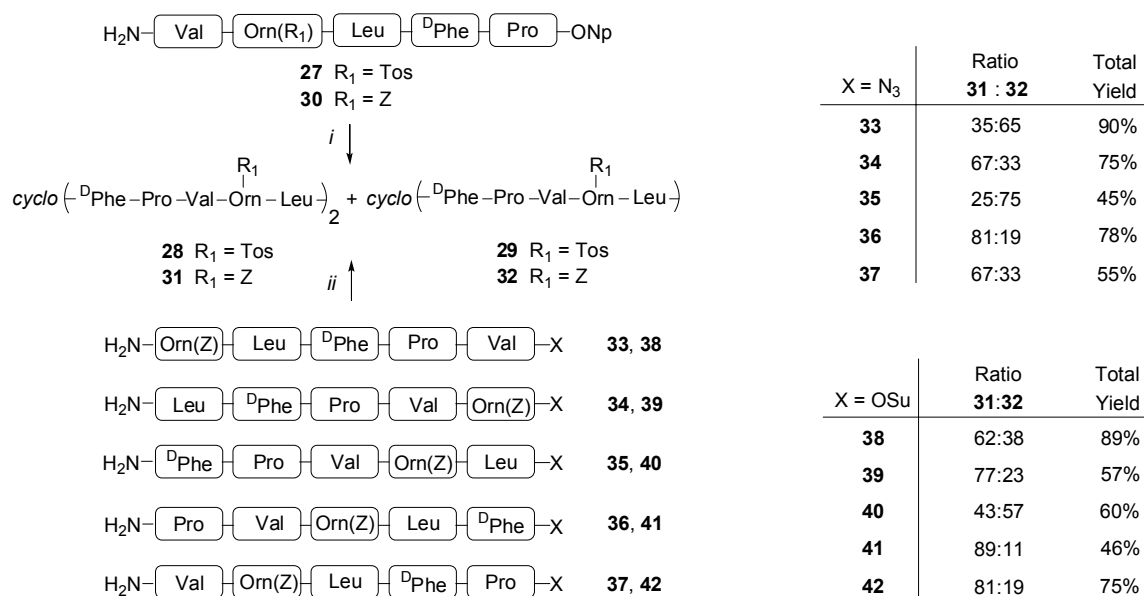


Scheme 1: Reagents and conditions: (i) TEA, THF, 15 h, 65%; (ii) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, MeOH; (iii) AcOH, 5 M HCl, NaNO_2 , 0 °C; (iv) TEA, THF, 5 h, 84%; (v) a) NaOH/THF (1/1 v/v), 96%; b) chloroacetonitrile, TEA, THF, 45 h, 94%; (vi) a) TEA, THF, 67 h, 67%; b) H_2 , 10% Pd/C, 8 h, 71%; (vii) EtOAc, 48 h; (viii) H_2 , 10% Pd/C; (ix) a) CHCl_3 , TrCl, TEA, 5 h, 97%; b) 1 M NaOH, 1,4-dioxane, 1 h, 83%; (x) DCC, MeCN, 7 h, 80%; (xi) a) 0.5 M NaOH, 1,4-dioxane, 1 h, 76%; b) bis(*p*-nitrophenyl) sulfite, pyridine, 5 h, 92%; c) TFA, -5 °C, 15 min; (xii) a) DMF, pyridine, 5 h, 28%; b) Na, NH_3 , 70-90%.

4.2 Dimerization-cyclization strategies towards gramicidin S

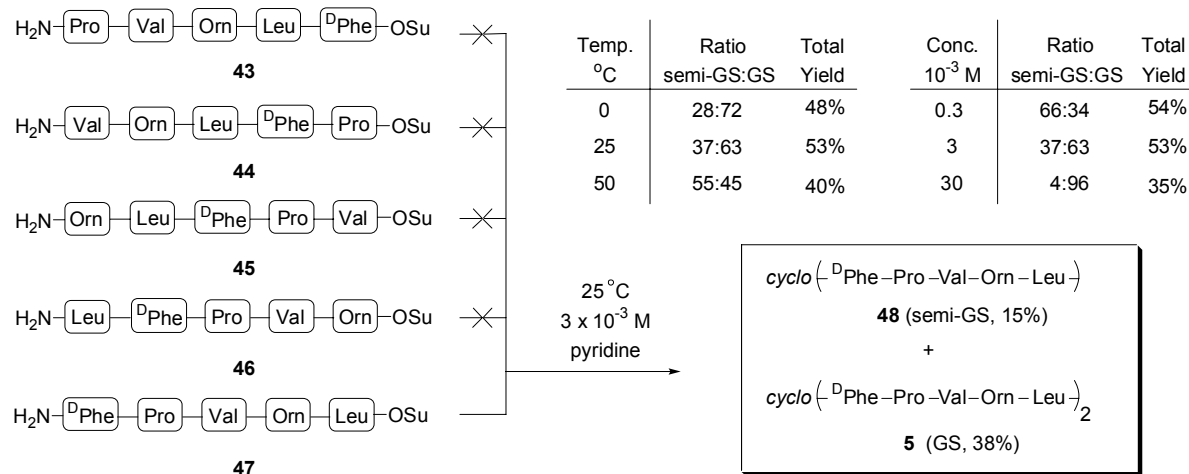
Shortly after their first successful synthesis of GS, Schwyzer and Sieber hypothesized that the macrocyclic structure of GS could also be constructed from two identical *p*-nitrophenylester precursor pentapeptides.⁴³ These precursors were envisaged to take on a pre-ordered conformation that forms intermolecular hydrogen bonds similar to the Hodgkin-Oughton model (*vide supra*). Dropwise addition of pentapeptide **27** to a solution of pyridine indeed resulted in formation of tosyl-protected GS **28** in 27% yield (Scheme 2). From their results, and taking into consideration the definitions of Pauling and Corey regarding the pleated sheet structure,⁴⁴ they concluded that there are structural periodicity rules that direct the cyclodimerization reaction. Namely, that when the final products contain $2(2n+1)$ residues (where $n = 1, 2, 3 \dots$) the dimerization followed by the cyclization of the precursor activated-esters is favoured. Further studies by Wishart and coworkers corroborated these results and refined the conditions under which β -sheet formation in cyclic peptides is promoted.⁴⁵

Upon reproducing the cyclodimerization reaction with *Z*-protected *p*-nitrophenylester **30**, Izumiya and coworkers observed the formation of both cyclic dimer **31** (*Z*-protected GS) in 12% yield and cyclic monomer **32** (*Z*-protected semi-GS) in 16% yield.⁴⁶ This prompted several studies toward the elucidation of the factors governing the mode of dimerization and cyclization.²⁶ It was found that active esters from C-terminal ^DPhe residues (**36** and **41**) predominantly formed cyclic dimers, whereas pentapeptides having a C-terminal Leu residue (**35** and **40**) favour intramolecular cyclization towards semi-GS **32**.⁴⁷ The azide active esters (**33-37**) and succinimide (**38-42**) active esters performed equally good in terms of total yield. In later studies, however, the succinimide ester activation became the method of choice as this entailed mild and simple experimental conditions.



Scheme 2: Reagents and conditions: (i) pyridine, 55 °C, 7 h, **28**, 27%; **29**, not reported; **31**, 12%; **32**, 16%; (ii) pyridine, 60 °C, for yields see tables.

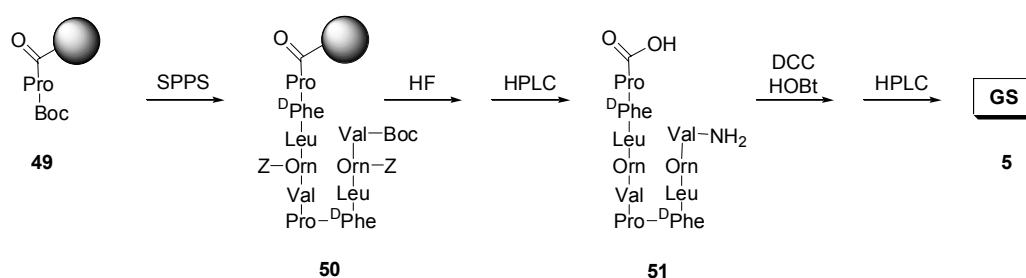
Tamaki *et al.* pointed out, that the above described mode of chemical dimerization and ensuing cyclization with protected pentapeptides is significantly different from that of the GS biosynthesis, in which the C-terminal Leu residue is appended from the GS synthetase.⁴⁸ They therefore chose to study the dimerization–cyclization of pentapeptide precursors having no protecting groups on the sidechains of the Orn residue in what they termed a biomimetic approach. Variation of the pentapeptide sequence (**43–47**), the concentration of peptide precursors in their cyclization medium and the reaction temperature resulted in semi-GS (**48**, 15%) and GS (**5**, 38%) in optimal yield and ratio (Scheme 3). It was found that in the biomimetic approach the only sequence that effectively produces GS was the sequence identical to the linear precursor pentapeptide found in the biosynthesis.



Scheme 3: Biomimetic synthesis of gramicidin S.

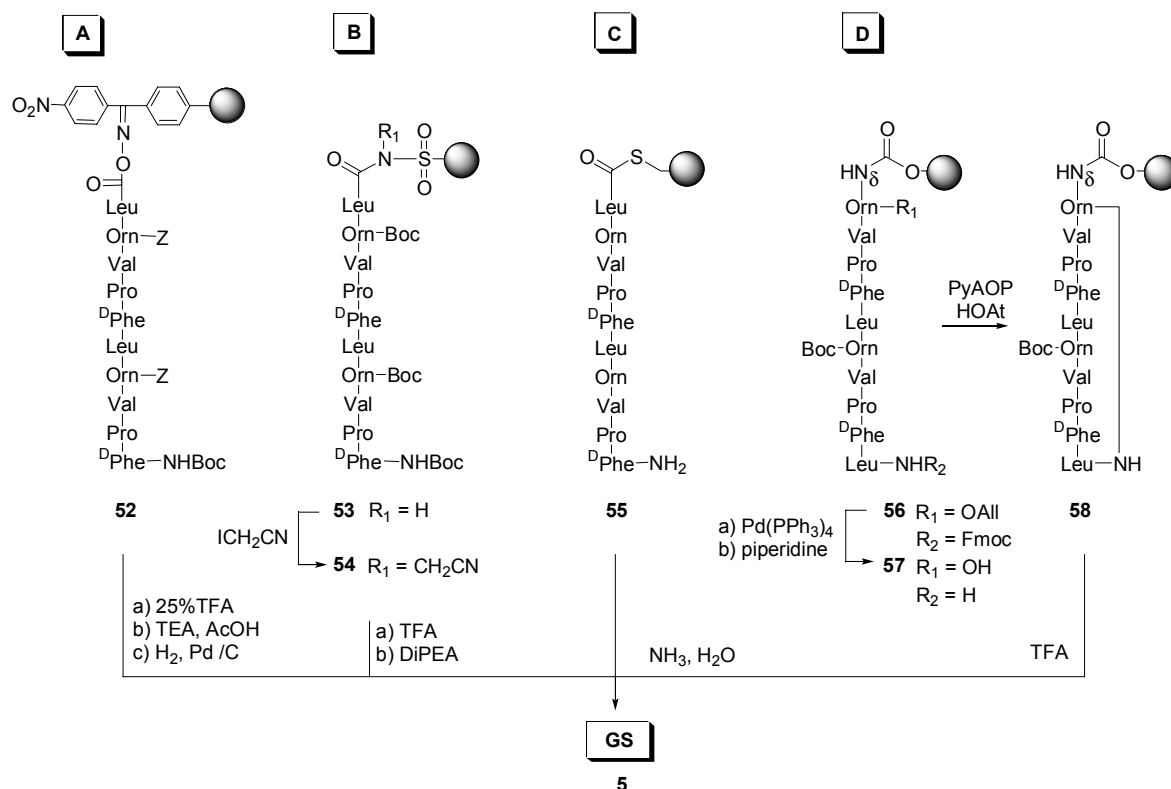
4.3 Solid phase strategies towards gramicidin S

After the advent of solid-phase peptide synthesis (SPPS), several protocols have been successfully applied to generate GS and analogues thereof. Early examples involve the assembly of linear, side-chain protected decapeptides by using the Merrifield resin in combination with Boc-chemistry. Ensuing cleavage from the solid support, cyclization and deprotection gave GS in moderate yields.⁴⁹



Scheme 4: Solid-phase synthesis of GS by Wishart *et al.*

A modification of this procedure was developed by Wishart *et al.* and entails the use of preloaded 4-hydroxymethylphenyl-acetamidomethyl (PAM) resin **49** in combination with Boc-chemistry (Scheme 4).⁵⁰ Acidolytic release of peptide **50** from the resin with concomitant removal of the Z-protection groups from the Orn residues and HPLC purification provided linear peptide **51**. Solution-phase cyclization and HPLC purification afforded GS in good yield.

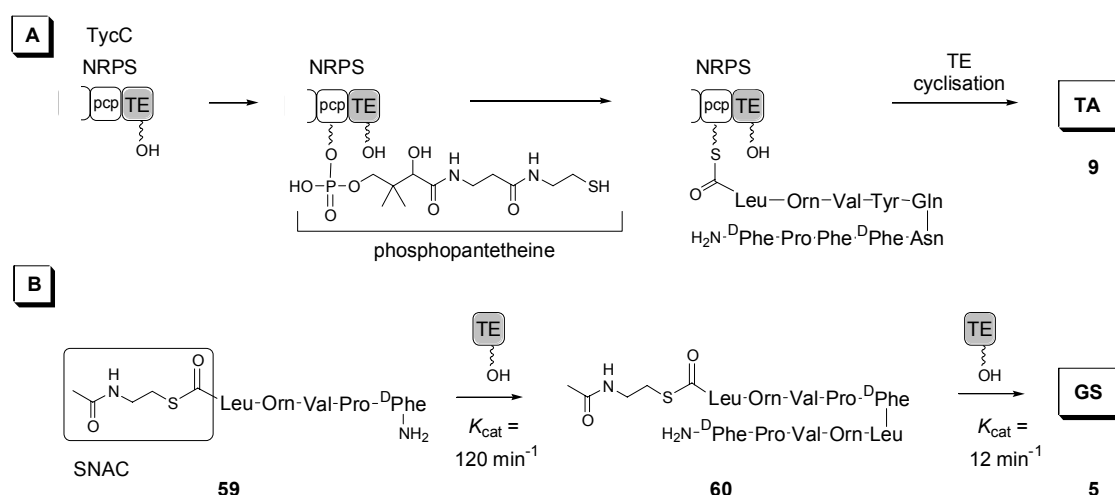


Scheme 5: Solid-phase cyclization using (A) oxime resin, (B) safety-catch resin, (C) chemoenzymatic approach, (D) side-chain linked approach.

Recent developments in resin-anchoring methods allowed the preparation of GS and analogues in either protected or unprotected form through exclusive solid-phase chemistry. Specifically, cyclization-cleavage protocols employing the Kaiser oxime linker (Scheme 5A, **52**) or the safety catch linker (Scheme 5B, **53**) have proven effective in the synthesis of GS-like peptides.^{51,52} With the application of a thioester linker (Scheme 5C), precursor **55** was found to cyclize into the desired head-to-tail product quantitatively when treated with an ammonia solution without abortive thioester hydrolysis.⁵³ Finally, Andreu *et al.* chose to anchor the side chain of an Orn-residue to the polymer and assemble the decapeptide (Scheme 5D) using Fmoc-chemistry.⁵⁴ By selectively removing both *N*- and *C*-terminal protections in **56**, the cyclization of **57** towards **58** proceeded on-resin under pseudodilution conditions provided by the polymeric matrix.

4.4 Chemoenzymatic synthesis towards gramicidin S

The thioesterase (TE) domain is the final catalytic domain of the NRPS that is involved with the cyclization and product release of tyrocidine A (TA, **9**), as is depicted in Scheme 6. To determine whether the TE domain can independently catalyze peptide cyclization, Walsh and coworkers replaced the C-terminal phosphopantetheinyl peptide, the natural substrate of the TE domain, with a synthetic peptide N-acetylcysteamine thioester (peptide-SNAC).⁵⁵ The decapeptide-SNAC corresponding to the TA sequence was shown to be recognized by isolated TE and efficient cyclization of the decapeptide was observed. Furthermore, they demonstrated that the isolated TE domain from the tyrosidine NRPS was also capable of catalyzing the dimerization of pentapeptide-SNAC precursor **59** and subsequent cyclization of decapeptide-SNAC precursor **60** towards GS (Scheme 6B). Having set the stage for merging natural product biosynthesis with solid-phase chemistry, a library of SNAC-decapeptides was constructed. From the ensuing cyclization studies it became apparent that the chemoenzymatic strategy is sufficiently robust for the incorporation of nonproteinogenic residues into the decapeptide scaffold.⁵⁶



Scheme 6: Biosynthesis of TA (**A**), and chemoenzymatic synthesis of GS (**B**).

5.1 Amino acid substitutions in the β -sheet region of gramicidin S

To evaluate the importance of the specific amino acid residues and their relative position in GS for structural stability and biological activity, a plethora of GS analogues have been synthesized in which single amino acids have been substituted. Most of these modifications are reviewed by Izumiya *et al.*²⁶ and Ovchinnikov *et al.*⁵⁷ Some prominent examples that have since appeared in literature and several trends that can be discerned from these data are discussed here. In some studies the cyclodecapeptide GS has been used either as structural or as biological model system, and structure-activity correlations are not always provided. Both

proteinogenic and nonproteinogenic amino acid residues have been incorporated in a β -strand of the GS analogues (see Figure 9). 4-Fluorophenylalanine (4F-Phe) has been used as highly sensitive reporter in ^{19}F -NMR to investigate the structure and dynamics of the peptide backbone of **61** both in solution and membrane-associated state.⁵⁸ 1-Pyrenylalanine (Pya) has similarly been used as a conformational probe to examine the twist present in separate β -strands in GS analogues **64-66**.⁵⁹ Although hexafluorovaline (Hfv) was introduced as racemic mixture at the valine positions of native GS, the resulting diastereomeric mixture could be separated and the [4,4']-L-Hfv GS analogue **62** obtained showed reduced antimicrobial activity.⁶⁰ The incorporation of aminomyristic acid (Amy) was envisaged to enhance the affinity of GS analogue **63** towards membrane environments.⁶¹ Although an increased ability to perturb phospholipid bilayers was observed for GS analogue **63**, it showed no antimicrobial activity.

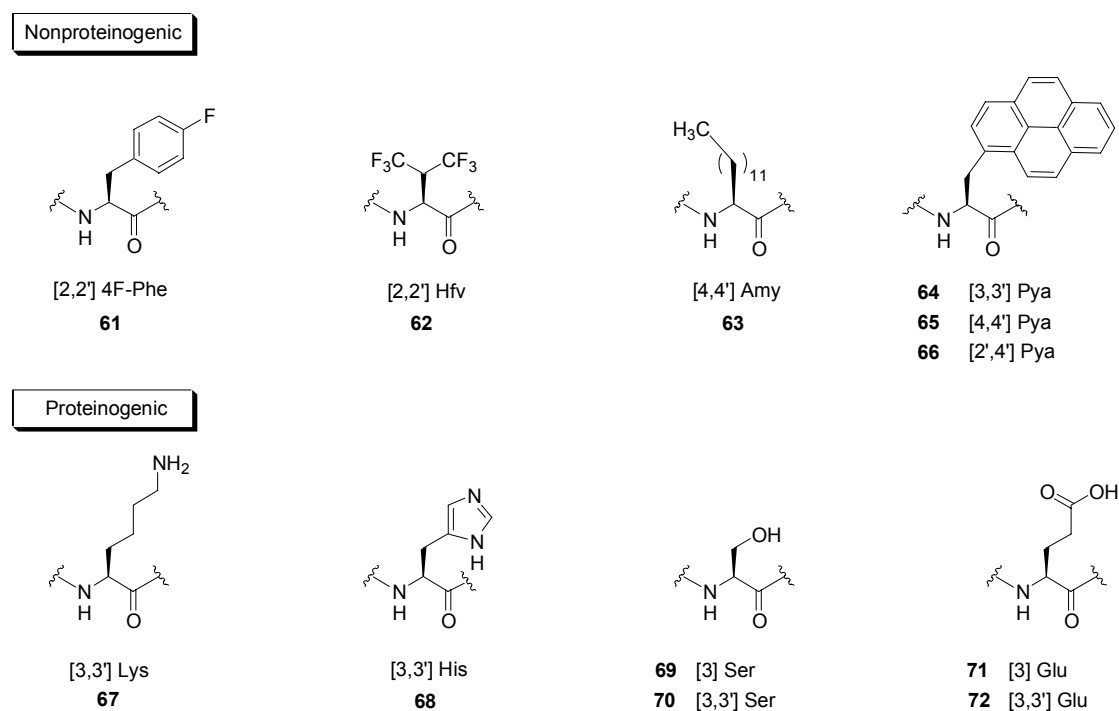


Figure 9: Amino acid residues incorporated in the β -sheet region of GS (prefixes between brackets denote the position in which the specific amino acid has been inserted).

Amino acid substitution in the β -sheet of GS with proteinogenic residues (**67-72**) has been most frequently performed at the [3,3'] position, thereby replacing the Orn residues. Notably, the [3,3']-Lys modified GS analogue **68** showed structural and biological properties identical to native GS.⁶² These residues have since been considered interchangeable and are used as such in more elaborate modifications discussed in the following paragraphs. The [3,3']-His GS analogue **68**, with its weaker basicity, was shown to be considerably less active as were the serine (**69** and **70**) or glutamic acid (**71** and **72**) substitutions at that same position.^{63,64} This demonstrates the importance of the basic residues to provide GS with its amphiphilic character.

5.2 Amino acid substitutions in the turn region of gramicidin S

Reports on single amino acid substitutions in the reverse turn region of GS have predominantly focussed on the [5,5']-^DPhe residue replacements (see Figure 10). Only two examples have recently appeared in literature in which the [1,1']-Pro residues were replaced with aminoproline (*S*-Amp, **73** and *R*-Amp, **74**) residues. The additional cationic moieties in the turn region resulted in poor antibiotic activity. However, GS analogues **73** and **74** could be employed synergetically to sensitize G^- bacteria towards GS.⁶⁵ Another cationic amino acid, 2,3-D-diaminopropionic acid (^DDap) similarly resulted in an altered antibiotic spectrum for peptide **75**, when compared to native GS.⁶⁶ Namely, the tetracationic GS analogue **75** showed activity against G^- bacteria, whilst activity against G^+ bacteria could not be observed.⁶⁷

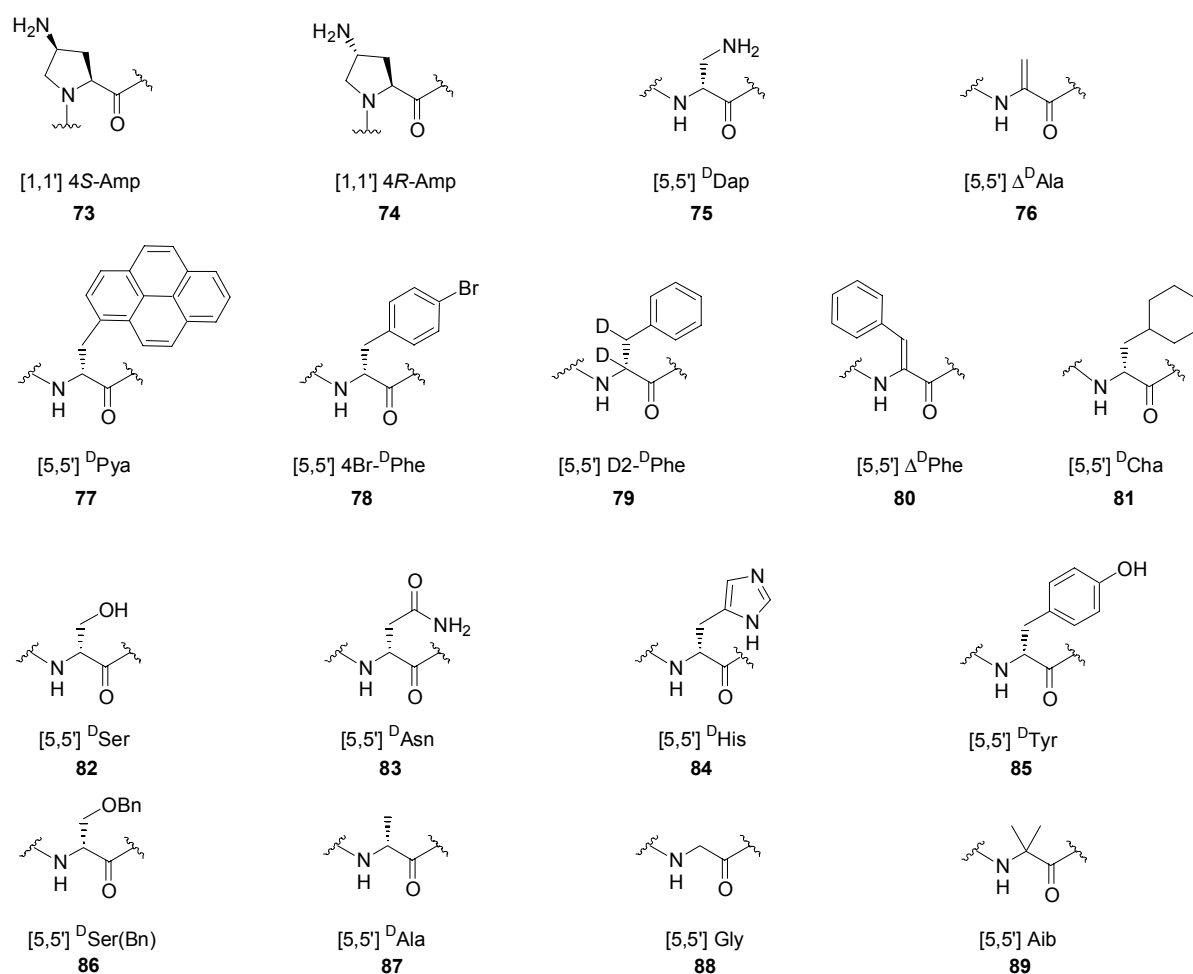


Figure 10: Amino acid residues incorporated in the reverse turn region of GS (prefixes between brackets denote the position in which the specific amino acid has been inserted).

Substituting the ^DPhe residues of GS with ^DSer, ^DAsn, ^DHis or ^DTyr residues (**82-85**) did not interfere with β -sheet formation.⁵⁰ However, the capacity of the resulting GS analogues to curb bacterial proliferation was impaired.^{50,68} Interestingly, when the D-serine was protected as a benzylether (^DSer(OBn), **86**), the biological activity was again on par with native GS.⁶⁹

The GS analogues that have the ^DPhe residues replaced with aromatic moieties such as D-pyrenylalanine (^DPya , **77**),^{51b} 4-bromo-D-phenylalanine (4Br- ^DPhe , **78**),^{49d} (2*R*,3*R*)-2,3-D2-phenylalanine (D2- ^DPhe , **79**),⁷⁰ and 2,3-dehydro-D-phenylalanine ($\Delta^D\text{Phe}$, **80**)⁷¹ all showed β -sheet formation and antimicrobial activities that were closely related to GS. The nonaromatic isostere D-cyclohexylalanine (^DCha) exhibited a reduced ability to perturb phospholipid bilayer when incorporated at the [5,5']-positions of GS analogue **81**, whereas in the D-alanine (^DAla , **87**), D-dehydroalanine ($\Delta^D\text{Ala}$, **76**), glycine (Gly, **88**) and 2-aminoisobutyric acid (Aib, **89**) analogues the antimicrobial activities were largely abolished.

5.3 Peptidomimetic compounds incorporated in gramicidin S

In the field of peptidomimetic research, peptidic structures are replaced by nonproteinogenic groups that mimic or stabilize common secondary structure elements.⁷² A second aim in peptidomimetic design is to correctly position pharmacophores that are required for biological activity. After the design and synthesis, the capacity of specific peptidomimetics to nucleate or propagate folding in peptides, or present functional groups in a specific orientation needs to be evaluated. Over the years GS, with its well-defined secondary structure and known biological activity, has become a standard peptide to demonstrate the ability of conformationally constrained mimetics to act as reverse turn inducers. For example, Sato *et al.* synthesized the bicyclic thioindolizine derivative **90** (Figure 11) from L-glutamic acid and L-cysteine.⁷³ Upon substitution of both $^D\text{Phe-Pro}$ dipeptide sequences of GS with **90**, the resulting GS analogue showed physical and biological characteristics comparable to those of GS. This confirmed the design of peptidomimetic **90** as an effective replacement of the native type II' β -turns in GS.

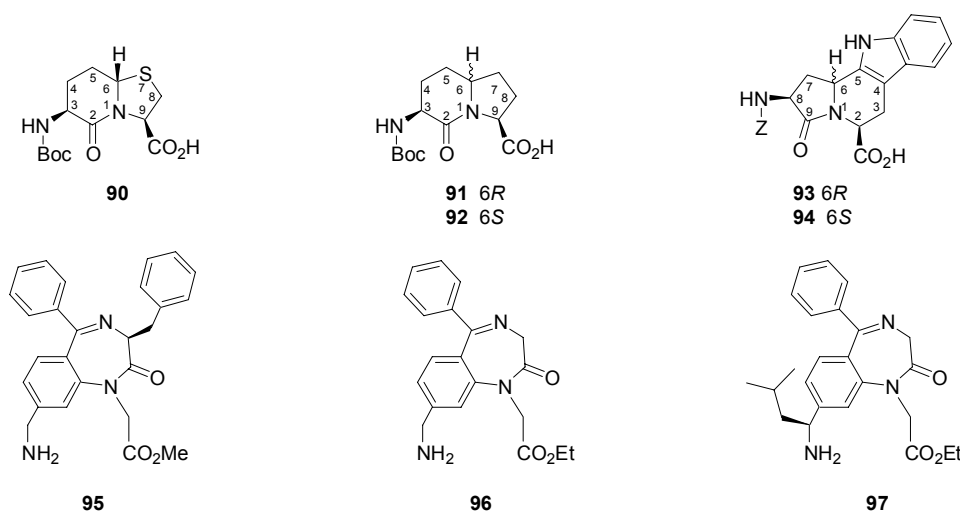
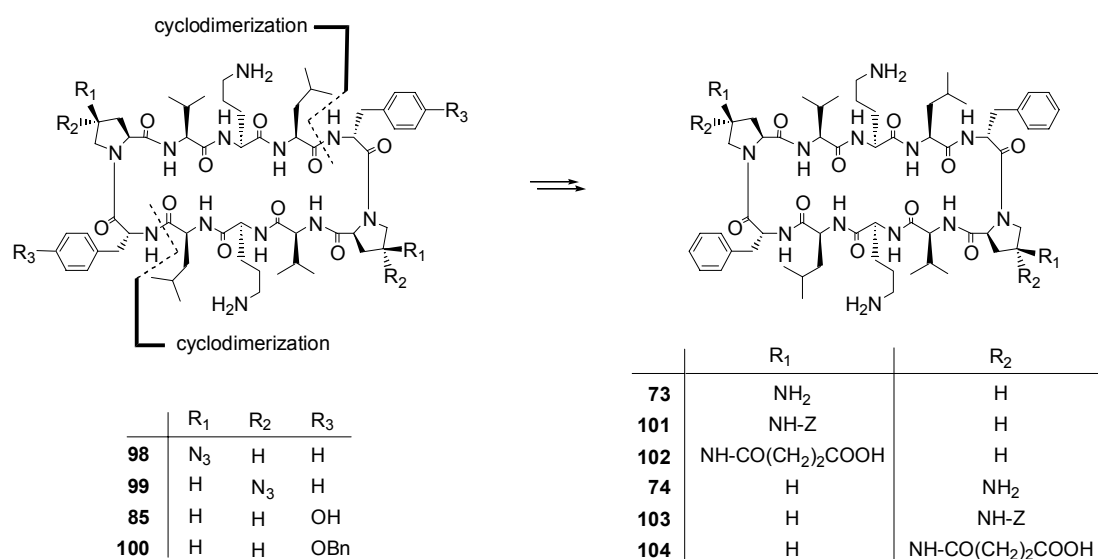


Figure 11: Reverse turn mimetics that replace the $^D\text{Phe-Pro}$ dipeptide (**90-94**) or Leu- $^D\text{Phe-Pro-Val}$ tetrapeptide sequence (**95-97**) in GS.

In a later report by Ripka and coworkers, the single incorporation of the thioindolizine structure **90** and ensuing NMR-spectral analysis provided additional support to that claim.⁷⁴ In a similar approach, the 5,6-fused azabicycloalkanes **91** and **92** having different ring-fusion stereochemistry were evaluated on their propensity to induce a reverse turn structure.⁷⁵ Incorporation of the 6*S*-diastereoisomer **92** resulted in peptides that exhibited physical properties that resemble native GS the closest, albeit that a loss in biological activity was recorded. The related 5,6-fused bicyclic motif **93** (6*R*-indolizine) with appended heteroaromatic units had been predicted to be a suitable type II' β -turn surrogate whereas **94** (6*S*-indolizine) would not be.⁷⁶ Incorporation of **93** and **94** in GS-like peptides conclusively established those predictions made by González-Muñiz and coworkers.⁵⁴ Benzodiazepines **95-97** were probed for their peptidomimetic ability to substitute a single Leu-^DPhe-Pro-Val tetrapeptide sequence in GS. However, the resulting GS analogues that contain benzodiazepines **95-97** do not adopt a defined secondary structure as evidenced by NMR spectral line-broadening and the peptides displayed a low antimicrobial activity.⁷⁷

6. Aim and outline of the Thesis

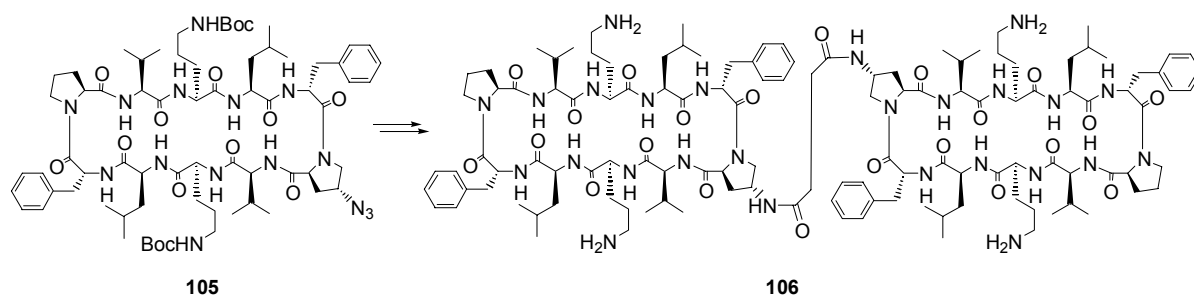
The work described in this Thesis was aimed at the synthesis of novel analogues of the cationic antimicrobial peptide gramicidin S with nonproteinogenic amino acid residues incorporated in the reverse turn regions. To establish the structure-activity relationships of these GS analogues, structural characterization was performed with the aid of ¹H NMR and X-ray crystallographic analysis. Furthermore, the biological activity of these GS analogues was examined through antimicrobial and hemolytic assays.



Scheme 7: Turn modified GS analogues from a biomimetic synthesis strategy.

In **Chapter 2**, the synthesis of GS analogues that have the Pro-residues replaced with 4*S*- or 4*R*-azidoproline (**73** and **74**, respectively) or the ^DPhe-residues replaced with ^DTyr residues (**85** and **100**) is described. These GS analogues with additional functionalities in the reverse turn region were constructed by employing a biomimetic synthesis approach, as is shown in Scheme 7. Ensuing transformation of the azide-functionalities provided GS analogues with cationic (**73**, **74**), hydrophobic (**101**, **103**) and anionic (**102**, **104**) moieties in the reverse turn region. The ability of these GS analogues to adopt a β -sheet structure was investigated by ¹H NMR analysis and the biological activity was probed by antimicrobial and hemolytic assays.

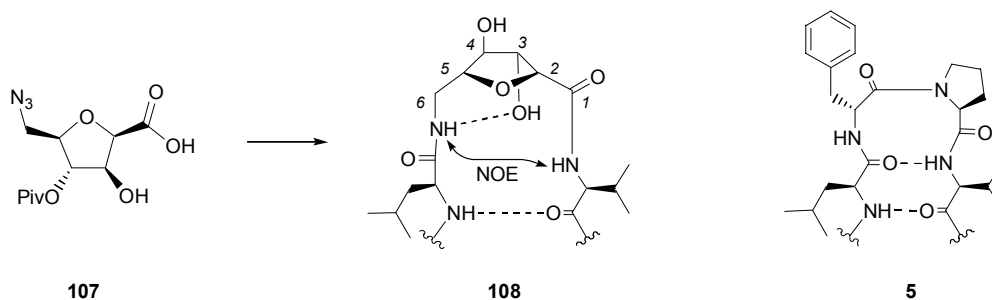
The exact mechanism by which many β -sheet CAPs induce membrane-permeability has not yet been resolved. However, the accumulation of these CAPs on lipid bilayers is thought to be an essential process that precedes pore formation. Manipulation of the balance between association and dissociation of GS analogues on the lipid bilayers might therefore shed light on the manner by which bacterial cell lysis is ultimately induced. It was envisaged that this equilibrium can be influenced by covalently linking GS analogues. The design and synthesis of GS dimers is described in **Chapter 3**. The synthesis of asymmetrically substituted GS analogues, using an Fmoc-based SPPS strategy in combination with a solution-phase cyclization strategy, gave for example access to the Azp-containing GS monomer **105** (Scheme 8). This could subsequently be transformed into dimer **106** of which, together with other GS dimers, the biological relevance was explored through antimicrobial and hemolytic assays. Conductivity measurements to probe ion channel forming properties are also described.



Scheme 8: Example of a GS dimer that was obtained from an Azp-functionalized GS analogue.

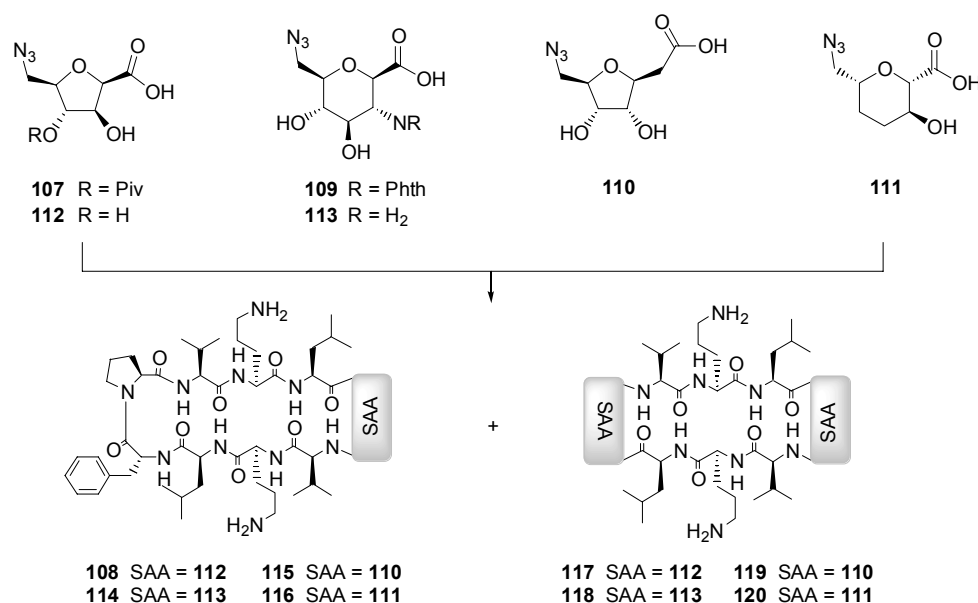
In **Chapter 4**, the synthesis of sugar amino acid (SAA) dipeptide isostere **107**, based on a 2,5-anhydroglucitol scaffold, and its ensuing incorporation in the reverse turn region of GS analogue **108** is disclosed (see Scheme 9). The C₃-hydroxyl function that originates from the parent sugar of the furanoid SAA is shown to act as H-bond acceptor. This feature induces an unusual reverse turn structure in the GS analogue **108**. Namely, the amide bond that connects the Leu residue with the SAA has flipped compared to the analogous amide bond in the native type II' β -turn of GS, as was gauged from ¹H NMR and X-ray crystallographic data. Furthermore, the molecular packing of GS analogue **108** in the single crystal X-ray structure,

revealed a hexameric beta-barrel-like assembly. The arrangement of six crystallographically equivalent β -sheets with a hydrophobic periphery and hydrophilic core is reminiscent of the pore-like structure reported by Dodson and coworkers.³¹



Scheme 9: A furanoid sugar amino acid that induces an unusual reverse turn structure in GS.

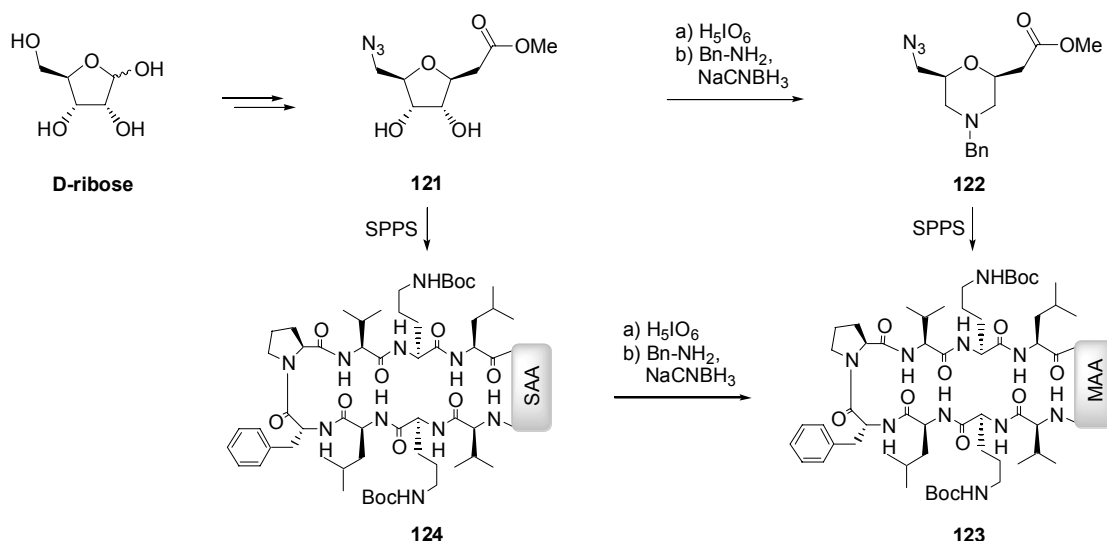
Chapter 5 discloses that the Fmoc-based solid-phase peptide synthesis protocol that is described in Chapter 3 and Chapter 4, could also be employed for the generation of eight gramicidin S analogues having nonproteinogenic sugar amino acid residues **107**, **109**, **110**, and **111** (see Scheme 10) incorporated in a single (**108**, **114-116**) and in both (**117-120**) reverse turn regions of GS. Perusal of the ^1H NMR data from the deprotected peptides revealed that the β -sheet structure was predominantly maintained. The antimicrobial and hemolytic properties of GS analogues **108**, **114-120** are presented.



Scheme 10: Sugar amino acids and their incorporation in the reverse turns of GS analogues.

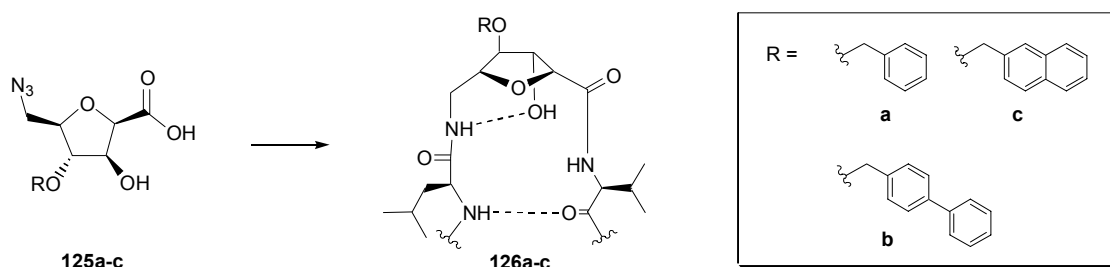
In **Chapter 6** of this Thesis, a synthetic strategy is described that concerns the decoration of SAAs having a *cis*-diol system on their furanoid core structure. In a two-step oxidative glycol cleavage / reductive amination protocol, the ϵ -sugar amino acid **121** was transformed into ϵ -morpholine amino acid (MAA) **122**, as is depicted in Scheme 11. This strategy was shown to be amenable for incorporation of several different amines, giving access to diversely functionalized ϵ -MAAs. The application of MAAs as peptidomimetic compounds was

demonstrated by replacing a single reverse turn in GS by an ε -MAA. Furthermore, the ε -MAA-containing GS analogue **123** is shown to be accessible by subjecting SAA-containing peptide **124** to the two-step glycol cleavage / reductive amination procedure. In order to obtain diastereoisomerically pure δ -MAAs, an alternative route is described that prevented epimerisation of δ -SAAs during the glycol cleavage step.



Scheme 11: Synthesis of a morpholine amino acid from a sugar amino acid and incorporation in GS.

The crystal structure of GS analogue **108** (Scheme 9) that is presented in Chapter 4, revealed that both the peptide backbone geometry as well as the amino acid side-chain functionalities were altered compared to native GS. To probe the factors that determine biological activity, the synthesis of sugar amino acids **125a-c** (see Scheme 12) was undertaken (see **Chapter 7**). It was envisaged that upon incorporation of SAA **125a-c** into their corresponding GS analogues **126a-c**, the appended aromatic groups should enhance the mimicry towards the original ^{D} Phe-Pro reverse turn (**5**, scheme 9). ^1H NMR analysis indicated that the GS analogues **126a-c** adopt a β -sheet conformation that feature a similar reverse turns as that described in Chapter 4. Through antimicrobial and hemolytic assays it was established that the GS analogues have a comparable biological activity to the native GS, thereby underscoring the peptidomimetic ability of decorated SAAs.



Scheme 12: Sugar amino acids adorned with aromatic groups that are incorporated in GS analogues.

In **Chapter 8** the results that are described in this Thesis are summarized and some future directions towards SAA-containing GS analogues and synthetic strategies towards novel β -sheet antibiotics are discussed.

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Chapter 2

Synthesis and Biological Evaluation of Novel Turn-modified Gramicidin S Analogues

Abstract: *The synthesis of novel gramicidin S analogues having additional functionalities in the turn region by employing a biomimetic approach is described. The preservation of β -sheet character in all analogues was established by NMR spectroscopy and the biological activity of the new GS analogues was evaluated.*¹

Introduction

The cationic antimicrobial peptide gramicidin S (GS, **1**, Figure 1), isolated from *Bacillus brevis*,² is active against a wide range of bacteria and fungi.^{3,4} The continual emergence of antibiotic resistance has spurred an interest in the generation of new GS analogues with improved antimicrobial activities. In this respect, a plethora of GS derivatives in which the β -strand region is modified has been described over the last decades.^{3,5} These examples include modulation of the amino acid composition as well as enlarging the β -strand region (*i.e.* the synthesis of GS homologues).^{6,7} Perusal of the latter studies revealed a number of factors influencing the bactericidal efficiency of β -strand modified GS analogues such as amphiphilicity, hydrophobicity, nature and number of cationic residues, backbone size and conformation. However, the wealth of information gathered has, to date, not resulted in the generation of a synthetic, clinically applicable antibiotic based on GS.

Modulation of the turn regions in GS is a relatively unexplored area of research. It is likely that substitution of the turn region amino acids, especially the proline residue, in most cases will lead to loss of β -sheet character and concomitant loss of antimicrobial activity.⁴ It was envisaged that the development of a strategy that allows the introduction of additional functionalities to the β -turn region of GS, without interfering with its intrinsic β -sheet character, would provide a potential entrance towards new GS-based antibiotics. In this chapter the results are reported on the generation of turn-modified GS analogues, in which the

two ^DPhe residues are replaced by benzylated D-tyrosines (*i.e.* ^DTyr(Bn), peptide **3**, Figure 1), as well as derivatives where Pro is substituted for either 2S,4R-azidoproline (R-Azp, peptide **4**) or 2S,4S-azidoproline (S-Azp, peptide **5**). Furthermore, the transformation of the azide residues into secondary amines (**6** and **7**, having additional cationic functionality in the turn region), benzyloxycarbamates (**8** and **9**, with bulky hydrophobic residues) and succinylamides (**10** and **11**, featuring carboxylic acid turn region elements) is presented. The secondary structure of GS analogues **3-11** and their capacity to arrest proliferation of various Gram-positive and -negative bacterial strains were compared to GS and the known GS analogue **2**.

Results and Discussion

Several synthetic strategies towards GS and its analogues have been reported in the literature (see **Chapter 1** for a comprehensive overview). Tamaki and coworkers followed a biomimetic approach in which the specific pentameric sequence H₂N-^DPhe-Pro-Val-Orn-Leu-ONSu yielded GS after cyclodimerization. The ability of this particular sequence to form GS was attributed to a preorganisation of the activated decameric linear peptide forming a β-hairpin structure.⁸ Importantly, the replacement of specific amino acid residues in the synthetic sequence (*e.g.* 4Br-^DPhe instead of ^DPhe) did not interfere with the dimerization-cyclization reaction.⁹ It was therefore decided to study the efficiency of this biomimetic synthesis for the construction of GS analogues **2-5**.

Commercially available 4-methylbenzhydrylamine (MBHA) resin (**12**, Scheme 1) was equipped with the acid-labile 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker¹⁰ under the agency of Castro's reagent¹¹ and *N,N'*-diisopropylethylamine (DiPEA) and condensed with Fmoc-Leu-OH using *N,N'*-diisopropylcarbodiimide (DIC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) to give the functionalized resin **13**. The immobilized pentapeptide sequences **14a-e** were synthesized *via* standard peptide chemistry, employing, next to standard amino acid building blocks, the readily available Fmoc-2S,4R-azidoproline (in **4**) and Fmoc-2S,4S-azidoproline (in **5**).^{12,13,14}

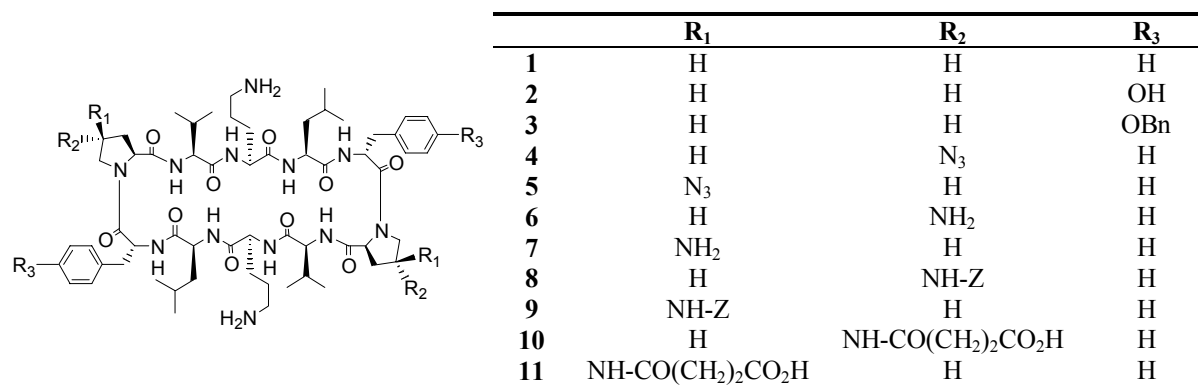
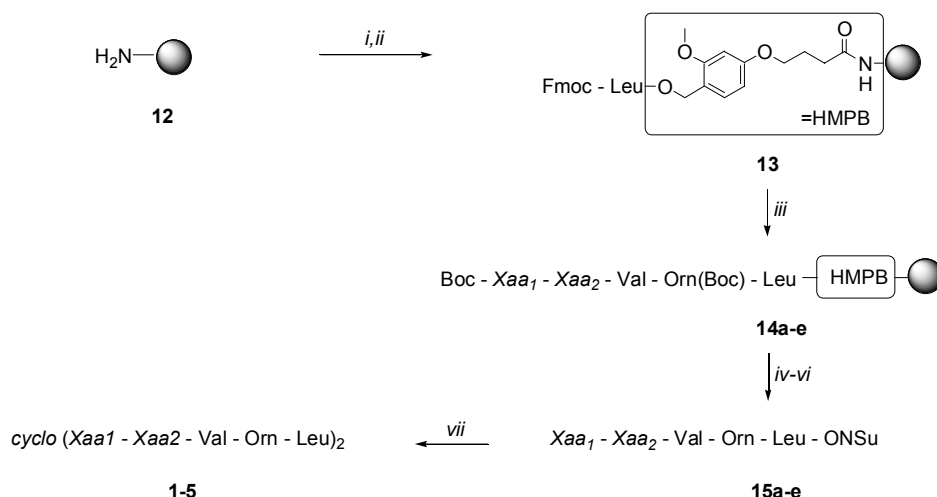
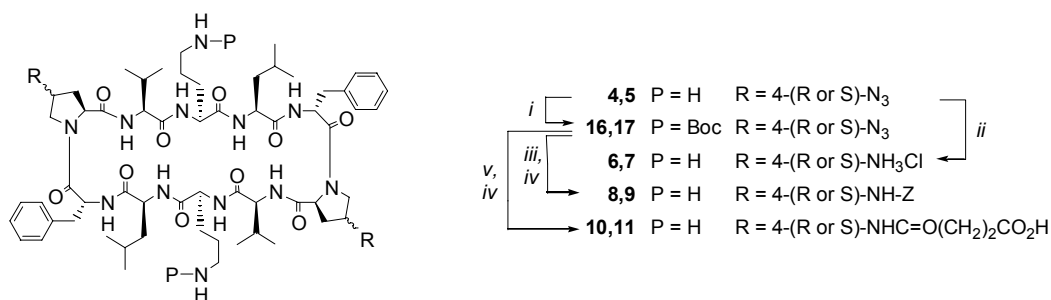


Figure 1: Gramicidin S, and the analogues discussed in this Chapter.



Scheme 1: Reagents and conditions: (i) HMPB, BOP, DiPEA, NMP; (ii) Fmoc-Leu-OH, DIC, DMAP (5 mol%), DCM; (iii) Repetitive deprotection: 20% piperidine in NMP, condensation: Fmoc-aa-OH or Boc-*Xaa*₁-OH, BOP, HOBt, DiPEA, NMP; **a** *Xaa*₁ = ^DPhe, *Xaa*₂ = Pro; **b** *Xaa*₁ = ^DTyr, *Xaa*₂ = Pro; **c** *Xaa*₁ = ^DTyr(Bn), *Xaa*₂ = Pro; **d** *Xaa*₁ = ^DPhe, *Xaa*₂ = *R*-Azp; **e** *Xaa*₁ = ^DPhe, *Xaa*₂ = *S*-Azp; (iv) 1% TFA in DCM; (v) HONSu, EDC, DCM; (vi) 50% TFA in DCM; (vii) **15a-e**, pyridine.

After cleavage (1% TFA/DCM) from the solid support the Boc-protected pentamers were condensed with HONSu under the agency of EDC to give their respective *N*-succinimic esters. After removal of the Boc-protective groups (TFA/DCM, 1/1, v/v) the activated pentapeptides **15a-e** were subjected to cyclodimerization by slow addition to a pyridine solution up to a final concentration of 3×10^{-3} M at 25°C.⁸ The resulting cyclic decamers were identified using LCMS and purified by semi-preparative HPLC to give GS and **2-5** in yields of approximately 5%, based on the initial loading of resin **13**. The crude cyclodimerization mixture contained, besides the expected products (*i.e.* the cyclic monomer and dimer described by Tamaki *et al.*), several other unidentified fragments that displayed an identical ESI-MS-profile. Apparently, the formation of cyclization products involving the ornithine side chain can also occur once the linear decameric active ester is formed. Notwithstanding the formation of these undesired side products, the biomimetic synthetic sequence provides an easy and rapid access to the construction of C₂-symmetric GS analogues **2-5**.



Scheme 2: Reagents and conditions: (i) Boc₂O, DiPEA, MeCN; (ii) 10% Pd/C, H₂, CHCl₃/MeOH (1/1 v/v); (iii) a) PMe₃, 1,4-dioxane/MeCN/H₂O (20/20/1 v/v/v); b) Z-Cl, DiPEA, DMF; (vi) TFA/DCM (1/1 v/v); (v) a) PMe₃, 1,4-dioxane/MeCN/H₂O (20/20/1 v/v/v); b) succinic anhydride, TEA, DMF.

As the next research objective, we set out to functionalize *R/S*-Azp containing GS analogues **4** and **5**. Treatment of azides **4** and **5** (Scheme 2) with 10% Pd/C under hydrogen atmosphere in the presence of CHCl_3 furnished the positively charged aminoproline (Amp) derivatives **6** and **7** in a respective yield of 58% and 60%, after HPLC purification. Alternatively, protection of the ornithine side chains (Boc_2O , **4** to **16** and **5** to **17**, 89% and 86%, respectively) allowed the selective modification of the azidoproline derivatives, as follows. Staudinger reduction¹⁵ of the azides in **16** and **17**, followed by condensation of the resulting secondary amines with either benzyl chloroformate or succinic anhydride, and final acidic removal of the Boc protective groups afforded target compounds **8-11** in good yields (**8**, 42%; **9**, 36%; **10**, 78%; **11**, 66%; after HPLC purification).

Having cyclic peptides **1-11** in hand, attention was focused on their structural evaluation by NMR. The resonance assignment of compounds **1-11** was unambiguously accomplished using two-dimensional NMR experiments (*i.e.* COSY, TOCSY). Several methods for the interpretation of the acquired ^1H NMR spectra can be applied to establish secondary structure elements in peptides. The presence of the $^{\text{D}}\text{Phe}$ and $^{\text{D}}\text{Tyr}$ residues in the turn regions was indicated by the small vicinal spin-spin coupling constants ($^3J_{\text{HN}\alpha} < 4\text{Hz}$), as was postulated by Ramachandran *et al.*¹⁶ As can be seen from the coupling constants for peptides **1-11** (Figure 2) the $^3J_{\text{HN}\alpha}$ of the Leu, Orn and Val residues (ranging between 8.5 and 9.0 Hz) correspond to a β -sheet structure.¹⁷ The values of the coupling constants for all residues are largely comparable with the corresponding values for GS.

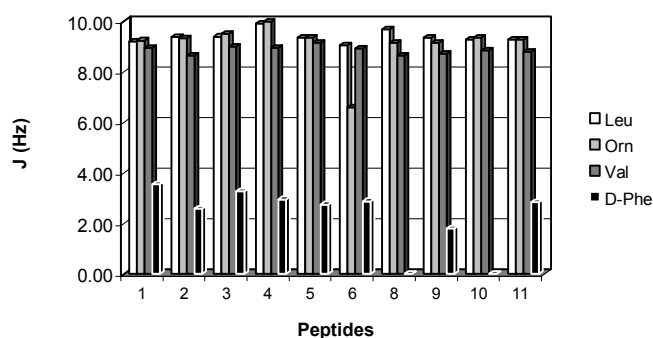


Figure 2: Coupling constants ($^3J_{\text{HN}\alpha}$) are given in Hertz (Hz). In the ^1H spectrum of peptide **7**, no splitting pattern of amide resonance signals could be observed. Peptides **8** and **10** showed no observable $^3J_{\text{HN}\alpha}$ for the $^{\text{D}}\text{Phe}$ residues.

The perturbation of chemical shift is defined by Wishart and co-workers¹⁸ as the difference between the measured chemical shift for the H_α of an amino acid and the H_α chemical shift value of the same residue reported for a random coil peptide. The presence of three or more consecutive residues having $\Delta\delta\text{H}_\alpha > 0.1$ ppm signifies an extended β -strand conformation. As can be seen from the data displayed in Figure 3, the α -protons of the Leu-Orn-Val sequence of all presented peptides (*i.e.* **1-11**) clearly show idiosyncratic secondary chemical shifts.¹⁹

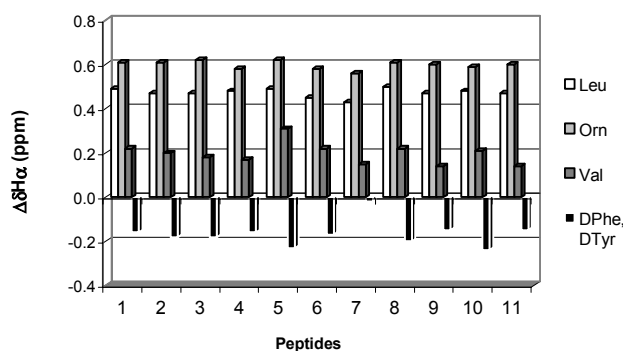


Figure 3: Chemical shift perturbation: $\Delta\delta H_{\alpha}$ = observed δH_{α} – random coil δH_{α} . For entries **2** and **3** the random coil value for tyrosine was used. As the substitution pattern on the pyrrolidine moiety influences the chemical shift of the H_{α} , no reference values for the Azp residues were available and were therefore omitted. The random coil value reported for lysine was used for ornithine.²⁰

The location of the ^DPhe and ^DTyr residues in the turn region is illustrated by a negative value of the chemical shift perturbation (*i.e.* $\Delta\delta H_{\alpha} < -0.1$ ppm). In summary, the vicinal coupling constants and perturbation of chemical shift of compounds **2-11** are reminiscent of those found in GS. Therefore, peptides **2-11** have a β -sheet structure which is most likely similar to that of GS.

The assessment of the antibacterial activity of peptides **2-11** and GS was performed using a standard minimal inhibitory concentration (MIC) test on several Gram-positive and Gram-negative bacterial strains. The results, listed in Table 1, show activity for GS and peptide **2** that are in agreement with the literature data.⁴ Azides **4** and **5** as well as peptide **3**, containing benzylated ^DTyr , have activity profiles comparable to gramicidin S. However, peptides **6-11** display a considerable loss of activity. The exocyclic amines of Amp, adding positive charge to the turn regions of **6** and **7** retain a small activity for *S. epidermidis*. Supplementary negative charge, introduced by the succinyl-group, leads to some activity for peptide **10** against *S. epidermidis*, *E. faecalis* and *E. coli*. and for **11** against *P. aeruginosa*.

Compounds	<i>S. aureus</i> ^a		<i>S. epidermidis</i> ^a		<i>E. faecalis</i> ^a		<i>E. coli</i> ^b		<i>P. aeruginosa</i> ^b		<i>B. cereus</i> ^a	
	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d
1	4	8	4	4	8	16	32	32	n.d.	>64	n.d.	8
2	32	32	8	8	32	32	64	>64	n.d.	>64	n.d.	8
3	8	16	4	8	8	32	>64	>64	n.d.	>64	n.d.	16
4	4	8	2	4	8	8	32	32	n.d.	>64	n.d.	>64
5	4	8	4	8	16	16	>64	>64	n.d.	>64	n.d.	16
6	>64	>64	32	32	>64	>64	>64	>64	>64	>64	>64	>64
7	>64	>64	64	64	64	>64	>64	>64	>64	>64	>64	>64
8	>64	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
9	>64	>64	32	64	64	64	>64	64	>64	>64	>64	>64
10	>64	>64	>64	32	32	32	>64	32	>64	>64	>64	64
11	>64	>64	64	>64	64	>64	64	>64	64	32	64	64

Table 1: Antimicrobial activity (MIC in $\mu\text{g/ml}$). Measurements were executed using standard agar dilution techniques. ^a Gram-positive ^b Gram-negative ^c 3 ml / 25 well plates ^d 100 μl / 96 microtiter plates. (n.d. = not determined).

In contrast to the introduction of hydrophobic moieties on the tyrosine residues, as in **3**, added hydrophobicity to the proline residue (*i.e.* **8** and **9**) largely abolishes all activity. These findings underscore the speculation of Izuyama *et al.*,³ that large groups on the ^DPhe-position have a stabilizing effect on the β -turn, thereby enhancing the antimicrobial activity. However, as the exact process of membrane disruption is not fully understood,²¹ the existence of β -sheet structure alone in the presented GS analogues can not be used for the prediction of potential antimicrobial activity.

Conclusion

The biomimetic synthesis of GS and analogues **2-5** was successfully employed. Modification of the fully assembled azide containing peptides **4** and **5** led to several GS analogues (**6-11**) having hydrophobic and hydrophilic functionalities in the turn region. The conservation of β -sheet character was confirmed for all peptides (**1-11**) using standard NMR techniques. Examination of the antimicrobial activity of the aforementioned peptides showed a lowered bactericidal effect for compounds **6-11**. Apparently, modification of the proline residue is counterproductive with respect to antibacterial activity even when the β -sheet character is preserved. The highest antimicrobial activity was observed for azides **4** and **5**, reflecting the small tolerance for turn region modifications. Surprisingly, the benzylated ^DTyr analogue **3** was more active than its unprotected counterpart **2**. Thus, the introduction of large aromatic entities in the ^DPhe region holds promise for the future development of GS-based antibiotics.

Experimental Section

The SPPS was performed on an ABI 433A (Applied Biosystems) automated peptide synthesizer supplied with the FastMoc[®] peptide synthesis protocol. ¹H NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer at 298 K. Chemical shifts (δ) are tabulated in ppm, relative to the solvent peak of CD₃OH (3.30 ppm), unless stated otherwise. LC/MS analysis was performed on a Jasco HPLC-system (simultaneous detection at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI). An analytical Alltima C₁₈ column (Alltech, 4.6 mmD $\times$ 250 mmL, 5 μ particle size) was used in combination with buffers A: H₂O, B: MeCN and C: 0.5% aq. TFA. For RP-HPLC purification of the peptides, a BioCAD “Vision” automated HPLC system (PerSeptiveBiosystems, inc.) equipped with a semi-preparative Alltima C₁₈ column (Alltech, 10.0 mmD $\times$ 250 mmL, 5 μ particle size) was used. The applied buffers were A: H₂O, B: MeCN and C: 1.0% aq. TFA.

cyclo-(^DPhe-Pro-Val-Orn-Leu)₂ (1): RP-HPLC purification (linear gradient of 3.5 CV; 40 $\rightarrow$ 75% B; R_t 3.2 CV) followed by lyophilization gave GS in a yield of 5.9 mg (5.2 μ mol, 5%). LC/MS analysis: R_t 20.48 min (linear gradient 50 $\rightarrow$ 90% B in 20 min); m/z = 1142.0 [M+H]⁺, 571.6 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^DTyr-Pro-Val-Orn-Leu)₂ (2): RP-HPLC purification (linear gradient of 4.0 CV; 30→65% B; R_t 3.5 CV) furnished, after lyophilization, unprotected peptide **2** in a yield of 3.8 mg (3.2 μmol, 3%). LC/MS analysis: R_t 10.15 min (linear gradient 25→90% B in 20 min); *m/z* = 1173.8 [M+H]⁺, 587.5 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^DTyr(Bn)-Pro-Val-Orn-Leu)₂ (3): RP-HPLC purification (linear gradient of 4.0 CV; 40→85% B; R_t 4.0 CV) furnished, after lyophilization, benzyl protected peptide **3** in a yield of 2.4 mg (1.7 μmol, 2%). LC/MS analysis: R_t 23.73 min (linear gradient 50→90% B in 20 min); *m/z* = 1354.1 [M+H]⁺, 677.8 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^DPhe-2*S*,4*R*-Azp-Val-Orn-Leu)₂ (4): RP-HPLC purification (linear gradient of 3.5 CV; 50→70% B; R_t 2.9 CV) gave, after lyophilization, azide **4** in a yield of 3.8 mg (3.1 μmol, 3%). LC/MS analysis: R_t 20.43 min (linear gradient 50→90% B in 20 min); *m/z* = 1223.9 [M+H]⁺, 612.6 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^DPhe-2*S*,4*S*-Azp-Val-Orn-Leu)₂ (5): RP-HPLC purification (linear gradient of 4.0 CV; 40→75% B; R_t 3.8 CV) gave, after lyophilization, azide **5** in a yield of 5.1 mg (4.2 μmol, 4%). LC/MS analysis: R_t 21.15 min (linear gradient 50→90% B in 20 min); *m/z* = 1224.0 [M+H]⁺, 612.6 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^DPhe-2*S*,4*R*-Amp-Val-Orn-Leu)₂ (6): The unprotected azide **4** (23 mg, 19 μmol) was dissolved in chloroform (2 mL) and methanol (2 mL) and a catalytic amount of 10% Pd/C was added. The resulting mixture was placed under an atmosphere of hydrogen and stirred for 16 h. The suspension was filtered over a plug of Hyflow Super Gel[®] and concentrated *in vacuo*. RP-HPLC purification (linear gradient of 3.5 CV; 30→55% B; R_t 1.6 CV) and freeze-drying of the combined collected fractions, furnished 12.93 mg of peptide **6** (11 μmol, 58%). LC/MS analysis: R_t 13.64 min (linear gradient 20→60% B in 20 min); *m/z* = 1172.0 [M+H]⁺, 586.6 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^DPhe-2*S*,4*S*-Amp-Val-Orn-Leu)₂ (7): The unprotected azide **5** (17 mg, 14 μmol) was treated similarly to **4**, to give, after RP-HPLC purification (linear gradient of 3.5 CV; 30→55% B; R_t 1.4 CV) and freeze-drying of all collected fractions, 9.89 mg of peptide **7** (8.4 μmol, 60%). LC/MS analysis: R_t 12.85 min (linear gradient 20→60% B in 20 min); *m/z* = 1172.0 [M+H]⁺, 586.6 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^DPhe-2*S*,4*R*-Amp(Z)-Val-Orn-Leu)₂ (8): To a solution of peptide **16** (17 mg, 12 μmol) in 1,4-dioxane (2 mL) and MeCN (2 mL) was added 100 μL (100 μmol) of PMe₃ (1 M in toluene). The mixture was stirred for 3 h after which H₂O (200 μL) was added and the mixture was allowed to stir for 16 h. All volatiles were evaporated under reduced pressure, and benzylchloroformate (7 μL, 48 μmol) and *N,N'*-diisopropylethylamine (12 μL, 72 μmol) in DMF (2 mL) was added to the residue. The solution was stirred for 6 h, concentrated, redissolved in DCM (2 mL) and cooled to 0°C after which TFA (2 mL) was added. The resulting mixture was warmed to room temperature over a period of 30 min. Evaporation of all solvents and RP-HPLC purification of the residue (linear gradient of 3.0 CV; 60→90% B; R_t 1.9 CV) gave, after lyophilization, the Z-protected peptide **8** in 7.2 mg (5.0 μmol, 42%). LC/MS analysis: R_t 13.96 min (linear gradient 50→90% B in 20 min); *m/z* = 1440.2 [M+H]⁺, 720.7 [M+H]²⁺. For ¹H NMR; see Table 2.

Table 2: Chemical shift (δ in ppm)

Peptide	Residue	α NH	α	β_d	β_u	γ_d	γ_u	δ_d	δ_u	NH
GS	Leu	8.80	4.66	1.55	1.41	1.50	-	0.87	0.87	-
	Orn	8.70	4.97	2.05	1.64	1.79	1.79	3.05	2.91	7.80
	Val	7.73	4.17	2.26	-	0.96	0.86	-	-	-
	Pro	-	4.35	2.00	1.67	1.71	1.59	3.73	2.48	-
	^D Phe	8.90	4.50	3.10	2.96			7.33 – 7.24		-
2	Leu	8.72	4.65	1.53	1.39	1.53	-	0.88	0.88	-
	Orn	8.68	4.97	2.01	1.64	1.74	1.74	2.99	2.81	7.81
	Val	7.71	4.15	2.26	-	0.95	0.88	-	-	-
	Pro	-	4.36	1.98	1.54	1.64	1.52	3.74	2.56	-
	^D Tyr	8.86	4.42	3.02	2.86			7.06 – 6.71		-
3	Leu	8.70	4.64	1.52	1.39	1.52	-	0.88	0.88	-
	Orn	8.67	4.98	2.02	1.60	1.74	1.74	3.01	2.84	7.81
	Val	7.68	4.13	2.23	-	0.93	0.86	-	-	-
	Pro	-	4.28	1.92	1.58	1.67	1.52	3.68	2.40	-
	^D Tyr	8.87	4.42	3.02	2.87			7.12 – 6.90		-
	Bn	-	4.87	-	-			7.42 – 7.25		-
4	Leu	8.74	4.65	1.51	1.38	1.51	-	0.87	0.87	-
	Orn	8.72	4.94	2.06	1.65	1.75	1.75	3.02	2.90	7.80
	Val	7.67	4.12	2.25	-	0.96	0.87	-	-	-
	Azp	-	4.41	2.25	1.88	3.95	-	3.95	2.54	-
	^D Phe	8.92	4.50	3.13	2.96			7.34 – 7.24		-
5	Leu	8.68	4.66	1.52	1.39	1.52	-	1.01	1.01	-
	Orn	8.64	4.98	2.04	1.61	1.77	1.77	3.04	2.89	7.82
	Val	7.72	4.26	2.24	-	1.01	0.89	-	-	-
	Azp	-	4.48	2.25	1.87	3.98	-	3.56	2.53	-
	^D Phe	8.86	4.43	3.09	2.94			7.31 – 7.23		-
6	Leu	8.72	4.62	1.47	1.37	1.47	-	0.85	0.85	-
	Orn	8.70	4.94	2.02	1.65	1.74	1.74	3.03	2.91	7.81
	Val	7.68	4.17	2.25	-	0.97	0.88	-	-	-
	Amp	-	4.57	2.48	1.99	3.76	-	4.31	2.84	-
	^D Phe	8.86	4.49	3.03	3.03			7.35 – 7.26		-
7	Leu	8.69	4.60	1.50	1.37	1.50	-	0.87	0.87	-
	Orn	8.64	4.92	2.03	1.68	1.77	1.77	3.02	2.94	7.81
	Val	7.71	4.10	2.24	-	1.00	0.89	-	-	-
	Amp	-	4.38	2.67	1.77	3.50	-	3.34	3.34	-
	^D Phe	8.96	4.64	3.12	2.94			7.38 – 7.16		-
8	Leu	8.73	4.67	1.56	1.41	1.56	-	0.91	0.91	-
	Orn	8.68	4.97	2.04	1.63	1.76	1.76	3.05	2.90	7.80
	Val	7.72	4.17	2.29	-	1.01	0.91	-	-	-
	Amp	-	4.40	2.29	1.75	4.06	-	4.13	2.29	7.03
	^D Phe	8.88	4.46	3.08	2.93			7.35 – 7.22		-
	Z	-	4.90	-	-			7.35 – 7.22		-
9	Leu	8.72	4.64	1.52	1.38	1.52	-	0.88	0.88	-
	Orn	8.63	4.96	2.06	1.62	1.76	1.76	3.06	2.88	7.79
	Val	7.64	4.09	2.22	-	0.93	0.85	-	-	-
	Amp	-	4.31	2.24	1.90	3.37	-	3.37	3.02	6.99
	^D Phe	8.87	4.51	3.10	2.92			7.42 – 7.16		-
	Z	-	4.88	-	-			7.42 – 7.16		-
10	Leu	8.70	4.65	1.46	1.38	1.46	-	0.88	0.88	-
	Orn	8.66	4.95	2.02	1.61	1.74	1.74	3.04	2.89	7.82
	Val	7.74	4.16	2.25	-	0.98	0.88	-	-	-
	Amp	-	4.42	2.27	1.69	4.20	-	4.20	2.27	7.96
	^D Phe	8.88	4.42	3.08	2.92			7.32 – 7.23		-
	Succinyl	-	-	2.52	2.52	2.36	2.36	-	-	-
11	Leu	8.74	4.64	1.51	1.38	1.51	-	0.89	0.89	-
	Orn	8.64	4.96	2.05	1.63	1.76	1.76	3.04	2.88	7.79
	Val	7.64	4.09	2.25	-	1.00	0.87	-	-	-
	Amp	-	4.30	2.40	1.76	3.47	-	3.23	3.23	7.95
	^D Phe	8.86	4.51	3.10	2.91			7.35 – 7.21		-
	Succinyl	-	-	2.52	2.52	2.37	2.37	-	-	-

cyclo-(^pPhe-2S,4S-Amp(Z)-Val-Orn-Leu)₂ (9): To a solution of peptide **17** (4 mg, 2.8 μmol) in 1,4-dioxane (2 mL) and MeCN (2 mL) was added 23 μL (23 μmol) of PMe₃ (1 M in toluene). The mixture was stirred for 3 h after which H₂O (200 μL) was added and allowed to stir for 16 h. All volatiles were evaporated under reduced pressure, and benzylchloroformate (2 μL, 12 μmol) and *N,N'*-diisopropylethylamine (3 μL, 17 μmol) in DMF (2 mL) were added to the residue. The solution was stirred for 6 h, concentrated, redissolved DCM (2 mL) and cooled to 0°C after which TFA (2 mL) was added. The resulting mixture was allowed to warm to room temperature over a period of 30 min. Evaporation of all solvents and RP-HPLC purification of the residue (linear gradient of 3.0 CV; 50→90 % B; R_t 2.4 CV) gave, after lyophilization, the Z-protected peptide **9** in 1.5 mg, (1.0 μmol, 36%). LC/MS analysis: R_t 16.02 min (linear gradient 50→90% B in 20 min); *m/z* = 1440.0 [M+H]⁺, 720.7 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^pPhe-2S,4R-Amp(Su)-Val-Orn-Leu)₂ (10): Azide **16** (17 mg, 12 μmol) was dissolved in 1,4-dioxane (2 mL) and MeCN (2 mL) and to the solution was added trimethylphosphine (100 μL, 1.0 M in toluene). The resulting mixture was stirred for 3 h, followed by addition of H₂O (200 μL) and stirred 16 h. All volatiles were removed by evaporation and succinic anhydride (4.8 mg, 48 μmol) and triethylamine (6.8 μL, 48 μmol) in *N,N'*-dimethylformamide (4 mL) were added to the residue. After stirring for 2 h the reaction was quenched with H₂O (200 μL) and concentrated. The resulting peptide were dissolved in DCM (5 mL) and cooled to 0°C, after which TFA (5 mL) was added. The mixture was allowed to warm to room temperature, stirred for 30 min and concentrated *in vacuo*. RP-HPLC purification of the residue (linear gradient of 3.0 CV; 30→50% B; R_t 2.7 CV) followed by lyophilization furnished 12.8 mg of **10** (9.3 μmol, 78%). LC/MS analysis: R_t 15.39 min (linear gradient 20→60% B in 20 min.); *m/z* = 1371.9 [M+H]⁺, 686.6 [M+H]²⁺. For ¹H NMR; see Table 2.

cyclo-(^pPhe-2S,4S-Amp(Su)-Val-Orn-Leu)₂ (11): Azide **17** (15 mg, 11 μmol) was subjected to the same reaction conditions as described for peptide **10**. RP-HPLC purification (linear gradient of 3.0 CV; 30→50% B; R_t 2.8 CV) followed by lyophilization furnished 9.4 mg of **11** (6.9 μmol, 66%). LC/MS analysis: R_t 14.30 min (linear gradient 20→60% B in 20 min.); *m/z* = 1372.0 [M+H]⁺, 686.7 [M+H]²⁺. For ¹H NMR; see Table 2.

Fmoc-Leu-HMPB-MBHA resin (13): 4-Methylbenzhydrylamine resin **12** (806 mg, 0.5 mmol) was suspended in 1,4-dioxane, evaporated to dryness (3 × 50 mL) and resuspended in NMP (25 mL). To the mixture, HMPB (360 mg, 1.5 mmol), BOP (663 mg, 1.5 mmol) and DiPEA (0.523 mL, 3.0 mmol) were added. The suspension was shaken 16 h, filtered and the resin was consecutively washed with DCM (2 × 20 mL), MeOH (20 mL) and DCM (2 × 20 mL). The resin was suspended in 1,4-dioxane, evaporated to dryness (3 × 50 mL) and resuspended in DCM (25 mL). Subsequent condensation of the first amino acid was effected by addition of Fmoc-Leu-OH (530 mg, 1.5 mmol), DIC (0.258 mL, 1.65 mmol) and DMAP (10 mg, 82 μmol) after which the reaction mixture was shaken for 2 hours. Washing of the resin and a second esterification cycle was performed as described above. The loading of the resin was determined to be 0.48 mmol × g⁻¹.

General procedure for peptide synthesis:

(a) Stepwise elongation: The immobilized peptides (**14a-e**) were synthesized using 210 mg (0.1 mmol) of resin **13**. The consecutive steps in each coupling cycle were: *i*. Deprotection: 20% piperidine in NMP (2 mL) 5 × 1 min *ii*. Coupling: the appropriate amino acid (0.5 mmol) was dissolved in NMP (1 mL) and subsequently 0.5 mmol (0.5 M BOP/0.5 M HOBt in NMP/DMF 1/1, v/v) and 1.5 mmol of DiPEA (1.25 M in NMP) were added. The resulting mixture was transferred to the reaction vessel and

shaken for 90 min *iii*. Capping: the resin was subjected to 1 min of shaking in a solution of 0.5 M acetic anhydride, 0.125 M DiPEA and 0.015 M HOBt in NMP (2 mL). The applied amino acids were Boc-^DPhe-OH, Boc-^DTyr-OH, Fmoc-2S,4R-Azp-OH, Fmoc-2S,4S-Azp-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Pro-OH and Fmoc-Val-OH. Double couplings were executed for Val.

(b) Cleavage from the resin: The resin (**14a-e**) was treated with 1% TFA in DCM (4 × 10 mL). All fractions were collected and after addition of toluene (50 mL) concentrated under reduced pressure. The mass of the fully protected peptides was established by mass spectroscopy (ESI-MS): **a** m/z = 830.4 [M+H]⁺, 852.6 [M+Na]⁺ **b** m/z = 830.7 [M+H]⁺, 852.6 [M+Na]⁺ **c** m/z = 895.7 [M+H]⁺, 917.7 [M+Na]⁺ **d** m/z = 805.7 [M+H]⁺, 827.5 [M+Na]⁺ **e** m/z = 789.9 [M+H]⁺, 811.7 [M+Na]⁺

(c) Activation of pentapeptides: The crude pentamers (100 μmol) were dissolved in DMF (2 mL) and cooled to 0°C. To this mixture were added N-hydroxy succinimide (23 mg, 200 μmol) in DCM (1 mL) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (38 mg, 200 μmol). The reaction mixture was then allowed to warm to room temperature and stirred 16 h. The resulting solution was concentrated and partitioned between DCM (50 mL) and H₂O (10 mL). The organic layer was dried (MgSO₄) and concentrated.

(d) Deprotection: The Boc-protection groups were removed by dissolving the peptide active esters in DCM (2 mL) followed by addition of TFA (2 mL) at 0°C. The reaction mixture was allowed to warm to room temperature, stirred for 30 min after which toluene (10 mL) was added and the solvents were removed *in vacuo*. The crude peptides (**15a-e**) were used in the following cyclodimerization reaction without further purification.

(e) Cyclodimerization procedure: The crude active esters **15a-e** were taken up in DMF (2 mL) and added dropwise to pyridine (30 mL). After stirring for 24 h, the resulting mixture was concentrated, analysed by LC/MS and purified by RP-HPLC to give peptides **1-5**.

cyclo-(^DPhe-2S,4R-Azp-Val-Orn(Boc)-Leu)₂ (16): To a solution of cyclic decamer **4** (53 mg, 37 μmol) in MeCN (5 mL) were added DiPEA (34 μL, 194 μmol) in CHCl₃ (1 mL) and di-*tert*-butyl dicarbonate (21 mg, 97 μmol). After stirring for 3 h, the solvents were removed *in vacuo* and the resulting residue was directly subjected to silica gel column chromatography (0→5% MeOH in EtOAc) to furnish **16** as a white amorphous solid (47 mg, 33 μmol, 89%). MS (ESI): m/z = 1424.2 [M+H]⁺, 1445.9 [M+Na]⁺ ¹H NMR (DMSO-*d*₆): δ = 8.94 (d, 1H, NH ^DPhe, *J* = 2.6 Hz), 8.55 (d, 1H, NH Orn, *J* = 8.8 Hz), 8.36 (d, 1H, NH Leu, *J* = 8.6 Hz), 7.26 (bs, 6H, H_{arom} ^DPhe, NH Val), 6.77 (m, 1H, δNH Orn), 4.70 (m, 1H, H_α Orn), 4.54 (m, 2H, H_α Leu, Amp), 4.39 (m, 2H, H_α ^DPhe, Val), 4.00 (m, 1H, H_γ Amp), 3.82 (m, 1H, H_δ Amp), 2.99 (m, 1H, H_β ^DPhe), 2.88 (m, 3H, H_δ Orn, H_β ^DPhe), 2.48 (m, 1H, H_δ Amp), 2.30 (m, 1H, H_β Amp), 2.01 (m, 1H, H_β Val), 1.67 (m, 1H, H_β Orn), 1.59 (m, 1H, H_β Amp), 1.43-1.15 (m, 15H, 3 × CH₃ Boc, 1 × H_β Orn, 2 × H_γ Orn, 2 × H_β Leu, 1 × H_γ Leu), 0.89-0.77 (m, 12H, 2 × H_δ Leu, 2 × H_γ Val).

cyclo-(^DPhe-2S,4S-Azp-Val-Orn(Boc)-Leu)₂ (17): Starting from **5** (51 mg, 35 μmol) peptide **17** was obtained as described for **16**, as a white amorphous solid (42 mg, 30 μmol, 86%). MS (ESI): m/z = 1424.2 [M+H]⁺, 1445.9 [M+Na]⁺ ¹H NMR (DMSO-*d*₆): δ = 8.81 (d, 1H, NH ^DPhe, *J* = 2.8 Hz), 8.57 (d, 1H, NH Orn, *J* = 8.9 Hz), 8.34 (d, 1H, NH Leu, *J* = 8.9 Hz), 7.27 (bs, 5H, H_{arom} ^DPhe), 7.18 (d, 1H, NH Val, *J* = 8.9 Hz), 6.80 (m, 1H, δNH Orn), 4.69 (m, 1H, H_α Orn), 4.55 (m, 3H, H_α Leu, H_α Val, H_α Amp), 4.35 (m, 1H, H_α ^DPhe), 4.11 (m, 1H, H_γ Amp), 3.41 (d, 1H, 1 × H_δ Amp, *J* = 11.5 Hz), 2.92 (m, 4H, 2 × H_δ Orn, 2 × H_β ^DPhe), 2.73 (m, 1H, 1 × H_δ Amp), 2.20 (d, 1H, 1 × H_β Amp, *J* = 13.3 Hz), 1.96 (m, 1H, H_β Val), 1.71 (m, 2H, 1 × H_β Amp, 1 × H_β Orn), 1.48-1.16 (m, 15H, 3 × CH₃ Boc, 1 × H_β Orn, 2 × H_γ Orn, 2 × H_β Leu, 1 × H_γ Leu), 0.87-0.80 (m, 12H, 2 × H_δ Leu, 2 × H_γ Val).

Biological Activity:

The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus cereus* (ATCC 11778). Bacteria were stored at -70°C and grown at 35°C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) overnight and diluted in 0.9% NaCl. Large plates (25 wells of 3 mL) as well as microtitre plates (96 wells of 100 μL) were filled with Mueller Hinton II Agar (Becton Dickinson, Cockeysville, USA) containing serial twofold dilutions of peptides **1-11**. To the wells was added 3 μL of bacteria, to give a final inoculum of 10^4 colony forming units (CFU) per well. The plates were incubated overnight at 35°C and the MIC was determined as the lowest concentration inhibiting bacterial growth.

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Chapter 3

Synthesis and Biological Evaluation of Gramicidin S Dimers

Abstract: *The design and synthesis of analogues of the cyclic β -sheet peptide gramicidin S (GS), having additional functionalities in their turn regions, is reported. The monomeric GS analogues were transformed into dimers and their activities towards biological membranes, through antimicrobial and hemolytic assays, were evaluated. Finally, conductivity measurements were performed to elucidate ion channel forming properties.¹*

Introduction

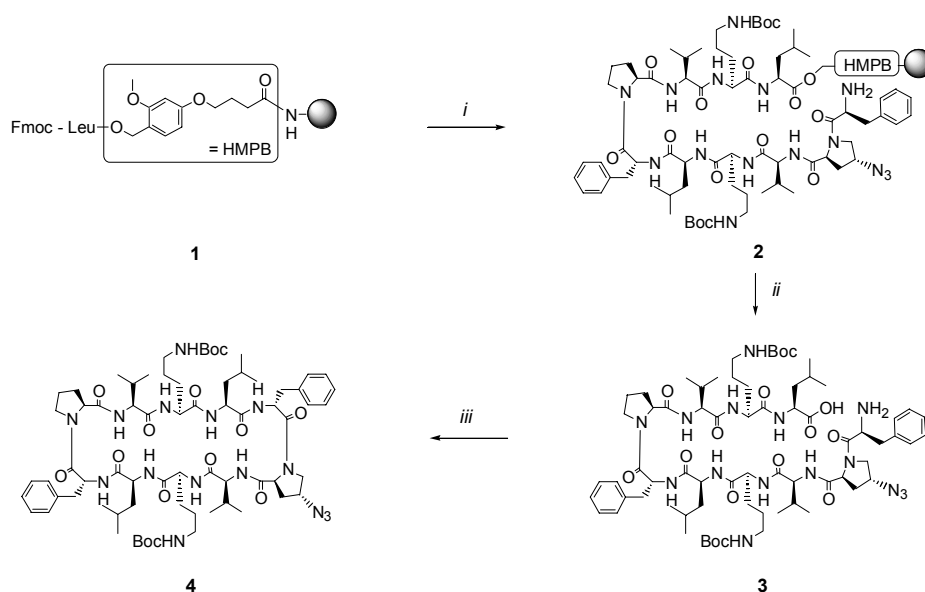
Cationic antimicrobial peptides (CAPs), a class of structurally diverse peptide-based compounds, are ubiquitously present in nature and are often important compounds of innate immune systems.² CAPs exert their biological activity by disturbing the integrity of the cell membrane of pathogenic organisms, and are thought to do so through direct interaction with the lipid bilayer.³ From the thought that, in most cases, there is no specific subcellular protein target involved in CAP-mediated cell lysis, it follows that the occurrence of pathogens with an acquired CAP-resistance may be unlikely. These considerations, to a large extent, explain current interest in CAPs and research efforts are focussed both on the elucidation of the molecular mechanisms that are at the basis of CAP-mediated membrane disturbance, and on the development of CAPs towards therapeutic agents.

Gramicidin A (GA) is a CAP that has attracted considerable attention over the years.⁴ This pentadecapeptide exerts its antibacterial properties by adopting a β -helical secondary

structure that associates into a dimer, thereby forming an active ion channel that traverses lipid bilayers. Schreiber and coworkers elegantly demonstrated that covalently linked GA monomers result in unimolecular channels spanning the membrane.⁵ More recently, unimolecular membrane-spanning ion channels that have a preference for specific ions were obtained by linking two GA monomers through tetrahydrofuran (THF)-based dipeptide isosters.⁶ The potential of synthetic dimers is further underscored by the requirement that accumulation of CAPs onto the lipid bilayer precedes pore formation. In this way, synthetic dimers can induce a shift in the dissociation/association equilibrium, resulting in favouring of the conducting over the non-conducting states of ion-channels. This enables the study of the molecular architecture of ion channels and creates an understanding of the dynamics involved. For example, Woolley and coworkers showed that tethered alamethicin monomers selectively stabilise specific conductance states,⁷ while Murata and coworkers have prepared bioactive dimers of the polyene antibiotic amphotericin B that enabled the study of pore assemblage.⁸ The design of novel GS analogues, may contribute to gain insight into the mechanism of its lytic effects, induce membrane specificity in order to curb its undesirable erythrocytic toxicity and to see whether defined channels can be resolved. In this chapter, the results are presented in the design, synthesis and biological evaluation of a set of dimeric GS analogues.

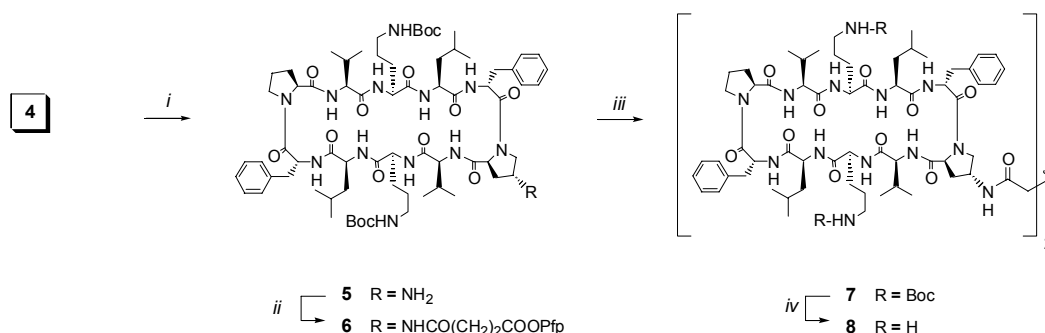
Results and Discussion

The synthesis of GS analogue **4** commences (Scheme 1) with the installation of the acid-labile HMPB-linker on MBHA-functionalized polystyrene. Subsequent esterification with Fmoc-Leu-OH using *N,N'*-diisopropylcarbodiimide (DIC) and a catalytic amount of 4-(dimethylamino)-pyridine (DMAP) furnished loaded resin **1** (0.50 mmol/g) as described in **Chapter 2**.⁹ Further elongation of the peptide was effected by standard SPPS (0.1 mmol scale), employing 20% piperidine in NMP for the liberation of the α -amine functionality followed by condensation with an commercially available Fmoc-protected amino acid building block or the readily accessible Fmoc-2*S*,4*R*-azidoproline (Azp)¹⁰ (3 equiv) effected by Castro's reagent¹¹ (3 equiv), *N*-hydroxybenzotriazole (HOBt, 3 equiv) and DiPEA (3.6 equiv). The immobilized decapeptide **2** was subsequently released from the resin by acidic cleavage (1% TFA in DCM). Next, the crude linear peptide **3** was dissolved in DMF and added dropwise, over a period of 60 min, to a vigorously stirred solution of benzotriazol-1-yl-oxytri-pyrrolidinophosphonium hexafluorophosphate (PyBOP, 5 equiv), HOBt (5 equiv) and DiPEA (15 equiv) to give a final concentration of 1.3×10^{-3} M of peptide. This mixture was then stirred overnight, concentrated *in vacuo*, applied to a SephadexTM size exclusion column and eluted with MeOH. The fractions containing Boc-protected **4** were pooled and concentrated, yielding a white amorphous solid in 86% yield.



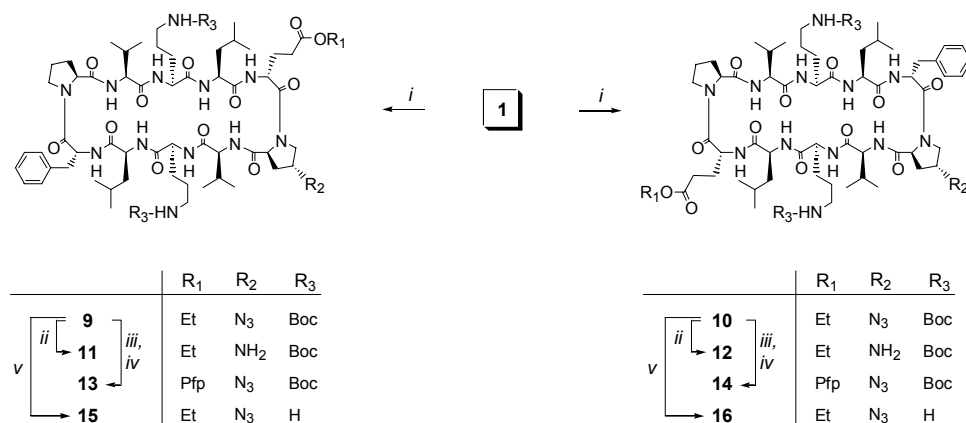
Scheme 1: *Reagents and conditions:* (i) Repetitive deprotection: piperidine/NMP (1/4 v/v), condensation: Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-^DPhe-OH, Fmoc-Leu-OH or Fmoc-Azp-OH (3 equiv), BOP (3 equiv), HOBT (3 equiv), DiPEA (3.6 equiv), NMP; (ii) TFA/DCM (1/99 v/v) 4 × 10 min; (iii) PyBOP (5 equiv), HOBT (5 equiv), DiPEA (15 equiv), DMF, 16 h, 86%.

The synthesis of GS dimer **8** (Scheme 2) was accomplished from GS analogue **4** as follows. Reduction of the azide moiety under Staudinger conditions gave free amine **5**, that was treated with succinic anhydride and subsequently condensed with pentafluorophenol (Pfp) under the agency of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to furnish **6** in 80% over the three steps. The activated ester in the monomeric GS analogue was next reacted with a slight excess of **5** to produce GS dimer **7**. Acidolysis of the Boc-protection groups still present on the Orn-residues followed by reversed-phase HPLC purification yielded the succinyl-tethered dimer **8** in 69%, the identity of which was confirmed by LC/MS analysis.



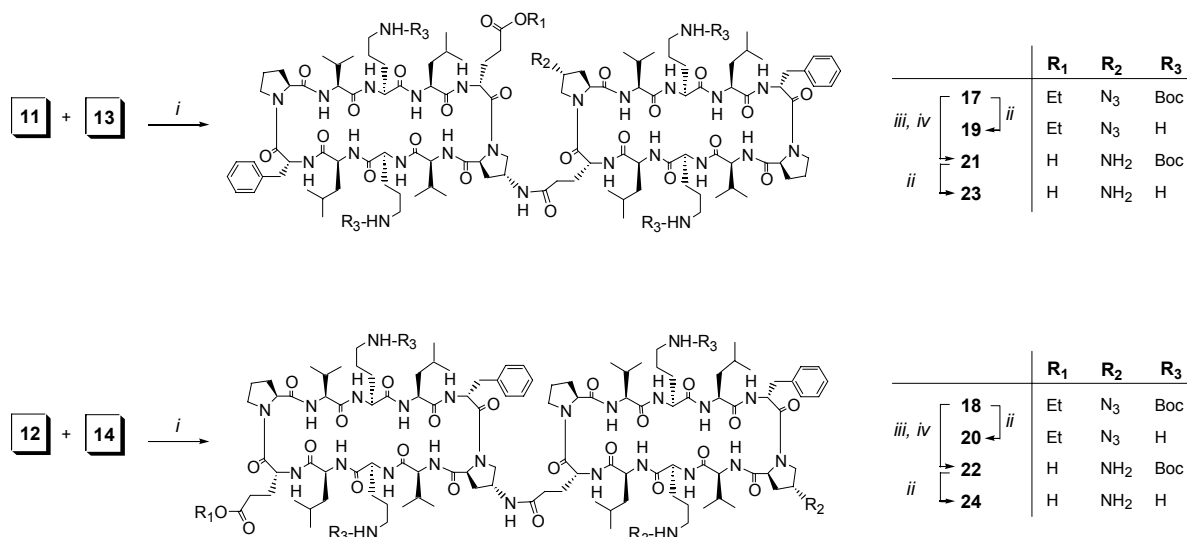
Scheme 2: *Reagents and conditions:* (i) PMe₃ (1 M in toluene, 8 equiv), 1,4-dioxane/MeCN (1/1 v/v), 6 h; (ii) a) succinic anhydride (4 equiv), TEA (4 equiv), DMF, 16 h. b) pentafluorophenol (2 equiv), EDC (2 equiv), DCM, 2 h, 80% in 3 steps; (iii) **5** (1.1 equiv), DiPEA (2 equiv), DMF, 48 h, 92%; (iv) TFA / DCM (1/1 v/v), 30 min, 69%.

Encouraged by these results, we set out to connect turn-modified GS analogues directly through newly introduced amino acid side-chain functionalities. For this purpose, a set of monomeric GS analogues, in which ^DPhe- and Pro-residues residing in the same or opposing turn regions are replaced by ^DGlu- and aminoproline (Amp)-residues, respectively, were prepared as follows (Scheme 3). Commencing from solid support **1**, fully protected cyclic peptides **9** and **10** were constructed as described for **4** in 82% and 92% yield, respectively. Staudinger reduction of the azide in **9** and **10** afforded the amines **11** and **12**, respectively. Saponification of the ester moiety in **9** and **10** revealed their carboxylic acid counterparts that were directly esterified with pentafluorophenol employing EDC to furnish **13** and **14**. GS monomers **11-14** were used without further purification in the following reaction steps. To facilitate characterization by LC/MS analysis and to evaluate the biological profile of the monomers, small aliquots of **9** and **10** were deprotected and purified by HPLC to produce **15** and **16** in their respective yields of 68% and 89%.



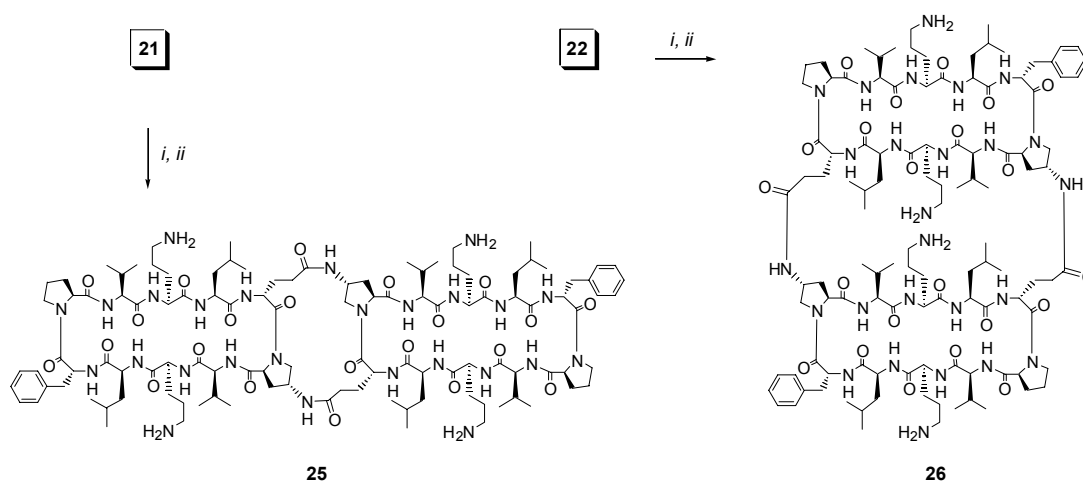
Scheme 3: *Reagents and conditions:* (i) As described for **4** (*vide supra*), **7**, 82% and **8**, 92%; (ii) PMe₃ (1 M in toluene, 8 equiv), 1,4-dioxane/MeCN (1/1 v/v), 6 h; (iii) 1 M NaOH, 1,4-dioxane, 4 h, then Amberlite IR-120 (H⁺); (iv) pentafluorophenol (2 equiv), EDC (2 equiv), DCM, 2 h; (v) TFA/DCM (1/1 v/v), 30 min, **15**, 68% and **16**, 89%.

Having set the stage for coupling of the separate monomeric building blocks, equimolar amounts of amine **11** and activated ester **13** were reacted to furnish dimer **17** in 45%, as is depicted in Scheme 4. Similarly, acylation of GS analogue **12** with Pfp-ester **14** gave side-chain linked dimer **18** in 85%. The Boc protective groups in **17** and **18** were subsequently removed to provide, after HPLC purification, their respective unprotected dimers **19** (66%) and **20** (31%). The azide and ester moieties in dimers **17** and **18** were also quantitatively transformed into the amine and carboxylic acid functionalities present in **21** and **22**. The latter peptides were subsequently deprotected to give dimers **23** (53%) and **24** (53%) as gauged by LC/MS analysis.¹²



Scheme 4: Reagents and conditions: (i) DMF, **17**; 45% and **18**; 85%; (ii) TFA / DCM (1/1 v/v), 30 min, **19**, 66%; **20**, 31%; **22**, 53% and **24**, 53%; (iii) PMe₃ (1 M in toluene, 8 equiv), 1,4-dioxane/MeCN (1/1 v/v), 6 h; (iv) 1 M NaOH, 1,4-dioxane, 4 h, then Amberlite IR-120 (H⁺) **21**, quant and **22**, quant (two steps).

As a final synthetic objective, it was investigated whether intramolecular cyclization of **21** and **22** was feasible. Condensation of the carboxylic acid and amine functionalities in **21** could be effected (Scheme 5) by dropwise addition of the peptide solution to a mixture of benzotriazole-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP), *N*-hydroxybenzotriazole (HOBT) and *N,N*-diisopropylethylamine (DiPEA) in DMF. Liberation of the Boc-protection groups yielded the rigid GS dimer **25** as the major product, in 25% yield over two steps. A similar intramolecular cyclization was performed on peptide **22**. Previous observations of β -sheet alignment in GS,^{13a} and the proposed “cross- β ” aggregates by Goodman and coworkers,^{13b} were expected to facilitate this cyclization reaction. However, the reaction conditions described for the cyclization of **21** proved to be marginally effective in yielding product **26** (15%).¹⁴



Scheme 5: Reagents and conditions: (i) BOP (5 equiv), HOBT (5 equiv), DiPEA (15 equiv), DMF, 16 h. (ii) TFA/DCM (1/1 v/v), 30 min, **25**, 25% and **26**, 15% (two steps).

Table 1: Antimicrobial activity (MIC in µg/ml).

Peptide	<i>S. aureus</i> ^a		<i>S. epidermidis</i> ^a		<i>E. faecalis</i> ^a		<i>B. cereus</i> ^a		<i>E. coli</i> ^b		<i>P. aeruginosa</i> ^b	
	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d
GS	8	8	4	4	n.d.	8	4-8	4	>64	32	>64	>64
8	>64	>64	64	>64	>64	>64	>64	>64	>64	>64	>64	>64
15	8	8	4	8	16	8-16	8	8	64	64->64	>64	>64
16	8	8	4	8	8	8-16	8	8	>64	64	>64	>64
19	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
20	64->64	>64	64->64	>64	>64	>64	>64	>64	>64	>64	>64	>64
23	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
24	>64	>64	64	32	>64	>64	>64	>64	>64	>64	>64	>64
25	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64

Measurements were executed using standard agar two-fold dilution techniques (n.d. = not determined).

^a Gram-positive ^b Gram-negative ^c 3 ml / 25 well plates ^d 100 µl / 96 microtiter plates.

Having the various GS dimers in hand, attention was focussed on the evaluation of their antimicrobial, hemolytic and conductance-increasing properties. The capacity of the GS dimers, and the monomers from which they are assembled, to arrest the proliferation of several Gram-positive and -negative bacterial strains was examined using a standard minimal inhibitory concentration (MIC) test (Table 1).¹⁵ From these results, it can be concluded that the modifications in the reverse turn, an Azp- for a Pro-residue and a ^DGlu- for ^DPhe-residue in monomers **15** and **16**, have no adverse effect on the antimicrobial properties compared to native GS. Conversely, the dimers had lost virtually all activity towards the tested Gram-positive and -negative strains with only dimer **24** displaying limited activity against *Staphylococcus epidermidis*.

A standard assay was applied to assess the hemolytic activity of the GS analogues towards human erythrocytes using a two-fold dilution series of the appropriate peptide and interpolating between 100% lysis induced by 1% Triton X-100 in saline and a blank. As can be gauged from the results in Table 2, all tested peptides, both GS monomers and GS dimers, cause considerable hemolysis in the 60 µM to 30 µM range, making them similarly toxic as or even more (as for **19**) toxic than the native peptide. However, most GS dimers in this assay display the propensity to lyse erythrocytes over a broader range of concentrations starting as low as 1.0 µM to 0.5 µM but often reaching 100% lysis only at 250 µM to 125 µM.

Table 2: Hemolytic activity

Peptide	% Hemolysis at the peptide concentration (µM)										
	250.0	125.0	62.5	31.3	15.6	7.8	3.9	2.0	1.0	0.5	0.2
GS			100 ± 2	80 ± 3	50 ± 1	24 ± 0	2 ± 2	0 ± 0	0 ± 1	0 ± 0	n.d.
8	100 ± 5	79 ± 9	50 ± 3	62 ± 5	65 ± 3	49 ± 6	32 ± 5	8 ± 4	8 ± 1	3 ± 3	n.d.
15	80 ± 5	90 ± 3	92 ± 3	52 ± 7	21 ± 3	3 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	n.d.
16		100 ± 5	66 ± 1	35 ± 7	25 ± 2	8 ± 0	0 ± 0	0 ± 1	0 ± 0	0 ± 0	n.d.
19				100 ± 1	93 ± 3	90 ± 31	77 ± 2	29 ± 3	14 ± 3	6 ± 2	0 ± 0
20		100 ± 2	87 ± 1	70 ± 9	62 ± 7	66 ± 2	44 ± 3	18 ± 3	6 ± 1	0 ± 0	n.d.
23	100 ± 0	98 ± 2	87 ± 7	76 ± 2	52 ± 2	26 ± 0	18 ± 2	10 ± 1	3 ± 4	0 ± 1	0 ± 0
24		100 ± 1	78 ± 3	52 ± 6	41 ± 5	23 ± 2	14 ± 1	2 ± 0	1 ± 0	0 ± 1	n.d.
25	96 ± 1	99 ± 4	82 ± 4	64 ± 4	51 ± 3	39 ± 4	26 ± 1	11 ± 1	1 ± 4	0 ± 0	n.d.

Measurements were executed using standard two-fold dilution techniques. (n.d. = not determined)

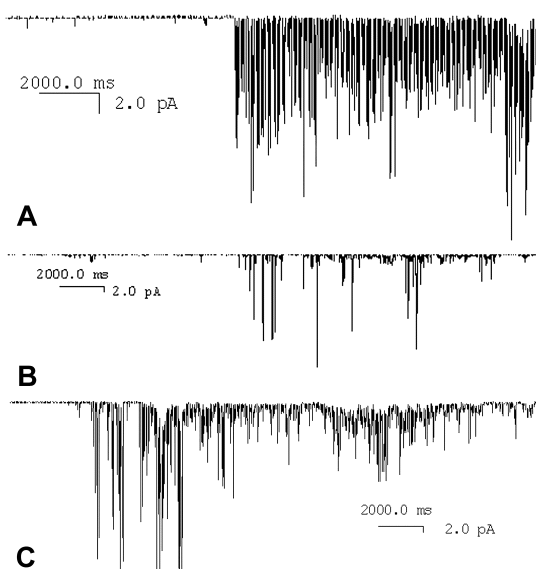


Figure 2: Conductivity traces for GS (A), dimer **8** (B) and dimer **25** (C) performed with 1M KCl in a DPhPC/DPhPGlycerol 4:1 membrane.

Finally, studies concerning the pore-forming properties of GS, succinyl-tethered GS dimer **8** and rigid GS dimer **25** have been conducted (Figure 2). Events of this type are commonly described as bursts and depending on the concentration, there is a minimal voltage required for these bursts. Below 10^{-6} molar, no effects were observed for GS and dimer **8**, but at 10^{-6} molar they induced rapid changes in conductances. Dimer **25** was slightly more active, displaying conductivity-increasing effects at 10^{-7} molar concentrations. Although all three compounds showed conductivity-increasing properties, no series of discrete single channels could be resolved.

Conclusion

A highly efficient strategy has been used for the synthesis of GS analogues that have been modified in the β -turn region, with ^{D} Phe residues being replaced by ^{D} Glu(OEt) and Pro-residues by Azp-residues. These GS monomers were subsequently covalently linked either *via* a succinyl-tether or directly through their side-chains to produce several differently functionalized GS dimers. Intramolecular cyclization also produced more conformationally restricted dimers albeit in moderate yields. As was previously observed with a GS dimer,¹⁶ no bactericidal effect against either Gram-positive or -negative strains could be detected. However, the GS dimers displayed a significant increase in hemolytic activity. Moreover, these compounds proved hemolytic over a broader range of concentrations which might suggest a different mode of action on the lipid bilayer. Upon studying the conductance-increasing properties of selected GS-dimers, membrane disruptive properties, but no discrete channels were observed.

Experimental Section

General: Reactions were monitored by TLC-analysis using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by spraying with 20% H₂SO₄ in EtOH, (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid or by spraying with a solution of ninhydrin (3 g/L) in EtOH / AcOH (20/1 v/v), followed by charring at ~150°C. Size exclusion chromatography was performed on Sephadex™ LH-20. For LC/MS analysis, a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical Alltima C₁₈ column (Alltech, 4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H₂O, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI) was used. For reversed-phase HPLC purification of the peptides, a BioCAD “Vision” automated HPLC system (PerSeptive Biosystems, inc.) equipped with a semi-preparative Alltima C₁₈ column (Alltech, 10.0 mmD × 250 mmL, 5μ particle size) was used. The applied buffers were A: H₂O, B: MeCN and C: 1.0% aq. TFA.

General procedure for peptide synthesis:

(a) Stepwise elongation: Resin **1** (200 mg, 0.5 mmol/g, 0.1 mmol) was submitted to nine cycles of Fmoc solid-phase synthesis with use of commercially available or readily accessible¹⁰ building blocks in the order: Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-^DPhe-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Azp-OH and Fmoc-^DPhe-OH. (a) deprotection with piperidine in NMP (1/4 v/v, 5 mL, 15 min); (b) washing with NMP (5 mL, 3 × 3 min); (c) coupling of the appropriate Fmoc amino acid (3 equiv, 0.3 mmol) in the presence of BOP (3 equiv, 134 mg, 0.3 mmol), HOBt (3 equiv, 44 mg, 0.3 mmol) and DiPEA (3.6 equiv, 68 μL, 0.36 mmol) which was preactivated for 2 min in NMP (5 mL) and shaken for 90 min; (d) washing with NMP (5 mL, 3 × 3 min). Couplings were monitored for completion by the Kaiser test,¹⁷ and Val-residues were standardly immobilized by applying a double coupling procedure.

(b) Cleavage from the resin: The *N*-terminal amine was liberated with piperidine in NMP (1/4 v/v, 5 mL, 15 min) followed by washing with NMP (5 mL, 3 × 3 min) and DCM (5 mL, 3 × 3 min). Afterwards, peptide **2** was treated with TFA/DCM (1/99, v/v, 10 mL, 4 × 10 min). The fractions were collected and coevaporated with toluene (50 mL) thrice, to give crude linear peptide **3** that was directly cyclized without further purification.

(c) Cyclization: Crude **3** was taken up in DMF (5 mL) and slowly added to a solution of PyBOP (5 equiv, 286 mg, 0.5 mmol), HOBt (5 equiv, 74 mg, 0.5 mmol) and DiPEA (15 equiv, 287 μL, 1.65 mmol) in DMF (80 mL) over the period of one hour and subsequently allowed to stir for 16 h. The mixture was concentrated and directly applied to a Sephadex™ LH-20 column (50.0 mmD × 1500 mmL) that was eluted with MeOH, to yield Boc-protected monomer **4** (120 mg, 86 μmol) in 86% as white amorphous solid.

(d) Deprotection: To confirm the identity of **4**, an aliquot was dissolved in DCM (2 mL) and the solution cooled to 0°C. TFA (2 mL) was added slowly and the mixture was stirred for 30 min, after which all volatiles were removed *in vacuo*. The identity of the deprotected peptide was established by LC/MS: R_t 13.94 min; linear gradient 10→90% B in 20 min.; *m/z* = 1183.0 [M+H]⁺, 592.4 [M+H]²⁺.

Monomer 5: Azide **4** (26 mg, 19 μmol) was dissolved in 1,4-dioxane (2 mL) and acetonitrile (2 mL), to which trimethylphosphine (0.15 mL, 0.15 mmol, 8 equiv, 1 M in toluene) was added. The mixture was stirred for 3 h, water (0.1 mL) was added and stirring was continued for another 3 h. Next, all solvents were removed *in vacuo* and the crude peptide was coevaporated with dry toluene thrice.

Monomer 6: Crude peptide **5** was dissolved in DMF (2 mL) and succinic anhydride (76 mg, 76 μ mol, 4 equiv) and triethylamine (76 mg, 76 μ mol, 4 equiv) were added. After stirring for 16 h, the solvent was evaporated and the mixture was directly applied to a LH-20 column that was eluted with MeOH. The peptide-containing fractions were pooled, evaporated and redissolved in DCM (2 mL). To the solution were added pentafluorophenol (6.3 mg, 34 μ mol, 2 equiv) and EDC (6.5 mg, 34 μ mol, 2 equiv) and stirring was continued for 2 h. The mixture was subsequently concentrated and partitioned between 0.1 N HCl and CHCl₃. The organic layer was dried (MgSO₄), filtered and concentrated, to furnish the activated ester **6** (22 mg, 14 μ mol) in 80% over 3 steps as an amorphous white solid.

Dimer 7: To Pfp-ester **6** (22 mg, 14 μ mol) in DMF (0.5 mL), a freshly prepared batch of amine **5** (22 mg, 16 μ mol, 1.1 equiv) in DMF (0.5 mL) was added and the resulting mixture was stirred for 48 h. The solvents were removed under reduced pressure and the mixture separated by size-exclusion chromatography using MeOH as eluent. The fractions containing peptide were pooled and evaporated to dryness to produce the title compound **7** (36 mg, 13 μ mol, 92%) as an amorphous white solid.

Dimer 8: The dimer **7** (18 mg, 6.4 μ mol) was deprotected as described in the general procedure to give crude **8**, that was analyzed by LC/MS (R_t 15.26 min; linear gradient 10→90% B in 20 min.; m/z = 2395.8 [M+H]⁺, 1198.6 [M+H]²⁺, 799.6 [M+H]³⁺) and purified by reversed-phase HPLC (linear gradient of 3.0 CV; 50→60% B; R_t 3.1 CV) to give dimer **8** (10.6 mg, 4.4 μ mol, 69%) as a fluffy white solid.

Monomer 9 and 10: From resin **1** (500 mg, 0.25 mmol), the peptides were assembled as described in the general procedure to furnish cyclic peptides **9** (299 mg, 0.21 mmol, 82%) and **10** (320 mg, 0.23 mmol, 92%) as white solids.

Monomer 11 and 12: Azides **9** (125 mg, 90 μ mol) and **10** (125 mg, 90 μ mol) were individually treated with PMe₃, as described for monomer **5**, to furnish crude amines **11** and **12** as white solids that were directly used in the next reaction step.

Monomer 13 and 14: Ethyl esters **9** (125 mg, 90 μ mol) and **10** (125 mg, 90 μ mol) were individually dissolved in EtOH (5 mL) and 1 M aq. NaOH (0.5 mL) was added. After stirring the mixtures for 2 h, TLC analysis showed completed conversion of starting material and Amberlite IR-120 (H⁺) was added. The neutral solutions were subsequently concentrated, coevaporated thrice with dry toluene and the crude acids were individually redissolved in DCM. To these were added pentafluorophenol (33 mg, 0.18 mmol, 2 equiv) and EDC (35 mg, 0.18 mmol, 2 equiv) and stirring was continued for 2 h. The mixture was diluted with CHCl₃ and extracted with 0.1 N HCl after which the organic layer was dried (MgSO₄), filtered and concentrated, to furnish the crude Pfp-esters **13** and **14** that were directly used in the next reaction step.

Monomer 15: To a cooled solution of cyclic peptide **9** (25 mg, 18 μ mol) in DCM (4 mL) was added TFA (4 mL) and the mixture was stirred for 30 min after which it was evaporated to dryness. The crude product was analyzed by LC/MS (R_t 18.53 min, linear gradient 10→90% B in 30 min; m/z = 1193.1 [M+H]⁺, 597.0 [M+H]²⁺), purified by RP-HPLC (linear gradient of 3.0 CV; 50→60% B; R_t 2.0 CV) and lyophilized, to produce peptide **15** (14.7 mg, 12 μ mol, 68%) as a white amorphous powder.

Monomer 16: The cyclic peptide **10** (30 mg, 22 μ mol) was similarly deprotected as for **15**, to give the crude peptide that was analyzed by LC/MS (R_t 18.48 min; linear gradient 10→90 % B in 30 min; m/z = 1193.1 [M+H]⁺, 597.0 [M+H]²⁺), purified by RP-HPLC (linear gradient of 3.0 CV; 50→60% B; R_t 2.3 CV) and lyophilized, to produce **16** (23.1 mg, 19 μ mol, 89%) as a white amorphous powder.

Dimer 17 and 18: Pfp-ester **13** was dissolved in DMF (2 mL) and a solution of amine **11** in DMF (2 mL) was slowly added. To this mixture, DiPEA (29 μ L, 0.18 mmol, 2 equiv) was added and the reaction was stirred 48 h. The solvents were subsequently removed *in vacuo* and the resulting mixture applied to a size-exclusion column that was eluted with MeOH. Pooling of the peptide-containing fractions gave dimer **17** (109 mg, 40 μ mol, 45%) as white amorphous solid. Monomer **14** was treated in an equal manner with amine **12** to furnish dimer **18** (207 mg, 76 μ mol, 85%) as a white amorphous solid.

Dimer 19 and 20: Dimers **17** (13 mg, 4.8 μ mol) and **18** (40 mg, 14.7 μ mol) were treated as described for **15**, to give crude **19** and **20**, respectively and were analyzed by LC/MS; **19**: R_t 15.97 min; linear gradient 10 $\rightarrow$ 90 % B in 20 min; m/z = 1157.5 $[M+H]^{2+}$, 771.8 $[M+H]^{3+}$, 597.4 $[M+H]^{4+}$ and **20**: R_t 18.71 min; linear gradient 10 $\rightarrow$ 90 % B in 30 min; m/z = 1157.6 $[M+H]^{2+}$, 772.0 $[M+H]^{3+}$, 597.4 $[M+H]^{4+}$. Subsequent purification by RP-HPLC of **19**: linear gradient of 3.0 CV; 55 $\rightarrow$ 70% B; R_t 2.2 CV and **20**: linear gradient of 3.0 CV; 50 $\rightarrow$ 60% B; R_t 2.9 CV, followed by freeze-drying, furnished **19** (7.4 mg, 3.2 μ mol, 66%) and **20** (10.4 mg, 4.5 μ mol, 31%) as white amorphous powders.

Dimer 21 and 22: Peptide **17** (55 mg, 20 μ mol) was dissolved in 1,4-dioxane (2 mL) and MeCN (2 mL) and PMe_3 (0.16 mL, 0.16 mmol, 8 equiv, 1 M in toluene) was added. The solution was stirred for 3 h, water (0.1 mL) was added and stirring was continued for another 3 h. All solvents were evaporated and the crude peptide was redissolved in EtOH (2 mL) and 1 M aq. NaOH (0.5 mL) was added. After stirring for 2 h, Amberlite IR-120 (H^+) was added and the neutral solution was subsequently concentrated and coevaporated thrice with dry toluene to quantitatively provide **21** (53 mg, 20 μ mol) as amorphous solid. Dimer **18** (20 mg, 7.5 μ mol) treated as described above to quantitatively furnish **22** (20 mg, 7.5 μ mol).

Dimer 23 and 24: Dimers **21** (15 mg, 5.4 μ mol) and **22** (20 mg, 7.5 μ mol) were treated as described for **15**, to give crude **23** and **24**, respectively and were analyzed by LC/MS; **23**: R_t 17.10 min; linear gradient 10 $\rightarrow$ 90 % B in 30 min; m/z = 1130.5 $[M+H]^{2+}$, 754.0 $[M+H]^{3+}$, 556.7 $[M+H]^{4+}$ and **24**: R_t 16.40 min; linear gradient 10 $\rightarrow$ 90 % B in 30 min; m/z = 1130.6 $[M+H]^{2+}$, 754.0 $[M+H]^{3+}$, 565.7 $[M+H]^{4+}$. Subsequent purification by RP-HPLC of **23**: linear gradient of 3.0 CV; 40 $\rightarrow$ 60% B; R_t 2.7 CV and **24**: linear gradient of 3.0 CV; 40 $\rightarrow$ 55% B; R_t 2.1 CV, followed by freeze-drying, furnished **23** (6.5 mg, 2.9 μ mol, 53%) and **24** (8.9 mg, 3.9 μ mol, 53%) as white amorphous powders.

Dimer 25: The crude **21** (45 mg, 20 μ mol) was taken up in DMF (3 mL) and slowly added to a solution of BOP (52 mg, 100 μ mol, 5 equiv), HOBT (14 mg, 100 μ mol, 5 equiv) and DiPEA (50 μ L, 300 μ mol, 15 equiv) in DMF (8 mL). The reaction was then stirred overnight, concentrated and the resulting mixture applied to a size-exclusion column that was eluted with MeOH, after which the peptide-containing fractions were combined and evaporated to dryness. Deprotection, as described for **15**, was followed by LC/MS analysis (R_t 19.10 min, linear gradient 10 $\rightarrow$ 90% B in 30 min; m/z = 1121.4 $[M+H]^{2+}$, 747.8 $[M+H]^{3+}$, 561.1 $[M+H]^{4+}$ of the crude product, purification by RP-HPLC (linear gradient of 4.0 CV; 50 $\rightarrow$ 65% B; R_t 3.4 CV) and lyophilization, to produce title compound **25** (8.4 mg, 4.5 μ mol, 25%) as a white amorphous powder over 2 steps.

Dimer 26: The crude **22** (20 mg, 7.5 μ mol) was treated as described for **25**, to give the crude product (LC/MS analysis: R_t 17.80 min, linear gradient 10 $\rightarrow$ 90% B in 30 min; m/z = 1121.8 $[M+H]^{2+}$, 748.4 $[M+H]^{3+}$) that was purified by RP-HPLC (linear gradient of 4.0 CV; 40 $\rightarrow$ 65% B; R_t 3.2 CV) and freeze-dried, to furnish in 2 steps the title compound **26** (2.5 mg, 1.1 μ mol, 15%) as a white amorphous powder.

Antimicrobial activity: The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Bacteria were stored at -70°C and grown at 35°C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) overnight and diluted in 0.9% NaCl. Microtiter plates (96 wells of 100 μL) as well as large plates (25 wells of 3 mL) were filled with Mueller Hinton II Agar (Becton Dickinson, Cockeysville, USA) containing serial two-fold dilutions of the peptides. To the wells were added 3 μL of bacteria, to give a final inoculum of 10^4 colony forming units (CFU) per well. The plates were incubated overnight at 35°C and the MIC was determined as the lowest concentration inhibiting bacterial growth.

Hemolytic Activity: The hemolytic activity of the peptides was determined in quadruple. Human blood was collected into EDTA-tubes and centrifuged to remove the buffy coat. The residual erythrocytes were washed three times in 0.85% saline. Serial two-fold dilutions of the peptides in saline were prepared in sterilized round-bottom 96-well plates (polystyrene, U-bottom, Costar) using 100 μL volumes (500-0.5 μM). Red blood cells were diluted with saline to 1/25 packed volume of cells and 50 μL of the resulting cell suspension was added to each well. Plates were incubated while gently shaking at 37°C for 4 h. Next, the microtiter plate was quickly centrifuged (1000 g, 5 min) and 50 μL supernatant of each well was transported into a flat-bottom 96-well plate (Costar). The absorbance was measured at 405 nm with a mQuant microplate spectrophotometer (Bio-Tek Instruments). The A_{blank} was measured in the absence of additives and 100% hemolysis (A_{tot}) in the presence of 1% Triton X-100 in saline. The percentage hemolysis is determined as $(A_{\text{pep}} - A_{\text{blank}}) / (A_{\text{tot}} - A_{\text{blank}}) \times 100$.

Conductivity measurements: Planar lipid membranes were prepared by painting a solution of diphytanoylphosphatidylcholin (DPhPC, Avanti Polar Lipids, Alabaster, AL) in n-decane (25 mg/ml) over the aperture of a polystyrene cuvette with a diameter of 0.15 mm.¹⁸ All experiments were performed at ambient temperature. The used electrolyte solutions at a concentration of 1M each were unbuffered. Probes were dissolved in methanol and added to the trans or cis side (containing the measuring electrode) of the cuvette. Current detection and recording was performed with a patch-clamp amplifier Axopatch 200B, a Digidata A/D converter and pClamp6 software (Axon Instruments, Foster City, MA). The acquisition frequency was 5 kHz. The data were filtered with a digital filter at 50 Hz for further analysis.

References and Notes

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Chapter 4

An Unusual Reverse Turn Structure Adopted by a Furanoid Sugar Amino Acid Incorporated in Gramicidin S

Abstract: *A new reverse turn, replacing one of the native type II' β -turns in the cyclic peptide gramicidin S, induced by a furanoid sugar amino acid is revealed. The C₃-hydroxyl function plays a pivotal role by acting as a H-bond acceptor, consequently flipping the amide bond between residues i and $i+1$, as was established by NMR and X-ray crystallographic analysis.¹*

Introduction

Peptides and proteins display an extraordinary structural diversity and are instrumental in numerous biological events. Correct folding of these biomolecules is imperative for their functioning. Key components contributing to the overall folding are secondary structure elements such as helices, sheets and turns.² Adoption of non-proteinogenic residues or sequences has appreciably contributed to further our understanding of the factors that are at the basis of secondary structure. Besides mimicking spatial arrangements found in polypeptides, peptide-like molecules have been designed with the aim to enhance resistance to proteolytic activity, to attain structural stabilization and to introduce additional functionalization sites.³ Synthetic peptide analogues are now widely recognized as important lead compounds, both in the development of new materials⁴ and in the generation of therapeutic agents.⁵

Increasing research efforts have been devoted to the study of reverse turns. In this common motif the polypeptide chain reverses its overall direction. The γ - and β -turn describe three and

four consecutive residues, respectively, in which the C=O of the first residue i is H-bonded to the NH of residue $i+2$ or $i+3$. Further classification of the turn motifs can be made on the basis of their peptide backbone geometry with specific angular and torsional parameters.⁶ Factors influencing turn motifs include hydrophobic interactions, conformational bias, side chain participation and intra- and interresidue interactions.

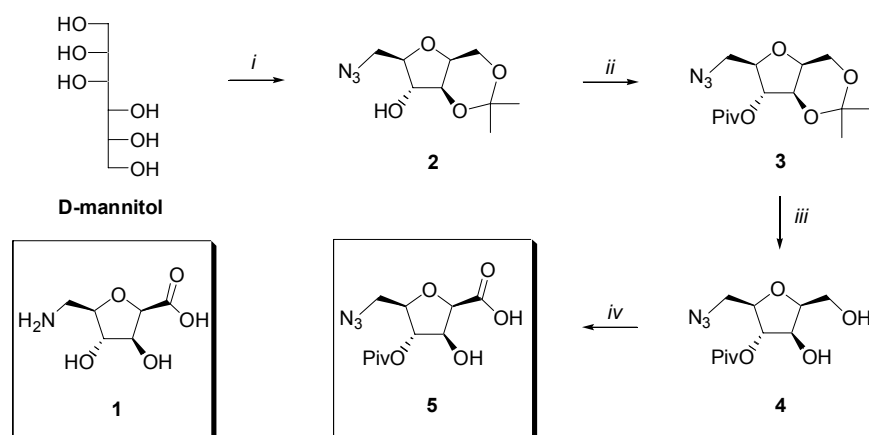
Recently, sugar amino acids (SAAs), carbohydrate derivatives featuring an amine and a carboxylic acid, have emerged as a promising new class of peptidomimetics.⁷ Oligopeptides containing SAA building blocks have been assembled with the aim to improve their biostability. Furthermore, examples of these structurally and functionally diverse molecular scaffolds have been found to induce well-defined secondary structures in oligomeric constructs, including reverse turns.^{8,9} An attractive feature of the use of carbohydrate-based peptidomimetics as turn motifs is the presence of additional functionalities on the furanose or pyranose core stemming from the parent sugar, enabling further functionalization. For instance, Smith, III *et al.* demonstrated the incorporation of a pyranoid SAA as β -turn inducer in a heptapeptide corresponding to the C-terminus of the R2 subunit of mammalian ribonucleotide reductase. The remaining hydroxyl functions were equipped with methylene carboxylate (mimicking aspartic acid) and isobutyl (mimicking leucine) functionalities, resulting in an artificial ensemble that closely resembles the native peptide sequence.¹⁰ Notably, the residual functionalities present at the parent core of SAAs may also prohibit the formation of the targeted secondary structural motif. The latter is exemplified by the finding of Chakraborty and co-workers that the incorporation of furanoid SAA **1** (Scheme 1) in short linear peptide sequences does not lead to regular β -turn structures.¹¹ Instead, one of the hydroxyl functionalities (C₃-OH) on the furanoid SAA proved to be actively involved in stabilizing the observed secondary structure by acting as hydrogen bond acceptor.

Our focus in the area of peptidomimetics is directed at the determination of the structural consequences of incorporating SAA building blocks in selected oligopeptides. Ultimately, we aim to attain tailor-made peptidomimetic building blocks able to induce the desired secondary structure combined with the opportunity to introduce extra functionalities on the turn region. To this end, we have selected gramicidin S (GS), a cyclic decapeptide antibiotic with the primary sequence *cyclo*-(Pro-Val-Orn-Leu-^DPhe)₂, as a suitable model peptide. GS adopts a C₂-symmetric amphiphillic antiparallel β -sheet structure¹² with two type II' β -turns having ^DPhe and Pro at positions $i+1$ and $i+2$, respectively, and is widely recognized as a good system to study the effect of potential artificial reverse turn inducers.¹³ We here report the in-depth study, through NMR and X-ray analysis, of synthetic GS analogue **10**, with SAA **1** as a replacement of one of the ^DPhe-Pro dipeptides in GS. NMR- and crystallographic analysis of **10** revealed the involvement of C₃-OH in the final overall secondary structure by inducing an unprecedented turn motif. The implications of this secondary structure element on the overall structure, as well as oligomeric assemblies of **10**, are disclosed.

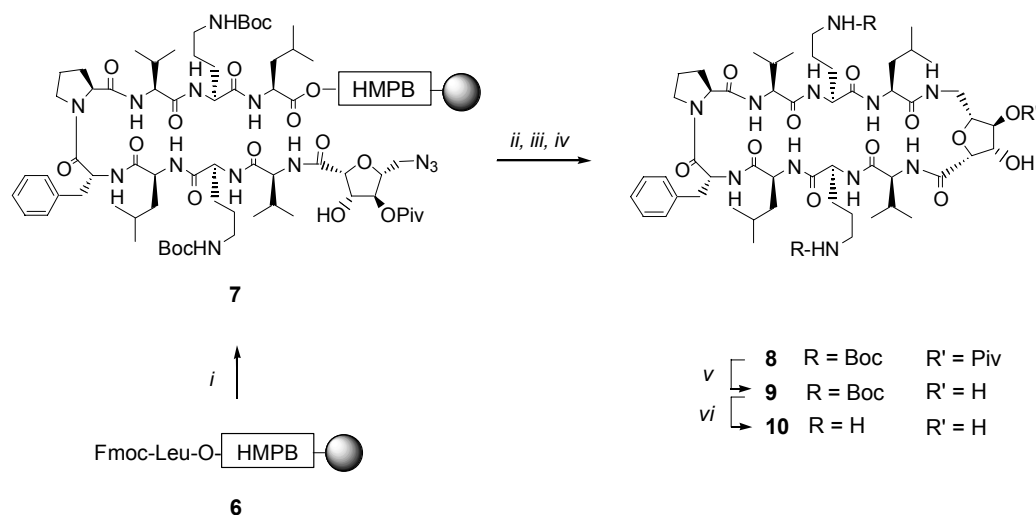
Results and Discussion

The synthesis of furanoid sugar amino acid **5** was accomplished as follows (Scheme 1). A four step procedure, developed by Timmer *et al.*,¹⁴ involving the acidic dehydration of D-mannitol, acetonation of the 1,3-*cis*-diol system and consecutive introduction of a primary azide easily furnished glucitol template **2**. The remaining hydroxyl functionality was protected with a pivaloyl ester and the isopropylidene group was released by acidic hydrolysis in 63% yield over the two steps. Selective oxidation of the primary hydroxyl moiety in diol **4** towards its respective aldehyde using Dess-Martin periodinane, followed by sodium chlorite mediated oxidation, produced protected SAA **5** in 52% yield.

Next, attention was focused on the incorporation of SAA **5** into the turn region of GS. Fmoc-Leu-OH was condensed with the 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB)-functionalized 4-methylbenzhydrylamine (MBHA)-resin under the agency of *N,N'*-diisopropylcarbodiimide (DIC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) to furnish **6** (Scheme 2). Standard Fmoc-based solid-phase peptide synthesis, as described in **Chapter 3** (condensating agents; Castro's reagent,¹⁵ *N*-hydroxybenzotriazole (HOBt) and *N,N'*-diisopropylethylamine (DiPEA), Fmoc cleavage; 20% piperidine in NMP) using the appropriate amino acid building blocks (Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-^DPhe-OH, Fmoc-Pro-OH and Fmoc-Leu-OH) followed by analogous condensation of azido acid **5** furnished immobilized nona-peptide **7**. At this stage, the azide functionality was converted to the corresponding amine employing Staudinger reduction conditions (PMe₃, 1,4-dioxane and H₂O). Mild acidic cleavage from the resin (1% TFA in CH₂Cl₂) afforded the partially protected linear peptide which was directly cyclized using Castro's reagent, HOBt and DiPEA under highly dilute conditions.



Scheme 1: Reagents and conditions: (i) See Timmer *et al.*¹⁴ (ii) pivaloyl chloride, pyridine, 16 h, quant; (iii) water/TFA (2/1, v/v), 16 h, 63%; (iv) a) Dess-Martin periodinane (1.1 equiv), DCM, 0°C, 45 min. b) NaH₂PO₄ (6 equiv) and NaClO₂ (10 equiv), *t*BuOH/water/2-methyl-2-butene (4/4/1 v/v/v), 16 h, 52 % (2 steps).



Scheme 2: Reagents and conditions: (i) Sequential coupling (Xaa or **5**, BOP, HOBT, DiPEA) and deprotection (piperidine/NMP 1/4 v/v) steps; (ii) PMe_3 , 1,4-dioxane, H_2O ; (iii) TFA/DCM (1/99 v/v); (iv) BOP, HOBT, DiPEA; (v) NaOMe, MeOH; (vi) TFA/DCM (1/1 v/v).

Purification by size exclusion chromatography (Sephadex™ LH-20) gave the homogeneous peptide **8** in 96% overall yield, based on **6**. Removal of the pivaloyl- and Boc-protecting groups (1% NaOMe in MeOH and 50% TFA in CH_2Cl_2 , respectively) and subsequent reversed phase HPLC purification finally gave GS analogue **10** in 59% yield.

The ^1H NMR resonance assignment of peptide **10** was accomplished using a combination of COSY, TOCSY and ROESY data sets. Subsequently, the obtained structural information was compared with the antiparallel β -sheet structure adopted by GS.¹² The structure of GS is characterized, apart from the two $^{\text{D}}\text{Phe-Pro}$ turn regions, by four H-bonds, two shared between the Leu_4 and Val_2 residues and two between the Leu_4 and Val_2 residues (Figure 1A). Besides numerous short range NOEs, the observation of interstrand NH-NH (Val_2 - Leu_9 and Val_7 - Leu_4), NH- $\text{H}\alpha$ (Val_7 - $^{\text{D}}\text{Phe}_5$ and Leu_9 - Orn_3) and $\text{H}\alpha$ - $\text{H}\alpha$ (Orn_3 - Orn_8) NOEs in **10** confirm the preservation of the overall β -sheet structure and indicate the presence of three H-bonds (Figure 1B).¹⁶ However, a strong NH-NH NOE between SAA_1 -NH and Val_2 -NH was observed, indicating their close proximity. The latter observation strongly suggests that residue SAA_1 does not induce a regular β -turn conformation.

With the aim to create a better understanding of the overall structure and the implications of the introduction of SAA_1 in one of the turn regions of GS, crystallographic data of **10** was obtained and analyzed. To this end, a solution of **10** in a 1:1 mixture of MeOH and H_2O in the presence of spermidine tri-HCl (or 1,5-diaminopentane di-HCl) was allowed to evaporate slowly under oil.

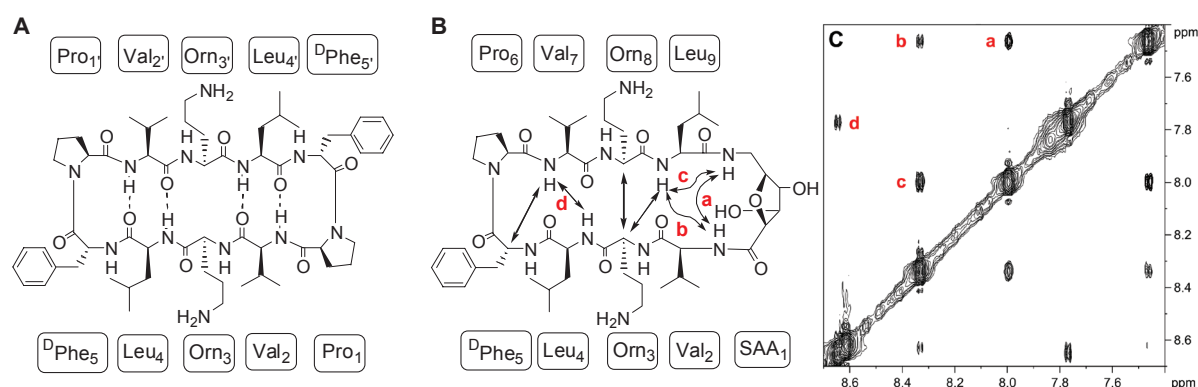


Figure 1. (A) GS; (B) Observed long range NOEs for **10**; (C) Amide region of the ROESY experiment of **10** (CD₃OH, 600 MHz).

The resulting needle-shaped crystals were subjected to X-ray diffraction analysis. The structure was solved and refined to 1.2 Å resolution (the diffraction limit of the crystals). As can be seen in Figure 2, peptide **10** adopts a pleated sheet structure with two H-bonds shared between the Leu₄ and Val₇ residues and one between Leu₉-NH and Val₂-carbonyl. The structure is similar to that reported for GS, but with a larger righthanded twist¹⁷ in the overall β -sheet. Interestingly, the SAA residue induces an unusual turn structure with the C₃-OH in close proximity to the SAA₁-NH (Figure 3). The protrusion of this hydroxyl function into the turn region, enabled by the C₃-*endo* conformation¹⁸ adopted by the furanose moiety, allows it to function as a H-bond acceptor. The structure that results from formation of a H-bond with SAA₁-NH is in full agreement with the data obtained from the NMR studies. As a consequence, the amide bond linking residues Leu₉ and SAA₁ is flipped, causing the SAA₁-NH to extend into the turn region leading to a novel secondary structure with a H-bond between a side chain functionality and the amide NH of the synthetic dipeptide isostere incorporated (Figure 3). The structure adopted by SAA **1** in compound **10** constitutes, to our knowledge, an unprecedented turn structure.

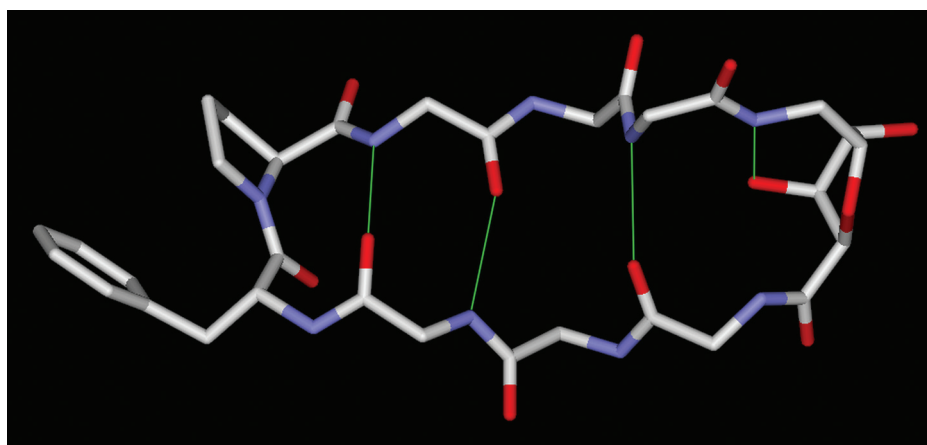


Figure 2. Pleated sheet structure of **10** with the intramolecular H-bonds depicted in green. Water molecules, Leu-, Val- and Orn-side chains as well as hydrogens were omitted for clarity.

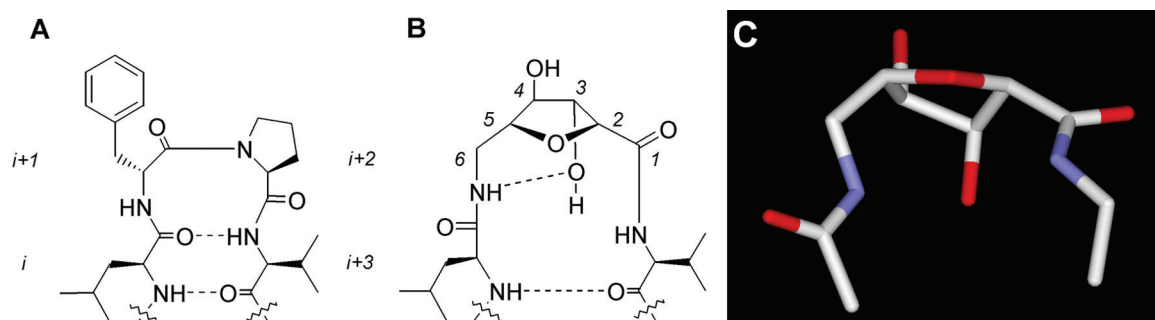


Figure 3. (A) Turn region of GS; (B) Turn region of **10**; (C) Crystal structure of turn region of **10**. Side chains and hydrogens were omitted for clarity.

Perusal of the molecular packing of **10** reveals the presence of cyclic assemblies of six crystallographically equivalent molecules, with the hydrophilic Orn side chain residues extending into the core and the Val, Leu and ^DPhe residues forming a hydrophobic periphery (Figure 4A). The structure is stabilized by intermolecular H-bonds between SAA₁-C=O and Orn₃-NH of one β-sheet with Orn₈-NH and Pro₆-C=O of the next, respectively (Figure 4B). This results in a novel hexameric β-barrel-like structure corresponding to a 12-stranded β-barrel of approximately 13 Å height (the length of the unit cell *c* axis). It has been reported that the parent cyclodecapeptide GS itself adopts oligomeric structures of a different nature. X-ray analysis of a crystal structure of a GS-urea-water complex revealed channel-like structures composed of six crystallographically equivalent GS molecules assembled in a double spiral of two left-handed helices.¹⁹

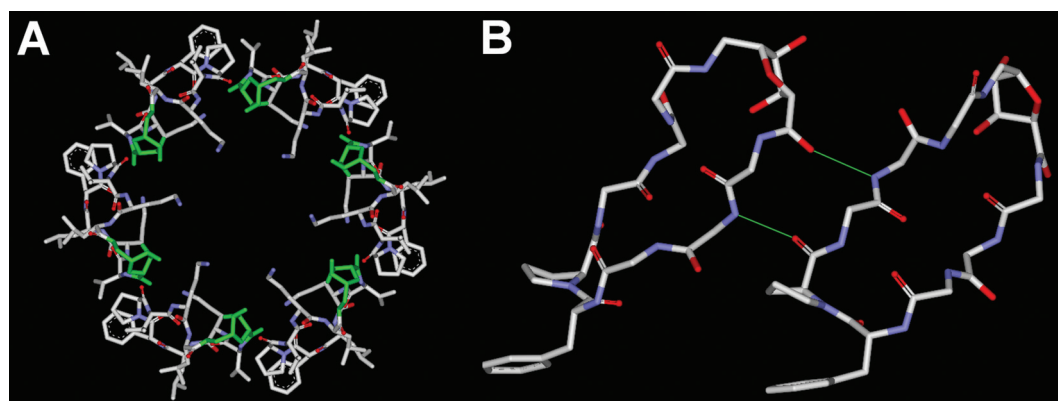


Figure 4. (A) Top view of the hexameric assembly of **10** with the SAA residues highlighted in green; (B) Side view of the assembly, showing two peptides **10** with intermolecular H-bonds depicted in green. Water molecules, Leu-, Val- and Orn-side chains and hydrogens were omitted for clarity.

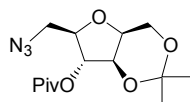
Conclusion

Furanoid sugar amino acid **5**, prepared from a 2,5-anhydroglucitol scaffold, was successfully incorporated into the turn region of GS, replacing a single ^DPhe-Pro dipeptide sequence. Structural analysis of this replacement, through ¹H NMR and single crystal X-ray diffraction

measurements, showed that the overall β -sheet structure in GS analogue **10** was maintained. However, the SAA induced a novel turn structure in which its C₃-hydroxyl functionality protrudes into turn region and is involved in H-bond formation with the amide bond between the Leu₉ and SAA₁-residues. Inspection of the molecular packing of **10** in the crystal structure showed an arrangement of six individual β -sheet peptides in an amphiphilic channel-like configuration. Thus, changes in the turn region, while of relatively small consequence on the secondary structure of the cyclic peptide itself (both GS and **10** adopt a pleated β -sheet) may have a profound effect on oligomeric assemblies thereof, at least in their crystal structures. Interestingly, β -barrels are found to be at the basis of the mode of action of many pore-forming proteins, including cytolytic bacterial toxins such as perfringolysin O and α -hemolysin.²⁰ The results presented here may therefore be of use for the future development of novel transmembrane channels and may contribute in the design of artificial β -barrel-like molecules based on cyclic peptides with applications such as bactericidal agents.²¹

Experimental Section

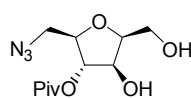
General: Reactions were performed under an inert atmosphere and at ambient temperature unless stated otherwise. Reactions were monitored by TLC-analysis using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by spraying with 20% H₂SO₄ in ethanol followed by charring at ~150°C or by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid followed by charring at ~150°C. Column chromatography was performed on Merck silicagel (0.040 – 0.063 nm) and size exclusion chromatography on Sephadex™ LH-20. Mass spectra were recorded on a PE/Sciex API 165 instrument with a custom-build Electrospray Ionisation (ESI) interface and HRMS (SIM mode) were recorded on a TSQ Quantum (Thermo Finnigan) fitted with an accurate mass option, interpolating between PEG-calibration peaks. For LC/MS analysis, a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical Alltima C₁₈ column (Alltech, 4.6 mmD × 250 mmL, 5 μ particle size) in combination with buffers A: H₂O, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI) was used. For RP-HPLC purification of the peptide, a BioCAD “Vision” automated HPLC system (PerSeptiveBiosystems, inc.) equipped with a semi-preparative Alltima C₁₈ column (Alltech, 10.0 mmD × 250 mmL, 5 μ particle size) was used. The applied buffers were A: H₂O, B: MeCN and C: 1.0 % aq. TFA. ¹H- and ¹³C NMR spectra were recorded on a Bruker AV-400 (400/100 MHz) and the peptide **10** was analyzed using a Bruker DMX 600 spectrometer equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard (¹H NMR) or CDCl₃ (¹³C NMR). Coupling constants are given in Hz. All presented ¹³C APT spectra are proton decoupled. Optical rotations were measured on a Propol automatic polarimeter (Sodium D line, λ = 589 nm) and ATR-IR spectra were recorded on a Shimadzu FTIR-8300 fitted with a single bounce DurasampliIR diamond crystal ATR-element.



2,5-Anhydro-6-azido-6-deoxy-1,3-O-isopropylidene-4-O-pivaloyl-D-glucitol (3):

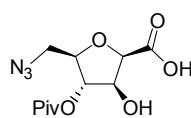
Azide **2** (100 mmol, 22.9 g) was coevaporated twice with pyridine and dissolved in pyridine (500 mL). After pivaloylchloride (PivCl) (1.2 equiv, 120 mmol, 14.7 mL) was added, the reaction mixture was stirred overnight before being concentrated. The residue was

dissolved in EtOAc and washed with 1M aq. HCl, water and brine. The EtOAc layer was dried over MgSO_4 and concentrated. Silica column chromatography (20% EtOAc in light PE) yielded fully protected glucitol **3** quantitatively (100 mmol, 31.4 g) as a transparent oil. ^1H -NMR (400 MHz, CDCl_3): δ = 4.85 (d, 1H, H_4 , $J_{4,5}$ = 1.7 Hz), 4.25 (d, 1H, H_3 , $J_{3,2}$ = 2.6 Hz), 4.10 (dd, 1H, H_{1a} , $J_{1a,2}$ = 2.5 Hz, $J_{1a,1b}$ = 13.4 Hz), 4.03 (dd, 1H, H_{1b} , $J_{1b,2}$ = 1.6 Hz, $J_{1b,1a}$ = 13.4 Hz), 3.98 (ddd, 1H, H_5 , $J_{5,4}$ = 1.7 Hz, $J_{5,6b}$ = 4.7 Hz, $J_{5,6a}$ = 7.8 Hz), 3.89 (ddd, 1H, H_2 , $J_{2,1b}$ = 1.6 Hz, $J_{2,1a}$ = $J_{2,3}$ = 2.6 Hz), 3.66 (dd, 1H, H_{6a} , $J_{6a,5}$ = 7.8 Hz, $J_{6a,6b}$ = 12.6 Hz), 3.50 (dd, 1H, H_{6b} , $J_{6b,5}$ = 4.7 Hz, $J_{6b,6a}$ = 12.6 Hz), 1.44 (s, 3H, CH_3 *i*Pr), 1.41 (s, 3H, CH_3 *i*Pr), 1.20 (s, 9H, 3 $\times$ CH_3 Piv) ^{13}C -NMR (100 MHz, CDCl_3): δ = 177.2 (C=O Piv), 97.6 (C_q *i*Pr), 83.6 (C_5), 80.1 (C_4), 73.6 (C_3), 73.3 (C_2), 60.1 (C_1), 52.7 (C_6), 38.5 (C_q Piv), 28.7 (CH_3 *i*Pr), 26.9 (CH_3 Piv), 18.8 (CH_3 *i*Pr). ATR-IR (thin film): 2977.9, 2096.5, 1732.0, 1481.2, 1375.2, 1280.6, 1143.7, 1091.6, 929.6, 846.7 cm^{-1} . $[\text{A}]_{\text{D}}^{23}$ +22.4 (c = 1.00, CHCl_3). MS (ESI): m/z 314.3 $[\text{M}+\text{H}]^+$, 336.1 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{14}\text{H}_{23}\text{N}_3\text{O}_5\text{NH}_4$ 331.1981, found 331.1968.



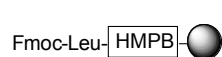
2,5-Anhydro-6-azido-6-deoxy-4-O-pivaloyl-D-glucitol (4):

Glucitol **3** (100 mmol, 31.4 g) was dissolved in a mixture of water/TFA (400 mL, 2/1, v/v). The resulting white suspension was stirred overnight to give a homogeneous yellow solution. The reaction mixture was concentrated and coevaporated with toluene before being purified by column chromatography (toluene→30% EtOAc in toluene) furnishing the title compound **4** (17.2 g, 63 mmol, 63%) as a transparent oil. ^1H -NMR (400 MHz, CDCl_3): δ = 4.81 (dd, 1H, H_4 , $J_{4,3}$ = 1.8 Hz, $J_{4,5}$ = 3.4 Hz), 4.28 (dd, 1H, H_3 , $J_{3,4}$ = 1.8 Hz, $J_{3,2}$ = 4.3 Hz), 4.05 (dd, 1H, H_2 , $J_{2,3}$ = 2.5 Hz, $J_{2,1}$ = 8.6 Hz), 3.97 (m, 3H, H_5 and 2 $\times$ H_1), 3.63 (d, 2H, 2 $\times$ H_6 , $J_{6,5}$ = 4.8 Hz), 1.20 (s, 9H, 3 $\times$ CH_3 Piv) ^{13}C -NMR (100 MHz, CDCl_3): δ = 178.3 (C=O Piv), 81.8 (C_5), 81.5 (C_4), 80.9 (C_2), 76.5 (C_3), 61.0 (C_1), 52.4 (C_6), 38.5 (C_q Piv), 26.8 (CH_3 Piv). ATR-IR (thin film): 3328.9, 2974.0, 2098.4, 1726.2, 1481.2, 1280.6, 1149.5, 1078.1, 1035.7 cm^{-1} . $[\text{A}]_{\text{D}}^{23}$ +41.6 (c = 1.00, CHCl_3). MS (ESI): m/z 273.9 $[\text{M}+\text{H}]^+$, 296.2 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{11}\text{H}_{19}\text{N}_3\text{O}_5\text{H}$ 274.1403, found 274.1409.

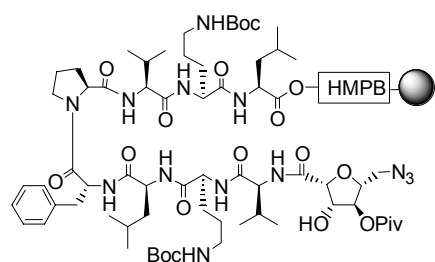


2,5-Anhydro-6-azido-6-deoxy-4-O-pivaloyl-D-gluconic acid (5):

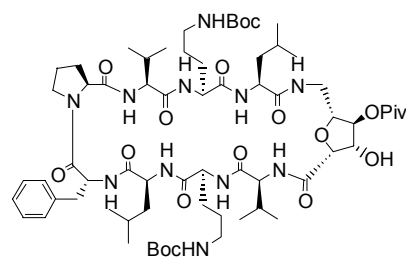
Diol **4** (5.8 g, 20 mmol) was coevaporated twice with toluene, dissolved in DCM (100 mL), placed under an argon atmosphere and cooled to 0°C, before Dess-Martin periodinane (9.35 g, 22 mmol, 1.1 equiv) was added under vigorous stirring. The reaction mixture was stirred for 30 min before a sat. aq. $\text{Na}_2\text{S}_2\text{O}_3$ / sat. aq. NaHCO_3 solution (100 mL, 7 / 3 (v/v)) was added and stirred for an additional 15 min. Then, the DCM layer was separated, washed with H_2O and brine, dried over MgSO_4 and concentrated. The residue was coevaporated with toluene and purified by column chromatography (toluene→5% EtOAc in toluene) yielding 6-azido-6-deoxy-4-pivaloyl-2,5-anhydro-D-glucose (4.97 g, 18.3 mmol, 92 %). The aldehyde (2.56 g, 9.4 mmol) was dissolved in a solution of *tert*-butanol (80 mL), 2-methyl-2-butene (20 mL) and water (80 mL), before NaH_2PO_4 (8.0 g, 56 mmol, 6 equiv) and NaClO_2 (8.0 g, 88 mmol, 10 equiv) were added. The reaction was stirred overnight, before the solution was acidified and extracted with EtOAc (2 $\times$). The EtOAc layers were dried over MgSO_4 , concentrated and purified by column chromatography (toluene→1% AcOH in EtOAc) yielding the title compound **5** (1.55 g, 5.38 mmol, 57% (52%, 2 steps)). ^1H NMR (400 MHz, CDCl_3): δ = 4.86 (dd, 1H, H_4 , $J_{4,3}$ = 1.0 Hz, $J_{4,5}$ = 2.0 Hz), 4.58 (d, 1H, H_2 , $J_{2,3}$ = 4.0 Hz), 4.41 (dd, 1H, H_3 , $J_{3,4}$ = 1.0 Hz, $J_{3,2}$ = 4.0 Hz), 4.00 (ddd, 1H, H_5 , $J_{5,4}$ = 2.0 Hz, $J_{5,6b}$ = 4.3 Hz, $J_{5,6a}$ = 5.5 Hz), 3.73 (dd, 1H, H_{6a} , $J_{6a,5}$ = 5.5 Hz, $J_{6a,6b}$ = 12.8 Hz), 3.63 (dd, 1H, H_{6b} , $J_{6b,5}$ = 4.3 Hz, $J_{6b,6a}$ = 12.8 Hz), 1.15 (s, 9H, 3 $\times$ CH_3 Piv). ^{13}C NMR (100 MHz, CDCl_3): δ = 177.9 (C=O Piv), 171.5 (COOH), 83.3 (C_5), 81.4 (C_2), 80.1 (C_4), 76.0 (C_3), 52.0 (C_6), 38.6 (C_q Piv), 26.8 (CH_3 Piv). ATR-IR (thin film): 3421.6, 2976.0, 2102.3, 1728.1, 1481.2, 1282.6, 1143.7, 1097.4, 1037.6 cm^{-1} . $[\text{A}]_{\text{D}}^{23}$ +30.0 (c = 1.00, CHCl_3). MS (ESI): m/z 288.2 $[\text{M}+\text{H}]^+$, 310.1 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{11}\text{H}_{17}\text{N}_3\text{O}_6\text{H}$ 288.1196, found 288.1240.



Fmoc-Leu-HMPB-MBHA resin (6): 4-methylbenzhydrylamine (MBHA) functionalized polystyrene resin (2.22 g, 0.9 mmol/g, 2.0 mmol) was shaken with NMP (30 mL, 3 $\times$, 3 min) followed by addition of a pre-activated mixture of 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) (3 equiv, 1.44 g, 6.0 mmol), benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (3 equiv, 2.652 g, 6.0 mmol) and *N,N*-diisopropylethylamine (DiPEA) (6 equiv, 2.09 mL, 12.0 mmol) in NMP (25 mL). Shaking was continued overnight after which the resin was washed with NMP (30 mL, 3 $\times$, 3 min) and DCM (30 mL, 3 $\times$, 3 min). Next, the resin was transferred to a flask, coevaporated with DCE (30 mL, 3 $\times$) and condensed with Fmoc-Leu-OH (3 equiv, 2.12 g, 6.0 mmol) under the influence of *N,N'*-diisopropylcarbodiimide (DIC) (3.3 equiv, 1.03 mL, 6.6 mmol) and a catalytic amount of 4-dimethylaminopyridine (DMAP) (40 mg, 0.33 mmol) for two hours. The resin was then filtered and washed with DCM (30 mL, 3 $\times$, 3 min) and subjected to a second condensation sequence, gaining fully loaded resin **6**. The loading of the resin was determined to be 0.50 mmol/g by spectrophotometric analysis.



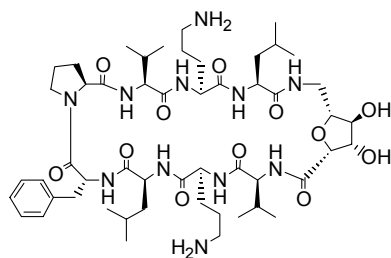
SAA(Piv)-Val-Orn(Boc)-Leu-^DPhe-Pro-Val-Orn(Boc)-Leu-HMPB-MBHA resin (7): Resin **6** (0.2 g, 0.5 mmol/g, 0.1 mmol) was submitted to seven cycles of Fmoc solid-phase synthesis with Fmoc-Orn₈(Boc)-OH, Fmoc-Val₇-OH, Fmoc-Pro₆-OH, Fmoc-^DPhe₅-OH, Fmoc-Leu₄-OH, Fmoc-Orn₃(Boc)-OH and Fmoc-Val₂-OH, respectively, as follows: a) deprotection with piperidine / NMP (1/4, v/v, 5 mL, 15 min); b) wash with NMP (5 mL, 3 $\times$, 3 min); c) coupling of the appropriate Fmoc amino acid (2.5 equiv, 0.25 mmol) in the presence of BOP (2.5 equiv, 0.25 mmol, 0.11 g), *N*-hydroxybenzotriazole (HOBt, 2.5 equiv, 0.25 mmol, 34 mg) and DiPEA (3 equiv, 0.3 mmol, 0.051 mL) which was preactivated for 2 min in NMP (5 mL) and shaken for 90 min; d) wash with NMP (5 mL, 3 $\times$, 3 min). Couplings were monitored for completion by the Kaiser test.²² Finally, the *N*-terminal amine was liberated by Fmoc-deprotection with piperidine / NMP (1/4, v/v, 5 mL, 15 min) followed by washing with NMP (5 mL, 3 $\times$, 3 min). To the resin bound octapeptide, a preactivated solution of SAA **5** (3.6 equiv, 105 mg, 0.366 mmol), BOP (6 equiv, 266 mg, 0.6 mmol), HOBt (6 equiv, 81 mg, 0.6 mmol) and DiPEA (6.5 equiv, 110 μ L, 0.65 mmol) in NMP (3 mL) was added and the resulting suspension was shaken for 16h. The resin was finally washed with NMP (5 mL, 3 $\times$, 3 min) to give title compound **7**.



cyclo-[SAA(Piv)-Val-Orn(Boc)-Leu-^DPhe-Pro-Val-Orn(Boc)-Leu] (8): Resin bound nonapeptide **7** was washed with 1,4-dioxane (5 mL, 3 $\times$, 3 min) and taken up in 1,4-dioxane (10 mL) to which trimethylphosphine (16 equiv, 1.6 mL, 1.6 mmol, 1 M in toluene) was added. Subsequently, the resin was shaken for 2 h, water (1 mL) was added and shaken for another 4 h. The resin was then washed with 1,4-dioxane (5 mL, 3 $\times$, 3 min) and DCM (5 mL, 3 $\times$, 3 min) after which the peptide was released from the resin by mild acidic cleavage (TFA/DCM, 1/99, v/v, 10 mL, 3 $\times$, 10 min). The fractions were collected and coevaporated with toluene (50 mL) for three times to give the crude linear peptidic construct which was cyclized directly without further purification.

For the cyclization of the crude linear peptide, it was taken up in DMF (5 mL) and added dropwise over the course of an hour to a solution of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (5 equiv, 270 mg, 0.5 mmol), HOBt (5 equiv, 67 mg, 0.5 mmol) and DiPEA (15 equiv, 254 μ L, 1.5 mmol) in DMF (70 mL) and allowed to stir for 16h. The solvent was removed *in vacuo* and the resulting mixture was applied to a Sephadex® size exclusion column (50.0 mmD $\times$ 1500 mmL) and eluted with MeOH yielding pure peptide **8** as white amorphous solid (128

mg, 96 μmol , 96%). $^1\text{H-NMR}$ (400 MHz, $\text{DMSO-}d_6$, 300 K): δ = 9.11 (bs, 1H, NH ^DPhe), 8.65 (d, 1H, NH_α Orn, $J_{\text{NH,H}\alpha}$ = 8.8 Hz), 8.57 (d, 1H, NH_α Orn, $J_{\text{NH,H}\alpha}$ = 8.6 Hz), 8.29 (d, 1H, NH Leu, $J_{\text{NH,H}\alpha}$ = 8.7 Hz), 7.97 (d, 1H, NH Leu, $J_{\text{NH,H}\alpha}$ = 7.6 Hz), 7.42 (m, 2H, NH SAA, NH Val), 7.28 (m, 1H, NH Val), 7.27–7.13 (m, 5H, H_{ar}), 6.86 (bs, 1H, NH_δ Orn), 6.55 (bs, 1H, NH_δ Orn), 5.90 (d, 1H, $\text{C}_3\text{-OH}$ SAA, $J_{\text{OH,H3}}$ = 4.8 Hz), 4.87 (m, 1H, H_α Orn), 4.73 (bs, 1H, H_2 SAA), 4.63 (m, 1H, H_α Leu), 4.53 (m, 1H, H_α Pro), 4.46 (m, 1H, H_α Val), 4.37 (d, 1H, H_4 SAA J = 3.3 Hz), 4.33 (m, 1H, H_α ^DPhe), 4.22 (m, 2H, H_α Val, H_α Orn), 4.16 (m, 3H, H_α Leu, H_3 SAA, H_5 SAA), 3.51 (m, 1H, $\text{H}_{\delta\text{d}}$ Pro), 3.39 (m, 1H, $\text{H}_{6\text{d}}$ SAA), 3.28 (m, 1H, $\text{H}_{6\text{u}}$ SAA), 2.98–2.78 (m, 6H, H_β ^DPhe , H_δ Orn), 2.42 (m, 1H, $\text{H}_{\delta\text{u}}$ Pro), 2.00 (m, 4H, H_β Val, H_β Pro), 1.75–1.25 (m, 16H, H_β Orn, H_γ Orn, H_β Leu, H_γ Leu, H_γ Pro), 1.33 (s, 18H, CH_3 Boc) 1.14 (s, 9H, CH_3 Piv) 0.91–0.66 (m, 24H, H_γ Val, H_δ Leu). ATR-IR (thin film): 3274.9, 2960.5, 2931.6, 2873.7, 1633.6, 1525.6, 1450.4, 1390.6, 1365.5, 1276.8, 1251.7, 1164.9, 1093.6, 1037.6, 910.3, 729.0, 702.0, 646.1 cm^{-1} . MS (ESI): m/z 1341.0 $[\text{M}+\text{H}]^+$, 1363.0 $[\text{M}+\text{Na}]^+$ HRMS: calcd for $\text{C}_{67}\text{H}_{109}\text{N}_{11}\text{O}_{17}\text{NH}_4$ 1357.8347, found 1357.8325.



cyclo-[SAA-Val-Orn-Leu- ^DPhe -Pro-Val-Orn-Leu] (10): Cyclic peptide **8** (64 mg, 48 μmol) was taken up in anhydrous MeOH (2.5 mL), sodium methoxide (7.7 equiv, 20 mg, 370 μmol) was added and the mixture was allowed to stir for 16h. Subsequently, the solution was neutralized using Amberlite[®] exchange resin (H^+ -form) and concentrated *in vacuo*. A portion of the deprotected cyclic peptide **9** (36 mg, 29 μmol) was directly dissolved in DCM

(5 mL) and cooled to 0 $^\circ\text{C}$. Then, trifluoroacetic acid (5 mL) was added and the mixture was allowed to warm to ambient temperature over a period of 30 min. To the solution was added toluene (15 mL) and concentrated. The resulting peptide was analyzed by LC/MS (R_t 17.56 min, linear gradient 20 $\rightarrow$ 60% B in 20 min; m/z = 1057.1 $[\text{M}+\text{H}]^+$, 529.1 $[\text{M}+\text{H}]^{2+}$) and purified by semi-preparative RP-HPLC (linear gradient of 4.0 CV; 30 $\rightarrow$ 50% B; R_t 4.0 CV). Lyophilization of the combined fractions furnished peptide **10** (22.0 mg, 17.1 μmol , 59%) as white amorphous powder. $^1\text{H-NMR}$ (600 MHz, CD_3OH): δ = 8.95 (d, 1H, NH $^D\text{Phe}_5$, $J_{\text{NH,H}\alpha}$ = 3.3 Hz), 8.64 (d, 1H, NH Leu₄, $J_{\text{NH,H}\alpha}$ = 9.1 Hz), 8.62 (d, 2H, NH_α Orn_{3,8}, $J_{\text{NH,H}\alpha}$ = 8.7 Hz), 8.33 (d, 1H, NH Leu₉, $J_{\text{NH,H}\alpha}$ = 8.5 Hz), 8.00 (t, 1H, NH SAA₁, $J_{\text{NH,6}}$ = 5.3 Hz), 7.83 (bs, 2H, NH_δ Orn₃), 7.80 (bs, 2H, NH_δ Orn₈), 7.77 (d, 1H, NH Val₇, $J_{\text{NH,H}\alpha}$ = 8.7 Hz), 7.46 (d, 1H, NH Val₂, $J_{\text{NH,H}\alpha}$ = 8.8 Hz), 7.40 – 7.21 (m, 5H, H_{ar}), 4.99 (m, 1H, H_α Orn₃), 4.67 (m, 1H, H_α Orn₈), 4.63 (m, 1H, H_α Leu₄), 4.53 (d, 1H, H_2 SAA₁, $J_{2,3}$ = 3.9 Hz), 4.48 (m, 1H, H_α $^D\text{Phe}_5$), 4.46 (m, 1H, H_α Leu₉), 4.33 (m, 1H, H_α Pro₆), 4.30 (m, 1H, H_α Val₂), 4.20 (dd, 1H, H_3 SAA₁, $J_{3,4}$ = 1.6 Hz, $J_{3,2}$ = 3.9 Hz), 4.10 (m, 1H, H_5 SAA₁), 4.03 (m, 1H, H_α Val₇), 3.93 (dd, 1H, H_4 SAA₁, $J_{4,3}$ = 1.6 Hz, $J_{4,5}$ = 1.6 Hz), 3.72 (m, 1H, $\text{H}_{\delta\text{d}}$ Pro₆), 3.59 (ddd, 1H, $\text{H}_{6\text{d}}$ SAA₁, $J_{6\text{d},5}$ = 3.8 Hz, $J_{6\text{d},\text{NH}}$ = 5.3 Hz, $J_{6\text{d},6\text{u}}$ = 14.3 Hz), 3.44 (ddd, 1H, $\text{H}_{6\text{u}}$ SAA₁, $J_{6\text{u},\text{NH}}$ = 5.3 Hz, $J_{6\text{u},6\text{d}}$ = 14.3 Hz), 3.08 (dd, 1H, $\text{H}_{\beta\text{d}}$ $^D\text{Phe}_5$, $J_{\beta\text{d},\beta\text{u}}$ = 12.6 Hz, $J_{\beta\text{d},\alpha}$ = 5.0 Hz), 2.99 (m, 1H, $\text{H}_{\delta\text{d}}$ Orn₃), 2.93 (m, 4H, H_δ Orn₈, $\text{H}_{\delta\text{u}}$ Orn₃, $\text{H}_{\beta\text{u}}$ $^D\text{Phe}_5$), 2.47 (m, 1H, $\text{H}_{\delta\text{u}}$ Pro₆), 2.28 (m, 1H, H_β Val₇), 2.07 (m, 1H, H_β Val₂), 1.98 (m, 2H, $\text{H}_{\beta\text{d}}$ Pro₆, $\text{H}_{\beta\text{d}}$ Orn₃), 1.84 (m, 1H, $\text{H}_{\beta\text{d}}$ Orn₈), 1.76 (m, 3H, $\text{H}_{\beta\text{u},\gamma}$ Orn₃), 1.68 (m, 2H, $\text{H}_{\beta\text{u},\gamma\text{d}}$ Pro₆), 1.67 (m, 2H, H_γ Orn₈), 1.65 (m, 3H, H_β , γ Leu₉), 1.63 (m, 1H, $\text{H}_{\beta\text{u}}$ Orn₈), 1.57 (m, 1H, $\text{H}_{\gamma\text{u}}$ Pro₆), 1.54 (m, 2H, $\text{H}_{\beta\text{d},\gamma}$ Leu₄), 1.40 (m, 1H, $\text{H}_{\beta\text{u}}$ Leu₄), 0.95 (m, 3H, $\text{H}_{\gamma\text{d}}$ Val₇), 0.93 (m, 6H, H_γ Val₂), 0.89 (m, 6H, H_δ Leu₄), 0.87 (m, 3H, $\text{H}_{\gamma\text{u}}$ Val₇), 0.85 (m, 6H, H_δ Leu₉). The amide region of the ROESY-experiment is depicted in Figure 1. ATR-IR (thin film): 3270.0, 3066.7, 2958.7, 2935.1, 2874.8, 1733.9, 1670.4, 1639.2, 1533.3, 1456.3, 1202.2, 1181.2, 1133.5, 1033.3, 837.4, 799.5, 748.6, 722.4, 702.4 cm^{-1} . HRMS: calcd for $\text{C}_{52}\text{H}_{85}\text{N}_{11}\text{O}_{12}\text{H}$ 1056.6457, found 1056.6382.

X-ray crystallographic data:

Lyophilized peptide **10** (1,40 mg, 1.09 μmol) was dissolved in 200 μL MeOH / H₂O (1/1, v/v), after which 5 μL of the solution was injected onto a 96-well microtiter plate that was previously filled with n-decane. To the sample, 1 μL spermidine tri-HCl (0.1 M) was added (addition of 1 μL 1,5-diaminopentane di-HCl (30% w/v) gave similar results), after which the microtiter plate was covered with a mixture of paraffine and silicon oil (10/9, v/v) and allowed to stand for a period of 2 weeks. The crystals that formed were then analyzed and the structure refined (Table 1).

A complete dataset was collected from one crystal (0.8 x 0.08 x 0.04 mm) at 100 K using a Bruker-Nonius FR591 rotating anode generator equipped with kappa-CCD2000 detector and MONTEL multilayer graded x-ray optics, CuK α radiation ($\lambda = 1.54184 \text{ \AA}$). Data were processed using HKL Denzo and Scalepack.²³ The structure was solved by direct methods (SIR-97)²⁴ and refined with full-matrix least-squares analysis on F^2 using SHELXL-97.²⁵ Due to the limited resolution of 1.2 $\text{\AA}$, local disorder and the presence of solvent channels in the crystal, hydrogens were not always added and some atoms were refined at multiple positions. Atoms with occupancies lower than unity, disordered side chains and solvent atoms were refined isotropically. Semi-empirical absorption correction from equivalents using SORTAV.²⁶ CCDC-216610 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223-336-033; or desposit@ccdc.cam.ac.uk).

Table 1: Crystal data and structure refinement for GS analogue **10**.

Identification code	gsd17e21
Empirical formula	C ₅₂ H ₈₅ N ₁₁ O ₁₂ 5.75(H ₂ O)
Formula weight	1149.64
Temperature	100 K
Wavelength	1.5418 Å
Crystal system,	Hexagonal
Space group	P6
Unit cell dimensions	a = 31.3930(4) Å α = 90° b = 31.3930(4) Å β = 90° c = 12.7243(2) Å γ = 120°
Volume	10860.0(3) Å ³
Z	6
Calculated density	1.055 Mg/m ³
Absorption coefficient	0.666 mm ⁻¹
F(000)	3700
Crystal size	0.8 x 0.08 x 0.04 mm
Theta range for data collection	3.47° to 50.32°
Limiting indices	0 ≤ h ≤ 27 0 ≤ k ≤ 26 -12 ≤ l ≤ 11
Reflections collected / unique	49791 / 7378 [R(int) = 0.075]
Completeness to theta = 50.32°	99 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.980 and 0.911
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	7378 / 38 / 709
Goodness-of-fit on F ²	1.072
Final R indices (F _o > 4σ(F _o))	R1 = 0.0982, wR2 = 0.2423
R indices (all data)	R1 = 0.1093, wR2 = 0.2590
Absolute structure parameter	0.0(4)
Extinction coefficient	0.0021(2)
Largest diff. peak and hole	0.624 and -0.549 eÅ ⁻³

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Chapter 5

Sugar Amino Acid Peptidomimetics Incorporated in Gramicidin S

Abstract: *The construction of eight gramicidin S analogues (13a-h) having nonproteinogenic sugar amino acid residues 1-4 incorporated in the turn regions is presented. Perusal of the ^1H NMR data of peptides 13a-h revealed that the overall β -sheet structure as in GS is preserved. The biological activity of these GS analogues was established through antimicrobial and hemolytic assays.*¹

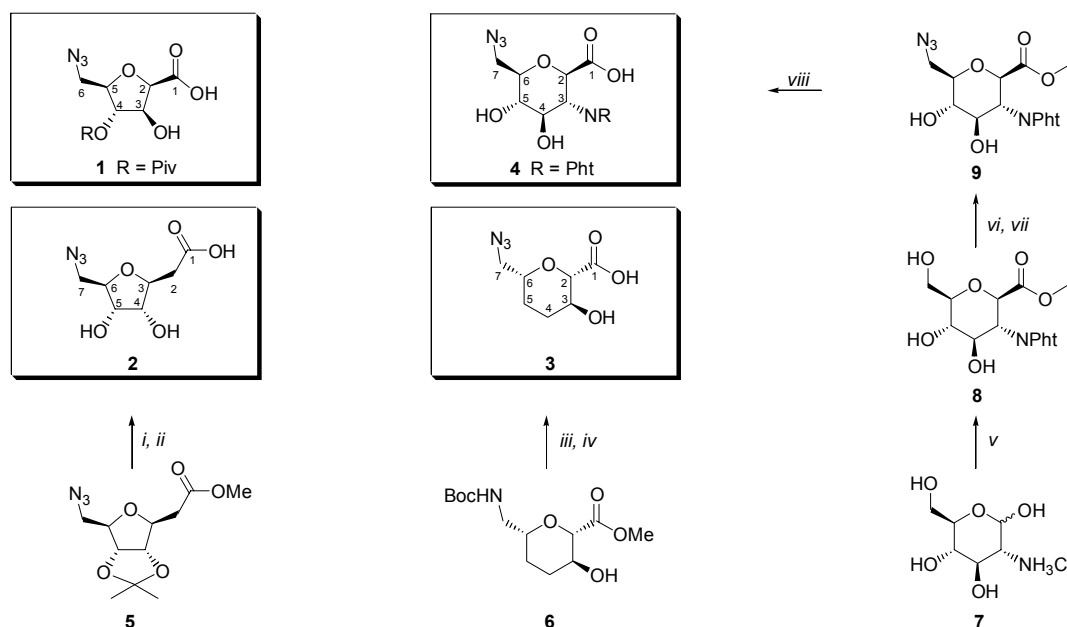
Introduction

Gramicidin S (GS) is a naturally occurring antimicrobial peptide that upon accretion on lipid bilayers inflicts a loss of barrier function of cellular membranes.² In delineating the factors that control the structure, and consequently the biological profile of antimicrobial peptides based on gramicidin S, the application of nonproteinogenic residues or sequences have been exceedingly beneficial. The incorporation of nonproteinogenic amino acids can have advantageous effects on structural stability, whereas newly introduced functionalities permit the attachment of potential pharmacophoric groups, as was shown in **Chapter 2**.³ Constrained peptidomimetics have been of special interest as they have the ability to induce distinct bioactive conformations and several examples have appeared in literature where peptide analogues replace the reverse turn in GS.⁴ A novel class of peptidomimetics that has recently attracted particular interest is the sugar amino acids (SAAs).⁵ These hybrid molecules consist of a carbohydrate core structure that has been endowed with the functional groups of an amino acid, thus enabling facile introduction into peptidic structures. The readily convertible substituents on these well-defined furanoid or pyranoid structures allow for further functionalization. However, the influence of the hydroxyls that originate from the parent sugar have been shown to participate in intramolecular hydrogen bonds formation. This is exemplified by findings described in the previous chapter, that the SAA does induce a locally distorted turn structure with a free hydroxyl group acting as H-bond acceptor. However, the incorporation of the furanoid SAA does not affect the overall pleated sheet structure of GS.⁶

In this chapter the synthesis of a set of turn modified analogues in which either a single or both reverse turn dipeptide sequences have been replaced with a sugar amino acid is described. Four SAA building blocks (**1**, **2**, **3** and **4**, Scheme 1) were selected and applied for the construction of GS analogues **13a-d**, which have a single type II' β -turn replaced and **13e-f**, which have both ^{D} Phe-Pro dipeptide sequences substituted (Scheme 2). Structural and functional data, including antimicrobial and hemolytic activity, of these novel GS analogues are presented.

Results and Discussion

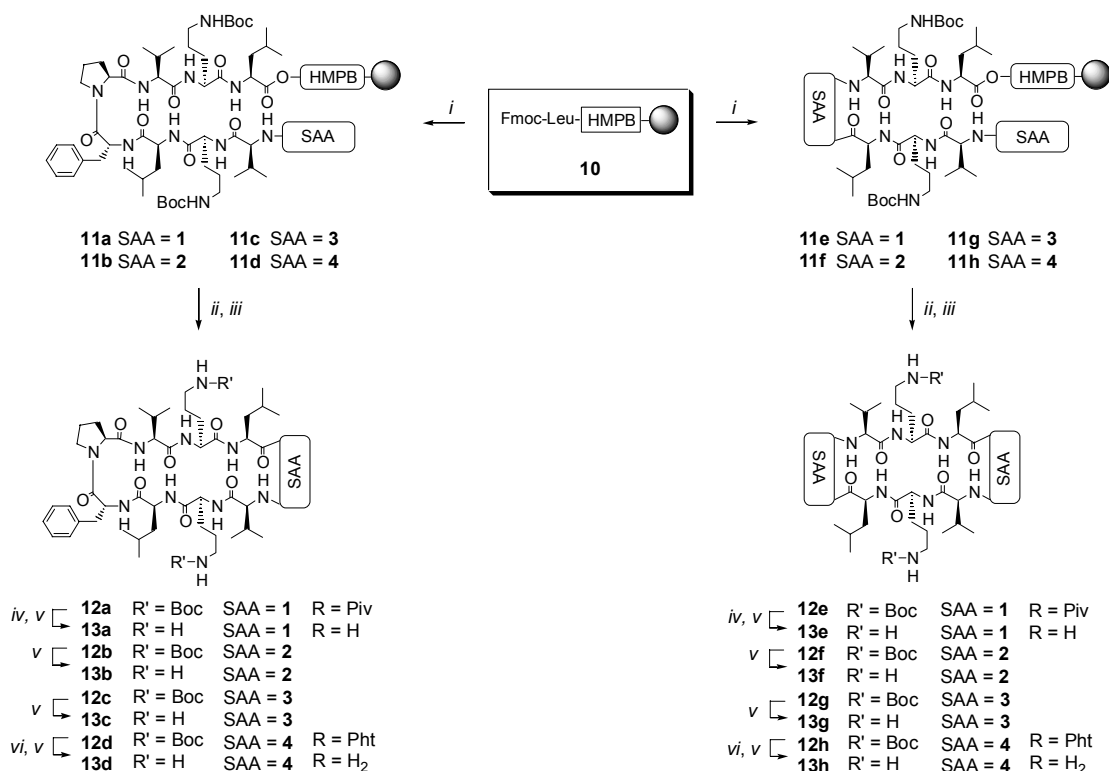
The synthesis of SAAs building blocks **1-4** is outlined in Scheme 1. The synthesis of furanoid SAA **1** was previously described in Chapter 4.⁶ Removal of the isopropylidene protection group in **5**⁷ by acidic methanolysis, followed by saponification of the methyl ester afforded **2** in 80% yield over the two steps. The partially deoxygenated gluconic amino acid **6**⁸ was transformed by acidic deblocking (50% TFA in DCM) of the Boc-protected amine, installation of the azide group by Cu-catalyzed diazo-transfer in a procedure developed by Wong and co-workers⁹ and saponification of the methyl ester, to give **3** in 58% yield over 2 steps. Finally, the novel β -D-glucosaminopyranosyl template **4** was prepared through adaptation of a synthetic strategy developed by Ichikawa and co-workers.¹⁰ Starting from D-(+)-glucosamine hydrochloride (**7**), the *N*-phthaloyl protected methyl ester **8** was obtained in a straightforward manner in 6 steps.



Scheme 1: Reagents and conditions: (i) 2 M HCl/MeOH (1/3 v/v), 16 h, 82%; (ii) 1 M NaOH/THF (1/1 v/v), 3 h, then Amberlite IR-120 (H^+), 98%; (iii) (a) TFA/DCM (1/1 v/v), 30 min; (b) TfN_3 (2 equiv), K_2CO_3 , $CuSO_4$ (cat.), H_2O , MeOH, 16 h, 58%; (iv) 0.2 M LiOH/1,4-dioxane (5/4 v/v), 3 h, then Amberlite IR-120 (H^+), 98 %; (v) See reference 10; (vi) TosCl (1.1 equiv), pyridine, 16 h, 73%; (vii) NaN_3 (10 equiv), DMF, 80 °C, 48 h, 85%; (viii) 1 M HCl/AcOH (1/1 v/v), 60 °C, 3 h, quant.

Regioselective tosylation of the primary hydroxyl function proceeded in 73% yield and subsequent nucleophilic displacement with sodium azide afforded the 2,6-dideoxy sugar **9** in 85%. Hydrolysis of the methyl ester under acidic conditions furnished SAA **4** quantitatively.¹¹

Having the sugar amino acid building blocks **1**, **2**, **3** and **4** in hand, attention was focused on their incorporation into GS, as is outlined in Scheme 2. The construction of the first four targets, having a single SAA substitution, comprises the stepwise elongation of the first seven amino acids, starting from Fmoc-protected leucine on a HMPB-functionalized MBHA-resin **10**, in a similar manner as described in **Chapter 3** and **4**. Ensuing condensation with SAAs **1**, **2**, **3** and **4** gave immobilized nonapeptides **11a-d**, respectively. To ensure complete condensation, an excess of 3 equivalents of coupling reagents (BOP, HOBT) and 2 equivalents of the SAA building block was employed. Next, the azide functionalities were subjected to Staudinger reduction to liberate the terminal amines. The resulting linear peptides were released from the solid support through acidolysis and cyclized according to the procedures described in **Chapter 3** and **4**. This led to the isolation of homogeneous, fully protected GS analogues **12a** (96%),⁶ **12b** (63%), **12c** (85%) and **12d** (78%), respectively.



Scheme 2: Reagents and conditions: (i) Fmoc deprotection: piperidine/NMP (1/4 v/v), azide deprotection: PMe₃ (16 equiv), 1,4-dioxane/H₂O (10/1 v/v); condensation: Fmoc-aa-OH (3 equiv) or SAA **1**, **2**, **3** and **4** (2 equiv), BOP (3 equiv), HOBT (3 equiv), DiPEA (3.3 equiv), NMP, 90 min; (ii) TFA/DCM (1/99 v/v), 4 × 10 min; (iii) PyBOP (5 equiv), HOBT (5 equiv), DiPEA (15 equiv), DMF, 16 h, **12a**, 96%; **12b**, 63%; **12c**, 85%; **12d**, 78%; **12e**, 36%; **12f**, 43%; **12g**, 72%; **12h**, 78%; (iv) NaOMe (16 equiv), MeOH, 16 h, then Amberlite IR-120 (H⁺); (v) TFA/DCM (1/1 v/v) 30 min; (vi) H₂NNH₂·H₂O (50 equiv), MeOH, 65 °C, 16 h.

The assembly of the final four targets, having a sugar amino acid scaffold in both turn regions, commenced with resin **10** that was sequentially elongated with Fmoc-Orn(Boc)-OH, Fmoc-Val-OH and the appropriate SAAs (*i.e.* **1**, **2**, **3** and **4**). Subjection of the thus obtained immobilized peptides to Staudinger reduction resulted in the formation of the terminal amines. Further elongation applying the proper amino acid building blocks gave the anchored linear peptides **11e-h**. Following the abovementioned three-step procedure for solid support release, cyclization and purification, the cyclic peptides **12e-h** were obtained in the respective yields of 36%, 43%, 72% and 78%. The protected GS analogues **12a-h** were transformed into their unprotected counterparts by basic methanolysis of the pivaloyl esters (in the case of **12a** and **12e**), hydrazinolysis of the *N*-phthaloyl amide (in the case of **12d** and **12h**) and finally treatment with 50% TFA in DCM. HPLC purification led to homogeneous cyclic peptides **13a-h** as gauged by LC/MS analysis.

At this stage, GS analogues **13a-h** were subjected to ^1H NMR studies and the results were compared with proton NMR data of native GS. The resonance assignment of the assembled GS analogues was undertaken by using a combination of COSY, TOCSY, and ROESY data sets. It was gratifying to establish that GS analogues **13a-h** showed large resonance dispersion, allowing for facile and unambiguous assignment of all residues. Perusal of the acquired data subsequently enabled the identification of the presence of secondary structure elements in those peptides. In this respect, it has been postulated that the vicinal spin-spin coupling constants can be indicative of turn and β -sheet structures.¹² For example, in native GS, the $^3J_{\text{HN}\alpha}$ values of the Val, Orn and Leu vary between 8.5 and 9.0 Hz and correspond to those found in β -sheet structures. Furthermore, the $^3J_{\text{HN}\alpha}$ of the $^{\text{D}}$ Phe residues are typically small (< 4 Hz) as they occupy a position in the turn regions.³ Therefore, the vicinal coupling constants found in peptides **13a-d** (Figure 1A) that are largely idiosyncratic to GS, strongly suggest that these analogues adopt a conformation closely related to that assumed by the native peptide. Only a small deviation of the coupling constants towards random coil values was observed in a single β -strand of GS analogue **13c**.

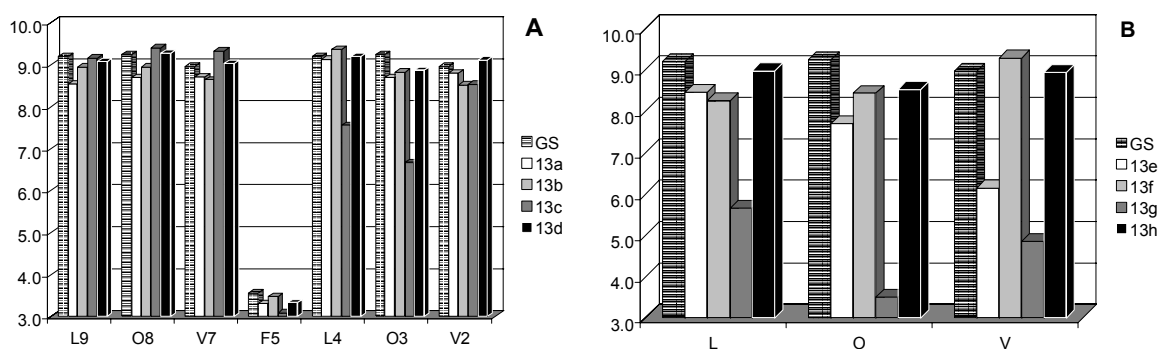


Figure 1: Coupling constants ($^3J_{\text{HN}\alpha}$) found in GS analogues **13a-d** (A) and **13e-h** (B) in Herz.

Consequently, as shown in **Chapter 4** for **13a**,⁶ these single SAA residues do not appear to interfere with β -sheet formation. Rather, they induce a turn conformation that can be locally distorted. For GS analogues **13e-h**, the spectra collapse into a unique set of resonances of four amino acid residues (*i.e.* Val, Orn, Leu and the appropriate SAA) that signify C_2 -symmetric peptides reminiscent of native GS. The furanoid ϵ -SAA **2** and pyranoid δ -SAA **4** in peptides **13f** and **13h**, respectively, display $^3J_{\text{HN}\alpha}$ values characteristic of a β -sheet structure (Figure 1B). However, in peptides **13e** and **13g**, featuring furanoid δ -SAA **1** and pyranoid δ -SAA **3**, spectral line-broadening was observed and the vicinal spin-spin coupling constants are considerably smaller compared to GS, representing a lesser degree of β -sheet formation.

An alternative ^1H NMR spectral analysis focuses on the position of the NMR lines of the individual amino acids. Wishart and co-workers have defined the perturbation of chemical shift as the difference between the measured chemical shift for the H_α of an amino acid and the H_α chemical shift value of the same residue reported for a random coil peptide.¹³ When three or more successive residues have $\Delta\delta\text{H}_\alpha > 0.1$ ppm, it can be assumed that the peptide exists in an extended β -strand conformation. In the case of GS analogues **13a-d**, the secondary chemical shifts follow a similar trend compared to the native peptide (Figure 2A). The large values found in the Leu-Orn-Val tripeptide sequences confirm that both are involved in β -strand formation, whereas the negative values of the Pro and $^{\text{D}}$ Phe residues imply the presence of a turn motif, further validating a β -sheet conformation for peptides **13a-d**. The chemical shift perturbation of the Leu, Orn and Val residues found in GS analogues **13e-h** (Figure 3B) show the largest values for peptides **13f** and **13h**. Positive values for peptides **13e** and **13g** were also observed although these proved to be considerably smaller, corroborating the data found in the $^3J_{\text{HN}\alpha}$ values.

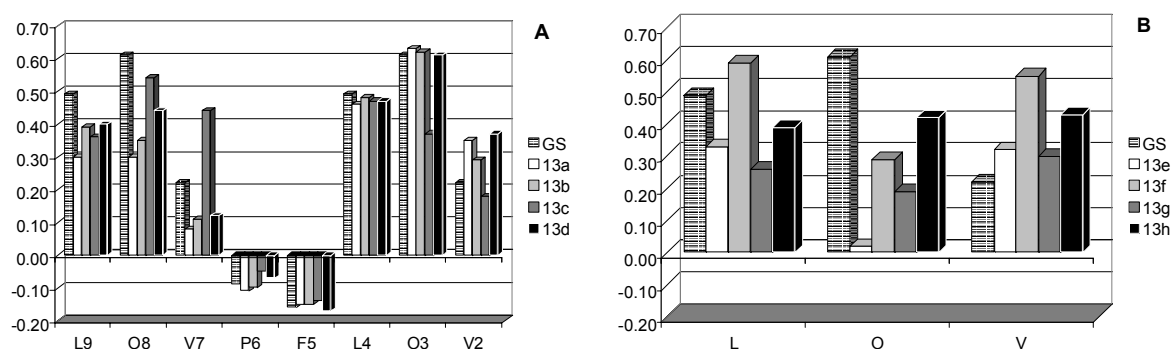


Figure 2: Chemical shift perturbation ($\Delta\delta\text{H}_\alpha = \text{observed } \delta\text{H}_\alpha - \text{random coil } \delta\text{H}_\alpha$) found in GS analogues **13a-d** (A) and **13e-h** (B).^{3,14,15}

The potential of peptides **13a-h** as antibacterial agents was assessed by employing a standard minimal inhibitory concentration (MIC) assay against four Gram-positive and two Gram-negative bacterial strains.³ The results of these tests demonstrate that GS analogues **13a-d** have substantially lost activity against the Gram-positive strains (Table 1).

Table 1: Antimicrobial activity (MIC in µg/mL).

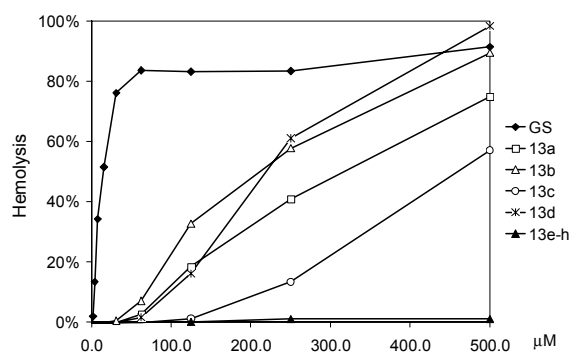
Peptide	<i>S. aureus</i> ^a		<i>S. epidermidis</i> ^a		<i>E. faecalis</i> ^a		<i>B. cereus</i> ^a		<i>E. coli</i> ^b		<i>P. aeruginosa</i> ^b	
	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d
GS	4	4	2	2	8	8	2	4	64->64	>64	>64	>64
13a	64	64	8-16	16-32	>64	>64	16	16-32	>64	>64	>64	>64
13b	>64	>64	32-64	32-64	>64	>64	32	64->64	>64	>64	>64	>64
13c	32	64	16	16	>64	>64	>64	16-32	>64	>64	>64	>64
13d	64	>64	16	16	>64	>64	16-32	64	>64	>64	>64	>64
13e	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
13f	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
13g	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
13h	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64

Measurements were executed using standard agar two-fold dilution techniques.

^a Gram-positive ^b Gram-negative ^c 3 mL / 25 well plates ^d 100 µL / 96 microtiter plates.

Generally, the *S. epidermidis* strain is the most sensitive towards lysis by these antimicrobial peptides. GS analogue **13c** proved to be the most active in this series. Peptides **13e-h** show a complete loss of activity against all bacterial strains in this assay.

The hemolytic activity towards human erythrocytes of **13a-h** was examined by a standard two-fold dilution assay of the appropriate peptide, interpolating between a blank measurement and 100% lysis induced by 1% Triton X-100 in saline. As can be seen in Figure 3, peptides **13a-d** displayed a reduced toxicity profile, showing appreciable lysis only around 500 µM, as compared to 32 µM for native GS. Furthermore, peptides **13e-h** lost all toxicity towards human erythrocytes. Since these results correlate with the abovementioned antimicrobial activity and the same trend for antimicrobial activity and hemolytic activity was observed, it can be concluded that the therapeutic value of the peptides presented here is limited.

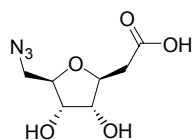
**Figure 3:** Hemolytic activity of GS analogues **13a-h**.

Conclusion

In summary, our practical synthetic strategy towards gramicidin S analogues has proven to be sufficiently versatile for the incorporation of nonproteinogenic sugar amino acids **1**, **2**, **3** or **4**, furnishing eight GS analogues **13a-h** in moderate to good yields, and necessitating only a single HPLC purification step. The ¹H NMR characterization of the GS analogues **13a-h** revealed that these peptides prevalently adopt a β-sheet secondary structure. Assaying their biological profile showed a deleterious effect on the antimicrobial activity with a similar decrease in toxicity towards human erythrocytes.

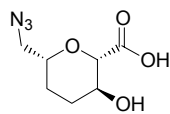
Experimental Section

General: Reactions were performed under an inert atmosphere and at ambient temperature unless stated otherwise. Reactions were monitored by TLC-analysis using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by spraying with 20% H₂SO₄ in ethanol followed by charring at ~150°C or by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid followed by charring at ~150°C. Column chromatography was performed on Merck silicagel (0.040 – 0.063 nm) and size exclusion chromatography on Sephadex™ LH-20. Mass spectra were recorded on a PE/Sciex API 165 instrument with a custom-build Electrospray Ionisation (ESI) interface. HRMS (SIM mode) were recorded on a TSQ Quantum (Thermo Finnigan) fitted with an accurate mass option, interpolating between PEG-calibration peaks. For LC/MS analysis, a Jasco HPLC-system equipped with an analytical Alltima C₁₈ column (Alltech, 4.6 mmD × 250 mmL, 5 μm particle size) in combination with buffers A: H₂O, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI) was used. For RP-HPLC purification of the peptides, a BioCAD “Vision” automated HPLC system (PerSeptiveBiosystems, inc.) equipped with a semi-preparative Alltima C₁₈ column (Alltech, 10.0 mmD × 250 mmL, 5 μm particle size) was used. The applied buffers were A: H₂O, B: MeCN and C: 1.0 % aq. TFA. ¹H- and ¹³C NMR spectra were recorded on a Bruker AV-400 (400/100 MHz) and the peptides were analyzed using a Bruker DMX 600 spectrometer equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard (¹H NMR) or CDCl₃ (¹³C NMR). Coupling constants are given in Hz. All presented ¹³C APT spectra are proton decoupled. Optical rotations were measured on a Propol automatic polarimeter (Sodium D line, λ = 589 nm) and ATR-IR spectra were recorded on a Shimadzu FTIR-8300 fitted with a single bounce DurasamplIR diamond crystal ATR-element.



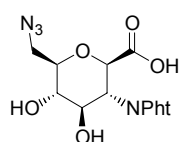
3,6-Anhydro-7-azido-2,7-dideoxy-D-allo-heptononic acid (2): Isopropylidene protected methyl ester **5** (5.03 g, 18.56 mmol) was dissolved in MeOH (75 mL) and 2 M aq. HCl (25 mL) was added, after which the solution was stirred overnight. The mixture was neutralized with 1 M aq. NaOH (50 mL), partially concentrated and extracted with EtOAc thrice. The organics were dried (MgSO₄), filtered and concentrated. Silica gel column chromatography (50%→100% EtOAc in light PE) gave the free diol as a clear oil (3.52 g, 15.25 mmol, 82%). ¹H NMR (400 MHz, CDCl₃): δ = 4.15 (ddd, 1H, H₃, J_{3,2a} = J_{3,2b} = 6.5 Hz, J_{3,4} = 6.3 Hz), 4.06 (dd, 1H, H₅, J_{5,6} = 5.4 Hz, J_{5,4} = 6.3 Hz), 4.00 (dd, 1H, H₆, J_{6,7} = 4.3 Hz, J_{6,5} = 5.4 Hz), 3.95 (dd, 1H, H₄, J_{4,5} = J_{4,3} = 6.3 Hz), 3.57 (dd, 1H, H_{7a}, J_{7a,6} = 3.4 Hz, J_{7a,7b} = 13.3 Hz), 3.31 (dd, 1H, H_{7b}, J_{7b,6} = 4.3 Hz, J_{7b,7a} = 13.3 Hz), 2.77 (dd, 1H, H_{2a}, J_{2a,3} = 6.5 Hz, J_{2a,2b} = 16.3 Hz), 2.69 (dd, 1H, H_{2b}, J_{2b,3} = 6.5 Hz, J_{2b,2a} = 16.3 Hz). ¹³C NMR (100 MHz, CDCl₃): δ = 172.3 (C=O), 82.8 (C₆), 79.1 (C₃), 74.6 (C₄), 72.1 (C₅), 52.1 (C₇), 52.0 (OMe), 37.9 (C₂). ATR-IR (thin film): 3396.4, 2956.2, 2098.4, 1728.1, 1438.8, 1400.2, 1274.9, 1172.6, 1097.4, 1037.6, 987.5, 910.3, 850.5, 829.3, 731.0 cm⁻¹. [α]_D²³ +80.4 (c = 1.0, CH₂Cl₂). MS (ESI): m/z 232.1 [M+H]⁺, 253.8 [M+Na]⁺, 463.0 [2M+H]⁺. The methyl ester (350 mg, 1.52 mmol) was dissolved in THF (4 mL) and 1 M aq. NaOH (2 mL) was added. After being stirred for 3 h, the mixture was neutralized with Amberlite IR-120 (H⁺), filtered and concentrated. Purification by column chromatography (0%→2% AcOH in EtOAc) gave **2** as clear oil (323 mg, 1.49 mmol, 98%). ¹H NMR (400 MHz, CD₃OD): δ = 4.16 (ddd, 1H, H₃, J_{3,2a} = 4.9 Hz, J_{3,4} = 5.3 Hz, J_{3,2b} = 8.4 Hz), 3.96 (m, 2H, H₅, H₆), 3.84 (dd, 1H, H₄, J_{4,3} = 5.3 Hz, J_{4,5} = 5.4 Hz), 3.51 (dd, 1H, H_{7a}, J_{7a,6} = 3.1 Hz, J_{7a,7b} = 13.2 Hz), 3.29 (dd, 1H, H_{7b}, J_{7b,6} = 4.4 Hz, J_{7b,7a} = 13.2 Hz), 2.67 (dd, 1H, H_{2a}, J_{2a,3} = 4.9 Hz, J_{2a,2b} = 15.7 Hz), 2.50 (dd, 1H, H_{2b}, J_{2b,3} = 8.4 Hz, J_{2b,2a} = 15.7 Hz). ¹³C NMR (100 MHz, CD₃OD): δ = 174.6 (C=O), 83.9 (C₆), 81.2 (C₃), 75.7 (C₄), 73.0 (C₅), 53.5 (C₇), 39.4 (C₂). ATR-IR (thin film): 3434.6, 2927.7, 2100.3, 1706.9, 1406.0, 1272.9, 1180.4, 1097.4, 1033.8, 977.8, 912.3, 827.4, 748.3 cm⁻¹. [α]_D²³ +54.4 (c = 1.0, MeOH). MS (ESI): m/z 217.9 [M+H]⁺, 241.0

$[M+Na]^+$, 435.1 $[2M+H]^+$, 457.1 $[2M+Na]^+$. HRMS: calcd for $C_7H_{11}N_3O_5H$ 218.07715, found 218.07724.

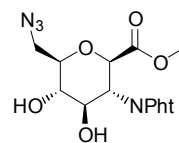


2,6-Anhydro-7-azido-3-hydroxy-4,5,7-trideoxy-L-ribo-heptonic acid (3): Methyl ester **6** (289 mg, 1.00 mmol) was dissolved in DCM (5 mL) and treated with triisopropylsilane (1.3 mmol, 266 μ L) and TFA (5 mL). After being stirred for 30 min, the solvents were removed *in vacuo*. The crude was coevaporated with toluene (5×5 mL) after which a solution of K_2CO_3 (1.5 equiv, 207 mg, 1.5 mmol) and $CuSO_4$ (3 mg, cat.) in H_2O (3.3 mL) was added, followed by MeOH (5 mL) and a freshly prepared solution of TfN_3 (2 equiv) in DCM. The reaction mixture was homogenized with additional MeOH and stirred overnight. The organics were removed by evaporation and the product was purified by silica column chromatography to produce the azide in 58% over 2 steps (0.58 mmol, 125 mg). ^1H NMR (300 MHz, $CDCl_3$): δ = 3.83 (s, 3H, CH_3), 3.77 (s, 1H, H_2), 3.61-3.59 (m, 1H, H_6), 3.48 (s, 1H, H_3), 3.39 and 3.24 ($2 \times$ dd, 2H, H_{7ab} , J = 6.1 and 6.8 Hz and J = 3.8 and J = 9.2 Hz), 1.79-1.68 and 1.62-1.43 (m, 4H, H_{4ab} and H_{5ab}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.4 (C_1), 79.8 (C_2), 76.9 (C_6), 67.2 (C_3), 54.3 (C_7), 52.6 (CH_3), 30.6 (C_4), 27.2 (C_5). ATR-IR (thin film): 2096.5, 1733.9, 1438.8, 1290.3, 1209.3, 1089.7, 1047.3 cm^{-1} . $[\alpha]_D^{20}$ +22.5 (c = 0.24, $CHCl_3$). MS (ESI): m/z 237.9 $[M+Na]^+$. HRMS: calcd for $C_8H_{13}N_3O_4NH_4^+$ 233.12443 found 233.12435.

Subsequently, a solution of the azide (50 mg, 0.23 mmol) in 1,4-dioxane / H_2O (1/1, v/v, 4 mL) was cooled to $0^\circ C$, treated with 1 M aq. LiOH (1.0 equiv, 0.23 mL) and the reaction mixture was allowed to warm to room temperature. After being stirred for 1 h, the reaction mixture was neutralized with Amberlite IR-120 (H^+), filtered and concentrated. The product was purified by silica column chromatography (0% $\rightarrow$ 15% MeOH in DCM) furnishing the title compound **3** quantitatively (46 mg, 0.23 mmol). ^1H -NMR (400 MHz, MeOD): δ = 3.64 (m, 1H, H_6), 3.57 (m, 2H, H_2 and H_4), 3.46 (dd, 2H, H_{7ab} , J = 4.0 and 7.6 Hz), 2.12 (m, 1H, H_{4a}), 1.73 (m, 1H, H_{5a}), 1.54-1.43 (m, 2H, $H_{4b,5b}$); ^{13}C -NMR (100 MHz, MeOD): δ = 179.4 (C_1), 82.1 (C_2), 77.6 (C_6), 69.2 (C_3), 55.8 (C_7), 32.3 (C_4), 28.5 (C_5). ATR-IR (thin film): 2100.3, 1589.2, 1431.1, 1292.2, 1085.8, 1045.3 cm^{-1} . $[\alpha]_D^{20}$ -3.8 (c = 0.16, MeOH). MS (ESI): m/z 201.9 $[M+H]^+$.



6-Azido-2,6-dideoxy-2-phthalimido-beta-D-glucopyranosyl formic acid (4): Methyl ester **9** (120 mg, 0.32 mmol) was dissolved in glacial acetic acid (4 mL) and 1 M aq. HCl (4 mL) was added. The reaction mixture was heated to $60^\circ C$ and stirred for 3 h until TLC analysis revealed complete consumption of starting material. All solvents were removed by repeated evaporation with toluene, to quantitatively furnish carboxylic acid **4** (115 mg, 0.32 mmol) as off-white foam. ^1H NMR (400 MHz, CD_3OD): δ = 7.87 - 7.80 (m, 4H, Phth), 4.94 (bs, 3H, $3 \times OH$), 4.73 (d, 1H, H_2 , $J_{2,3}$ = 10.6 Hz), 4.36 (dd, 1H, H_4 , $J_{4,3}$ = 10.6 Hz, $J_{4,5}$ = 9.0 Hz), 4.21 (dd, 1H, H_3 , $J_{3,4}$ = 10.6 Hz, $J_{3,2}$ = 10.6 Hz), 3.66 - 3.57 (m, 2H, H_6 and H_{7a}), 3.55 (dd, 1H, H_{7b} , $J_{7b,6}$ = 6.5 Hz, $J_{7b,7a}$ = 13.1 Hz), 3.41 (dd, 1H, H_5 , $J_{5,4}$ = $J_{5,6}$ = 9.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ = 171.6 (COOMe, C=O Phth), 135.5 (CH Phth), 131.4 (C_q Phth), 124.2 (CH Phth), 80.6 (C_6), 74.7 (C_2), 73.0 (C_4 , C_5), 55.7 (C_3), 52.6 (C_7). ATR-IR (thin film): 3348.2, 2102.3, 1772.5, 1701.1, 1386.7, 1234.4, 1112.9, 1058.8, 1010.6, 966.3, 873.7, 719.4 cm^{-1} . $[A]_D^{23}$ +17.6 (c = 1.0, $CHCl_3$). HRMS: calcd for $C_{15}H_{14}N_4O_7NH_4$ 380.1206, found 380.1213.



Methyl 6-azido-2,6-dideoxy-2-phthalimido-beta-D-glucopyranosyl formate (9): Triol **8** (3.26 g, 9.27 mmol) was dried by repeated coevaporation with pyridine and redissolved in pyridine (50 mL). The solution was stirred at $0^\circ C$ and *p*-toluenesulfonyl chloride (1.95 g, 10.2 mmol) was added, after which the mixture was stirred overnight at room temperature. Then, the reaction mixture was concentrated *in vacuo* and partitioned between water and EtOAc. The organic layer was washed successively with sat. aq. $NaHCO_3$, sat. aq. $CuSO_4$ and brine after which it was dried ($MgSO_4$) and evaporated. The crude

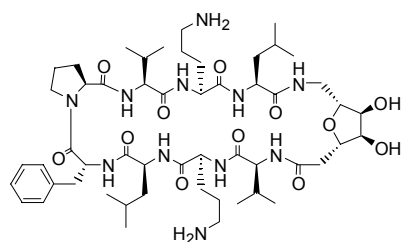
product was purified by silica column chromatography (40%→ 70% EtOAc in toluene) to afford the tosylate (3.42 g, 6.76 mmol, 73%) as a white foam. ^1H NMR (400 MHz, CDCl_3): δ = 7.79 - 7.67 (m, 6H, Tos, Phth), 7.32 (d, 2H, Tos), 4.65 (d, 1H, H_2 , $J_{2,3}$ = 10.6 Hz), 4.42 (dd, 1H, H_4 , $J_{4,3}$ = 10.6 Hz, $J_{4,5}$ = 9.0 Hz), 4.34 (dd, 1H, H_{7a} , $J_{7a,6}$ = 2.0 Hz, $J_{7a,7b}$ = 11.2 Hz), 4.29 (dd, 1H, H_{7b} , $J_{7b,6}$ = 5.4 Hz, $J_{7b,7a}$ = 11.2 Hz), 4.21 (dd, 1H, H_3 , $J_{3,4}$ = 10.6 Hz, $J_{3,2}$ = 10.6 Hz), 3.63 (m, 1H, H_6), 3.51 (s, 3H, OCH_3), 3.49 (m, 1H, H_5), 2.42 (s, 3H, CH_3 Tos). ^{13}C NMR (100 MHz, CDCl_3): δ = 168.1 (COOMe, C=O Phth), 144.9 (C_q Tos), 134.2 (CH Phth), 132.4 (C_q Tos), 131.3 (C_q Phth), 129.8, 128.1 (CH Phth), 77.2 (C_6), 73.4 (C_2), 71.6 (C_4), 70.6 (C_5), 68.9 (C_7), 53.5 (C_3), 52.5 (OMe), 21.5 (CH_3 Tos). ATR-IR (thin film) 3456.5, 2923.9, 1774.4, 1708.8, 1386.7, 1359.7, 1190.0, 1174.6, 1118.6, 1095.5, 966.3, 813.9, 719.4 cm^{-1} . $[\text{A}]_D^{23}$ +20.4 (c = 1.0, CHCl_3). MS (ESI): m/z 506.0 $[\text{M}+\text{H}]^+$, 528.3 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{23}\text{H}_{23}\text{NO}_{10}\text{SNH}_4$ 523.1386, found 523.1396.

The tosylate (3.42 g, 6.76 mmol) was then dissolved in DMF (35 mL) and NaN_3 (4.4 g, 67.6 mmol) was added. The reaction mixture was stirred at 80°C for 48 h and subsequently concentrated. The residue was diluted with water and extracted twice with EtOAc. The combined organic layers were successively washed with sat. aq. NaHCO_3 and brine, dried (MgSO_4) and concentrated. The crude product was applied to a silica gel column (60→ 80% EtOAc in light PE) to yield azide **9** (2.15 g, 5.72 mmol, 85%) as a white foam. ^1H NMR (400 MHz, CDCl_3): δ = 7.80 - 7.72 (m, 4H, Phth), 4.72 (d, 1H, H_2 , $J_{2,3}$ = 10.4 Hz), 4.39 (dd, 1H, H_4 , $J_{4,3}$ = 10.4 Hz, $J_{4,5}$ = 9.2 Hz), 4.25 (dd, 1H, H_3 , $J_{3,4}$ = 10.4 Hz, $J_{3,2}$ = 10.4 Hz), 3.59 – 3.41 (m, 4H, H_5 , H_6 , H_7), 3.55 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3): δ = 168.3, 168.2 (COOMe, C=O Phth), 134.3 (CH Phth), 131.4 (C_q Phth), 123.5 (CH Phth), 78.5 (C_6), 73.4 (C_2), 71.8 (C_4), 71.8 (C_5), 53.6 (C_3), 52.6 (OMe), 51.2 (C_7). ATR-IR (thin film): 3455.0, 2100.3, 1774.4, 1741.0, 1705.0, 1436.9, 1384.8, 1286.4, 1114.8, 1066.6, 1010.6, 964.3, 873.7, 719.4 cm^{-1} . $[\text{A}]_D^{23}$ +54.4 (c = 1.0, CHCl_3). MS (ESI): m/z 377.2 $[\text{M}+\text{H}]^+$, 398.9 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{16}\text{H}_{16}\text{N}_4\text{O}_7\text{NH}_4$ 394.1363, found 394.1340.

Assembly of GS analogues 12a-h: Resin-anchored peptides **11a-d** (100 μmol) were constructed from **10**, according to the general SPPS procedure described in **Chapter 3**. Reduction of the *N*-terminal azide was accomplished by washing the solid support with 1,4-dioxane (5 mL, 3× 3min) and dispersing it in 1,4-dioxane (10 mL), to which trimethylphosphine (16 equiv., 1.6 mL, 1.6 mmol, 1 M in toluene) was added. The resin was shaken for 2 h, water (1 mL) was added and shaking was continued another 4 h. The resin was washed with 1,4-dioxane (5 mL, 3× 3 min) and DCM (5 mL, 3× 3 min). The peptides were released from the resin, cyclized and purified as described in **Chapter 3** to yield the protected **12a**, 96%; **12b**, 63%; **12c**, 85% and **12d**, 78%, respectively, as amorphous white solids. The assembly of peptides **12e-h**, was performed in a similar manner to furnish **12e**, 36%; **12f**, 43%; **12g**, 72% and **12h**, 78%, respectively.

Deprotection of 12a-h: The pivaloyl protection groups in **12a** (32 mg, 24 μmol) and **12e** (12 mg, 10 μmol) were removed by dissolving the peptides in MeOH (5 mL), followed by addition of NaOMe (16 equiv, 20 mg, 370 mmol) and stirring overnight. The mixtures were neutralized with Amberlite IR-120 (H^+), filtered, concentrated and the crudes were used directly in the following Boc-deprotection step. For peptides **12d** (17 mg, 13 μmol) and **12h** (17 mg, 11.4 μmol), the phthaloyl protection groups were removed by dissolving the peptides in MeOH (5 mL), followed by addition of hydrazine-monohydrate (50 equiv, 28 μL , 0.57 mmol). After refluxing for 16 h, the solvents were evaporated and the crude compounds were used without further purification in the following Boc-deprotection. Removal of the Boc protection groups in the aforementioned peptides, as well as **12b** (14 mg, 11.0 μmol), **12c** (14 mg, 11 μmol), **12f** (6 mg, 5.0 μmol), **12g** (12 mg, 10.3 μmol) was performed according to the general procedure described in **Chapter 3**, to give **13a** (22.0 mg, 20.8 μmol , 87%), **13b** (8.1 mg, 7.6 μmol , 69%), **13c** (9.8 mg, 9.3 μmol , 85%), **13d** (12.5 mg, 11.5 μmol , 88%), **13e** (9.0 mg, 9.3 μmol , 93%), **13f** (4.2 mg, 4.2 μmol , 84%), **13g** (9.6 mg, 9.9 μmol , 96%) and **13h** (6.9 mg, 6.7 μmol , 59%), respectively, as white amorphous powders.

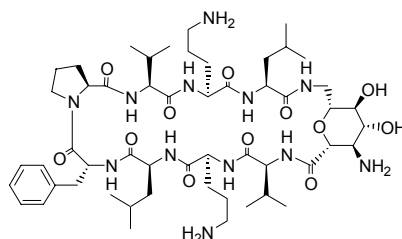
cyclo-[SAA₄-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu] (13a): Prepared as in Chapter 4.⁶



cyclo-[SAA₅-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu] (13b):

Analyzed by LC/MS (R_t 14.71 min; linear gradient 10→90% B in 20 min; m/z = 1070.8 $[M+H]^+$, 536.1 $[M+H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 40→50% B; R_t 1.9 CV). ¹H NMR (600 MHz, CD₃OH): δ = 8.90 (d, 1H, NH ^DPhe₅, $J_{NH,H\alpha}$ = 3.5 Hz), 8.68 (d, 1H, NH _{α} Orn₃, $J_{NH,H\alpha}$ = 8.1 Hz), 8.62 (d, 1H, NH Leu₄, $J_{NH,H\alpha}$ = 9.4 Hz), 8.61 (d, 1H, NH _{α} Orn₈, $J_{NH,H\alpha}$ = 8.9 Hz),

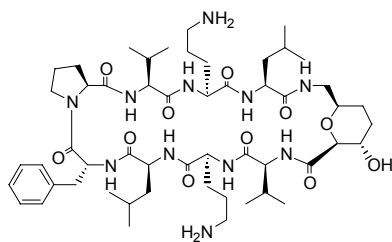
8.56 (d, 1H, NH Leu₉, $J_{NH,H\alpha}$ = 8.9 Hz), 8.07 (t, 1H, NH SAA₁, $J_{NH,7}$ = 6.1 Hz), 7.86 (bs, 2H, NH _{δ} Orn_{3,8}), 7.74 (d, 1H, NH Val₇, $J_{NH,H\alpha}$ = 8.6 Hz), 7.55 (d, 1H, NH Val₂, $J_{NH,H\alpha}$ = 8.5 Hz), 7.38 – 7.21 (m, 5H, H_{ar}), 4.98 (m, 1H, H _{α} Orn₃), 4.71 (m, 1H, H _{α} Orn₈), 4.65 (m, 1H, H _{α} Leu₄), 4.56 (m, 1H, H _{α} Leu₉), 4.51 (m, 1H, H _{α} ^DPhe₅), 4.34 (m, 1H, H _{α} Pro₆), 4.24 (m, 1H, H _{α} Val₂), 4.06 (m, 1H, H _{α} Val₇), 3.95 (m, 2H, H_{3,6} SAA₁), 3.86 (dd, 1H, H₅ SAA₁, $J_{5,4}$ = 5.2 Hz, $J_{5,6}$ = 3.0 Hz), 3.78 (dd, 1H, H₄ SAA₁, $J_{4,5}$ = 5.2 Hz, $J_{4,3}$ = 6.5 Hz), 3.72 (m, 1H, H _{$\delta\delta$} Pro₆), 3.36 (m, 1H, H_{7d} SAA₁), 3.31 (m, 1H, H_{7u} SAA₁), 3.07 (dd, 1H, H _{$\beta\delta$} ^DPhe₅, $J_{\beta\delta,\beta u}$ = 12.6 Hz, $J_{\beta\delta,\alpha}$ = 5.0 Hz), 3.02 (m, 1H, H _{$\delta\delta$} Orn₃), 2.98 (m, 1H, H _{$\delta\delta$} Orn₈), 2.96 (m, 3H, H _{δu} Orn₃, H _{δu} Orn₈, H _{βu} ^DPhe₅), 2.50 (m, 3H, H₂ SAA₁, H _{δu} Pro₆), 2.28 (m, 1H, H _{β} Val₇), 1.99 (m, 3H, H _{$\beta\delta$} Pro₆, H _{$\beta\delta$} Orn₃, H _{β} Val₂), 1.83 (m, 1H, H _{$\beta\delta$} Orn₈), 1.74 (m, 2H, H _{γ} Orn₃), 1.71 (m, 2H, H _{βu} , $\gamma\delta$ Pro₆), 1.67 (m, 1H, H _{βu} Orn₃), 1.66 (m, 2H, H _{γ} Orn₈), 1.64 (m, 3H, H _{β} , γ Leu₉), 1.59 (m, 1H, H _{γu} Pro₆), 1.56 (m, 2H, H _{$\beta\delta$} , γ Leu₄), 1.39 (m, 1H, H _{βu} Leu₄), 0.95 (m, 3H, H _{$\gamma\delta$} Val₇), 0.94 (m, 3H, H _{$\gamma\delta$} Val₂), 0.92 (m, 3H, H _{γu} Val₂), 0.90 (m, 6H, H _{δ} Leu₄), 0.88 (m, 3H, H _{γu} Val₇), 0.86 (m, 6H, H _{δ} Leu₉). ATR-IR (thin film): 3278.1, 3071.9, 2959.2, 2935.6, 2873.4, 1669.8, 1636.5, 1539.2, 1464.7, 1456.7, 1437.0, 1203.7, 1182.7, 1135.0, 1033.3, 1020.8, 837.1, 800.1, 722.6, 702.5 cm⁻¹. HRMS: calcd for C₅₃H₈₇N₁₁O₁₂H 1079.6608, found 1070.6521.



cyclo-[SAA₆-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu] (13c):

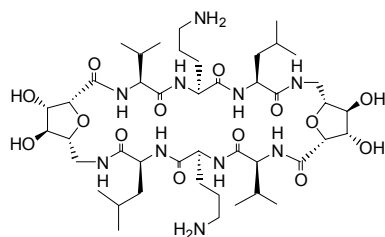
Analyzed by LC/MS (R_t 15.79 min; linear gradient 10→90% B in 20 min; m/z = 1054.8 $[M+H]^+$, 528.2 $[M+H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 35→55% B; R_t 2.7 CV). ¹H NMR (600 MHz, CD₃OH): δ = 8.99 (d, 1H, NH ^DPhe₅, $J_{NH,H\alpha}$ = 3.1 Hz), 8.76 (d, 1H, NH _{α} Orn₃, $J_{NH,H\alpha}$ = 6.7 Hz), 8.74 (d, 1H, NH Leu₄, $J_{NH,H\alpha}$ = 7.6 Hz), 8.56 (d, 1H, NH Leu₉, $J_{NH,H\alpha}$ = 9.2 Hz),

8.51 (d, 1H, NH _{α} Orn₈, $J_{NH,H\alpha}$ = 9.4 Hz), 8.11 (t, 1H, NH SAA₁, $J_{NH,7}$ = 6.3 Hz), 7.87 (bs, 2H, NH _{δ} Orn_{3,8}), 7.69 (d, 1H, NH Val₂, $J_{NH,H\alpha}$ = 8.5 Hz), 7.60 (d, 1H, NH Val₇, $J_{NH,H\alpha}$ = 9.3 Hz), 7.32 – 7.23 (m, 5H, H_{ar}), 4.90 (m, 1H, H _{α} Orn₈), 4.73 (m, 1H, H _{α} Orn₃), 4.64 (m, 1H, H _{α} Leu₄), 4.50 (m, 2H, H _{α} ^DPhe₅, H _{α} Leu₉), 4.39 (m, 2H, H _{α} Pro₆, H _{α} Val₇), 4.13 (m, 1H, H _{α} Val₂), 3.71 (m, 1H, H _{$\delta\delta$} Pro₆), 3.52 (m, 1H, H_{7d} SAA₁), 3.44 (2, 2H, H_{2,3} SAA₁), 3.41 (m, 1H, H₆ SAA₁), 3.08 (dd, 1H, H _{$\beta\delta$} ^DPhe₅, $J_{\beta\delta,\beta u}$ = 12.9 Hz, $J_{\beta\delta,\alpha}$ = 4.9 Hz), 3.07 (m, 1H, H_{7u} SAA₁), 3.01 (m, 1H, H _{$\delta\delta$} Orn₃), 2.93 (dd, 1H, H _{βu} ^DPhe₅, $J_{\beta\delta,\beta u}$ = $J_{\beta\delta,\alpha}$ = 12.9 Hz), 2.87 (m, 3H, H _{δ} Orn₈, H _{δu} Orn₃), 2.46 (m, 1H, H _{δu} Pro₆), 2.23 (m, 1H, H _{β} Val₇), 2.14 (m, 1H, H _{β} Val₂), 2.09 (m, 1H, H_{4d} SAA₁), 2.01 (m, 2H, H _{$\beta\delta$} Pro₆, H _{$\beta\delta$} Orn₃), 1.77 (m, 2H, H _{β} Orn₃), 1.71 (m, 1H, H_{5d} SAA₁), 1.62 (m, 5H, H _{$\beta\delta$} Leu₉, H _{$\beta\delta$} Leu₆, H _{$\beta\delta$} Orn₈, H _{βu} Pro₆, H _{γu} Orn₃), 1.52 (m, 7H, H _{βu} , $\gamma\delta$ Orn₈, H _{γ} Leu₄, H _{γ} Leu₉, H _{γ} Pro₆, H_{4u} SAA₁), 1.42 (m, 4H, H _{βu} , Leu₉, H _{βu} , Leu₆, H _{γu} Orn₈, H_{4u} SAA₁), 1.42 (m, 1H, H _{βu} Leu₄), 1.02 (d, 3H, H _{$\gamma\delta$} Val₂ $J_{\gamma,\beta}$ = 6.7 Hz), 0.97 (d, 3H, H _{γu} Val₂ $J_{\gamma,\beta}$ = 6.8 Hz), 0.92 (d, 6H, H _{γ} Val₇), 0.93 – 0.87 (m, 12H, H _{δ} Leu₄, H _{δ} Leu₉). ATR-IR (thin film): 3267.7, 3061.3, 2957.6, 2933.1, 2870.1, 1675.2, 1639.5, 1538.9, 1456.8, 1203.4, 1182.1, 1133.3, 1060.1, 1033.4, 838.3, 800.0, 749.1, 722.8, 701.7 cm⁻¹. HRMS: calcd for C₅₃H₈₇N₁₁O₁₁H 1054.6659, found 1054.6622.

**cyclo-[SAA₇-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu] (13d):**

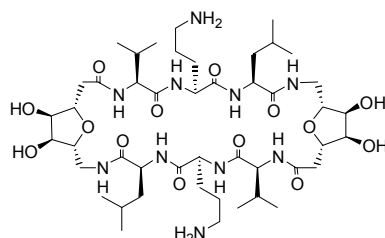
Analyzed by LC/MS (R_t 12.92 min; linear gradient 10→90% B in 20 min; m/z = 1086.0 $[M+H]^+$, 543.6 $[M+H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 30→50% B; R_t 2.8 CV). ^1H NMR (600 MHz, CD_3OH): δ = 8.92 (d, 1H, NH ^DPhe₅, $J_{NH,H\alpha}$ = 3.3 Hz), 8.69 (d, 1H, NH Leu₄, $J_{NH,H\alpha}$ = 9.2 Hz), 8.61 (d, 1H, NH _{α} Orn₃, $J_{NH,H\alpha}$ = 8.9 Hz), 8.58 (d, 1H, NH _{α} Orn₈, $J_{NH,H\alpha}$ = 9.3 Hz), 8.47 (d,

1H, NH Leu₉, $J_{NH,H\alpha}$ = 9.1 Hz), 8.09 (t, 1H, NH SAA₁, $J_{NH,7}$ = 6.1 Hz), 7.72 (d, 1H, NH Val₇, $J_{NH,H\alpha}$ = 9.0 Hz), 7.70 (d, 1H, NH Val₂, $J_{NH,H\alpha}$ = 9.1 Hz), 7.38 – 7.21 (m, 5H, H_{ar}), 4.97 (m, 1H, H _{α} Orn₃), 4.80 (m, 1H, H _{α} Orn₈), 4.64 (m, 1H, H _{α} Leu₄), 4.57 (m, 1H, H _{α} Leu₉), 4.49 (m, 1H, H _{α} ^DPhe₅), 4.34 (m, 1H, H _{α} Pro₆), 4.32 (m, 1H, H _{α} Val₂), 4.07 (m, 2H, H₂ SAA₁, H _{α} Val₇), 3.87 (m, 1H, H_{7d} SAA₁), 3.71 (m, 1H, H_{8d} Pro₆), 3.58 (dd, 1H, H₄ SAA₁, $J_{4,5}$ = $J_{4,3}$ = 9.2 Hz), 3.49 (m, 1H, H₆ SAA₁), 3.34 (m, 1H, H_{7u} SAA₁), 3.18 (dd, 1H, H₅ SAA₁, $J_{5,4}$ = $J_{5,6}$ = 9.2 Hz), 3.08 (dd, 1H, H _{βd} ^DPhe₅, $J_{\beta d,\beta u}$ = 12.6 Hz, $J_{\beta d,\alpha}$ = 5.0 Hz), 3.02 (m, 1H, H_{8d} Orn₃), 3.00 (m, 2H, H₈ Orn₈), 2.98 (m, 1H, H _{δu} Orn₃), 2.93 (m, 1H, H _{βu} ^DPhe₅), 2.80 (dd, 1H, H₃ SAA₁, $J_{3,2}$ = 10.2 Hz, $J_{3,4}$ = 9.2 Hz), 2.47 (m, 1H, H _{δu} Pro₆), 2.29 (m, 1H, H _{β} Val₇), 2.17 (m, 1H, H _{β} Val₂), 1.98 (m, 2H, H _{βd} Pro₆, H _{βd} Orn₃), 1.75 (m, 2H, H _{βu} Orn₃, H _{βd} Orn₈), 1.68 (m, 6H, H _{βd} Leu₉, H _{βu} Pro₆, H _{γ} Orn₃, H _{γ} Orn₈), 1.55 (m, 3H, H _{βu} Orn₈, H _{$\beta d,\gamma$} Leu₄), 1.52 (m, 4H, H _{γ} Pro₆, H _{$\beta u,\gamma$} Leu₉), 1.42 (m, 1H, H _{βu} Leu₄), 0.98 (d, 3H, H _{γd} Val₂, $J_{\gamma,\beta}$ = 6.8 Hz), 0.95 (d, 3H, H _{γd} Val₇, $J_{\gamma,\beta}$ = 6.6 Hz), 0.93 (d, 3H, H _{γu} Val₂, $J_{\gamma,\beta}$ = 6.8 Hz), 0.91 – 0.86 (m, 15H, H _{γu} Val₇, H₈ Leu₄, H₈ Leu₉). ATR-IR (thin film): 3273.4, 3066.7, 2954.8, 2936.6, 2872.3, 1672.1, 1645.9, 1539.5, 1454.0, 1437.0, 1203.7, 1182.5, 1133.5, 1033.2, 1021.5, 838.7, 799.9, 748.0, 722.9, 702.4 cm^{-1} . HRMS: calcd for C₅₃H₈₈N₁₂O₁₂H 1085.6717, found 1085.6691.



cyclo-[SAA₄-Val-Orn-Leu]₂ (13e): Analyzed by LC/MS (R_t 10.96 min; linear gradient 10→90% B in 20 min; m/z = 971.8 $[M+H]^+$, 486.6 $[M+H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 20→40% B; R_t 2.7 CV). ^1H NMR (600 MHz, CD_3OH): δ = 8.46 (d, 1H, NH _{α} Orn, $J_{NH,H\alpha}$ = 7.7 Hz), 8.00 (d, 1H, NH Leu, $J_{NH,H\alpha}$ = 8.5 Hz), 7.99 (t, 1H, NH SAA, $J_{NH,6}$ = 8.5 Hz), 7.89 (d, 1H, NH Val, $J_{NH,H\alpha}$ = 6.1 Hz), 4.57 (d, 1H, H₂ SAA, $J_{2,3}$ = 4.0 Hz), 4.50 (m,

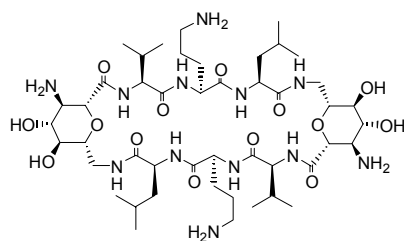
1H, H _{α} Leu), 4.38 (m, 1H, H _{α} Orn), 4.27 (m, 2H, H _{α} Val, H₃ SAA), 4.02 (m, 1H, H₅ SAA), 3.95 (s, 1H, H₄ SAA), 3.63 (m, 1H, H_{6d} SAA), 3.37 (m, 1H, H_{6u} SAA), 2.94 (m, 2H, H₈ Orn), 2.25 (m, 1H, H _{β} Val), 1.95 (m, 1H, H _{βd} Orn), 1.84 (m, 1H, H _{βu} Orn), 1.71 (m, 2H, H _{γ} Orn), 1.59 (m, 3H, H _{β,γ} Leu), 0.96 (m, 6H, H _{γ} Val), 0.89 (m, 6H, H₈ Leu). ATR-IR (thin film): 3279.5, 2961.1, 2933.9, 2875.5, 1648.7, 1528.3, 1435.9, 1202.6, 1182.4, 1135.4, 1042.1, 837.6, 799.8, 722.3 cm^{-1} . HRMS: calcd for C₄₄H₇₈N₁₀O₁₄H 971.5771, found 971.5736.



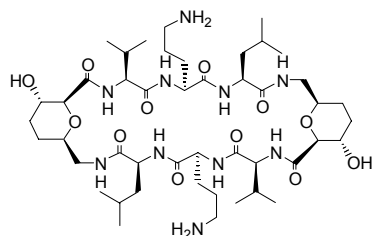
cyclo-[SAA₅-Val-Orn-Leu]₂ (13f): Analyzed by LC/MS (R_t 10.95 min; linear gradient 10→90% B in 20 min; m/z = 999.8 $[M+H]^+$, 500.7 $[M+H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 20→40% B; R_t 2.5 CV). ^1H NMR (600 MHz, CD_3OH): δ = 8.64 (d, 1H, NH Leu, $J_{NH,H\alpha}$ = 8.2 Hz), 8.50 (d, 1H, NH _{α} Orn, $J_{NH,H\alpha}$ = 8.4 Hz), 8.46 (t, 1H, NH SAA, $J_{NH,7}$ = 4.3 Hz), 8.04 (d, 1H, NH Val, $J_{NH,H\alpha}$ = 9.3 Hz), 4.76 (m, 1H, H _{α} Leu), 4.65 (m, 1H, H _{α} Orn),

4.50 (m, 1H, H _{α} Val), 4.06 (m, 2H, H_{4,6} SAA), 4.01 (m, 1H, H₃ SAA), 3.93 (dd, 1H, H₅ SAA, $J_{5,4}$ = $J_{5,6}$ = 4.6 Hz), 3.55 (m, 1H, H_{7d} SAA), 3.32 (m, 1H, H_{7u} SAA), 2.93 (m, 2H, H₈ Orn), 2.63 (dd, 1H, H_{2d} SAA, $J_{2d,3}$ = 3.3 Hz, $J_{2d,2u}$ = 15.3 Hz), 2.37 (dd, 1H, H_{2u} SAA, $J_{2u,3}$ = 7.3 Hz, $J_{2u,2d}$ = 15.3 Hz), 2.12 (m, 1H, H _{β} Val), 1.71 (m, 3H, H _{$\beta,\gamma d$} Orn), 1.61 (m, 3H, H _{γu} Orn, H _{$\beta d,\gamma$} Leu), 1.44 (m, 1H, H _{βu} Leu), 0.92 (m, 6H, H _{γ} Val), 0.88 (m, 6H, H₈ Leu). ATR-IR (thin film): 3279.4, 3072.3, 2957.8, 2930.1, 2872.2,

2857.6, 1663.5, 1642.8, 1539.4, 1534.0, 1437.0, 1202.3, 1182.8, 1134.3, 839.3, 800.5, 722.8 cm^{-1} . HRMS: calcd for $\text{C}_{46}\text{H}_{82}\text{N}_{10}\text{O}_{14}\text{H}$ 999.6084, found 999.6097.



cyclo-[SAA₆-Val-Orn-Leu]₂ (13g): Analyzed by LC/MS (R_t 12.02 min; linear gradient 10→90% B in 20 min; m/z = 967.7 $[\text{M}+\text{H}]^+$, 484.6 $[\text{M}+\text{H}]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 25→40% B; R_t 2.8 CV). ^1H NMR (600 MHz, CD_3OH): δ = 8.49 (d, 1H, NH_α Orn, $J_{\text{NH},\text{H}_\alpha}$ = 3.5 Hz), 8.34 (d, 1H, NH Leu, $J_{\text{NH},\text{H}_\alpha}$ = 5.6 Hz), 8.06 (t, 1H, NH SAA, $J_{\text{NH},6}$ = 3.2 Hz), 7.76 (d, 1H, NH Val, $J_{\text{NH},\text{H}_\alpha}$ = 4.8 Hz), 4.55 (m, 1H, H_α Orn), 4.43 (m, 1H, H_α Leu), 4.25 (m, 1H, H_α Val), 3.52 (m, 2H, H_2 , H_3 SAA), 3.47 (m, 2H, H_6 , H_7 SAA), 3.16 (dd, 1H, H_{7u} SAA, $J_{7u,7d}$ = 8.4 Hz, $J_{7u,6}$ = 1.7 Hz), 2.89 (m, 2H, H_δ Orn), 2.14 (m, 2H, H_β Val, H_{4d} SAA), 1.69 (m, 2H, $\text{H}_{\beta d}$ Orn, H_{5d} SAA), 1.61 (m, 4H, $\text{H}_{\beta u, \gamma d}$ Orn, $\text{H}_{\beta d, \gamma}$ Leu), 1.52 (m, 4H, $\text{H}_{\gamma u}$ Orn, $\text{H}_{\beta u}$ Leu $\text{H}_{5u, 4u}$ SAA), 0.99 (m, 6H, H_γ Val), 0.92 (d, 3H, $\text{H}_{\delta u}$ Leu $J_{\delta, \gamma}$ = 6.2 Hz), 0.89 (d, 3H, $\text{H}_{\delta d}$ Leu $J_{\delta, \gamma}$ = 6.1 Hz). ATR-IR (thin film): 3285.8, 3070.5, 2957.8, 2932.4, 2872.1, 1652.6, 1533.2, 1468.4, 1437.0, 1202.3, 1180.2, 1130.8, 837.1, 799.7, 722.1 cm^{-1} . HRMS: calcd for $\text{C}_{46}\text{H}_{82}\text{N}_{10}\text{O}_{12}\text{H}$ 967.6186, found 967.6191.



cyclo-[SAA₇-Val-Orn-Leu]₂ (13h): Analyzed by LC/MS (R_t 9.73 min; linear gradient 10→90% B in 20 min; m/z = 1029.8 $[\text{M}+\text{H}]^+$, 515.6 $[\text{M}+\text{H}]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 20→40% B; R_t 2.0 CV). ^1H NMR (600 MHz, CD_3OH): δ = 8.55 (d, 1H, NH_α Orn, $J_{\text{NH},\text{H}_\alpha}$ = 8.5 Hz), 8.47 (d, 1H, NH Leu, $J_{\text{NH},\text{H}_\alpha}$ = 9.0 Hz), 8.10 (t, 1H, NH SAA, $J_{\text{NH},7}$ = 6.3 Hz), 7.76 (d, 1H, NH Val, $J_{\text{NH},\text{H}_\alpha}$ = 8.9 Hz), 4.78 (m, 1H, H_α Orn), 4.56 (m, 1H, H_α Leu), 4.38 (m, 1H, H_α Val), 4.53 (d, 1H, H_2 SAA, $J_{2,3}$ = 10.6 Hz), 3.83 (m, 1H, H_{7d} SAA), 3.58 (dd, 1H, H_4 SAA, $J_{4,5}$ = 9.3 Hz, $J_{4,3}$ = 10.0 Hz), 3.48 (m, 1H, H_6 SAA), 3.39 (m, 1H, H_{7u} SAA), 3.18 (dd, 1H, H_5 SAA, $J_{5,6}$ = 9.1 Hz, $J_{5,4}$ = 9.3 Hz), 3.02 (m, 1H, $\text{H}_{\delta d}$ Orn), 2.98 (m, 1H, $\text{H}_{\delta u}$ Orn), 2.79 (dd, 1H, H_3 SAA, $J_{3,4}$ = 10.0 Hz, $J_{3,2}$ = 10.6 Hz), 2.19 (m, 1H, H_β Val), 1.71 (m, 4H, $\text{H}_{\beta, \gamma d}$ Orn, $\text{H}_{\beta d}$ Leu), 1.55 (m, 2H, $\text{H}_{\gamma u}$ Orn, H_γ Leu), 1.48 (m, 1H, $\text{H}_{\beta u}$ Leu), 0.98 (d, 3H, $\text{H}_{\gamma d}$ Val $J_{\gamma, \beta}$ = 6.8 Hz), 0.95 (d, 3H, $\text{H}_{\gamma u}$ Val $J_{\gamma, \beta}$ = 6.8 Hz), 0.89 (m, 6H, H_δ Leu). ATR-IR (thin film): 3280.4, 3072.9, 2957.5, 2932.8, 2872.4, 1671.5, 1647.8, 1544.5, 1437.6, 1203.3, 1186.7, 1136.2, 1084.3, 840.9, 800.1, 723.6 cm^{-1} . HRMS: calcd for $\text{C}_{46}\text{H}_{84}\text{N}_{12}\text{O}_{14}\text{H}$ 1029.6302, found 1029.6280.

Biological activity: The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Bacteria were stored at -70°C and grown at 35°C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) overnight and diluted in 0.9% NaCl. Microtiter plates (96 wells of 100 μL) as well as large plates (25 wells of 3 mL) were filled with Mueller Hinton II Agar (Becton Dickinson, Cockeysville, USA) containing serial two-fold dilutions of peptides **13a-h**. To the wells were added 3 μL of bacteria, to give a final inoculum of 10^4 colony forming units (CFU) per well. The plates were incubated overnight at 35°C and the MIC was determined as the lowest concentration inhibiting bacterial growth.

Hemolytic Activity: The hemolytic activity of the peptides was determined in quadruple. Human blood was collected into EDTA-tubes and centrifuged to remove the buffy coat. The residual erythrocytes were washed three times in 0.85% saline. Serial two-fold dilutions of the peptides **13a-h** in saline were prepared in sterilized round-bottom 96-well plates (polystyrene, U-bottom, Costar) using 100 μL volumes (500–0.5 μM). Red blood cells were diluted with saline to 1/25 packed volume of cells and 50 μL of the resulting cell suspension was added to each well. Plates were incubated while

gently shaking at 37 °C for 4 h. Next, the microtiter plate was quickly centrifuged (1000 g, 5 min) and 50 µL supernatant of each well was transported into a flat-bottom 96-well plate (Costar). The absorbance was measured at 405 nm using a mQuant micro plate spectrophotometer (Bio-Tek Instruments). The A_{blank} was measured in the absence of additives and 100% hemolysis (A_{tot}) in the presence of 1% Triton X-100 in saline. The percentage hemolysis is determined as $(A_{\text{pep}} - A_{\text{blank}})/(A_{\text{tot}} - A_{\text{blank}}) \times 100$.

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Chapter 6

Synthesis and Application of Carbohydrate-Derived Morpholine Amino Acids

Abstract: *The synthesis of a series of diversely functionalized ϵ -morpholine amino acids (MAAs, **5a-h**) starting from an ϵ -sugar amino acid and following a two-step oxidative glycol cleavage/reductive amination strategy, is described. In an alternative synthetic scheme, diastereoisomerically pure δ -MAAs (**12a,b**) were obtained. Oligopeptides containing MAAs were prepared either by direct incorporation of a MAA building block or by subjecting a fully assembled SAA-containing peptide to the two-step glycol cleavage/reductive amination procedure.¹*

Introduction

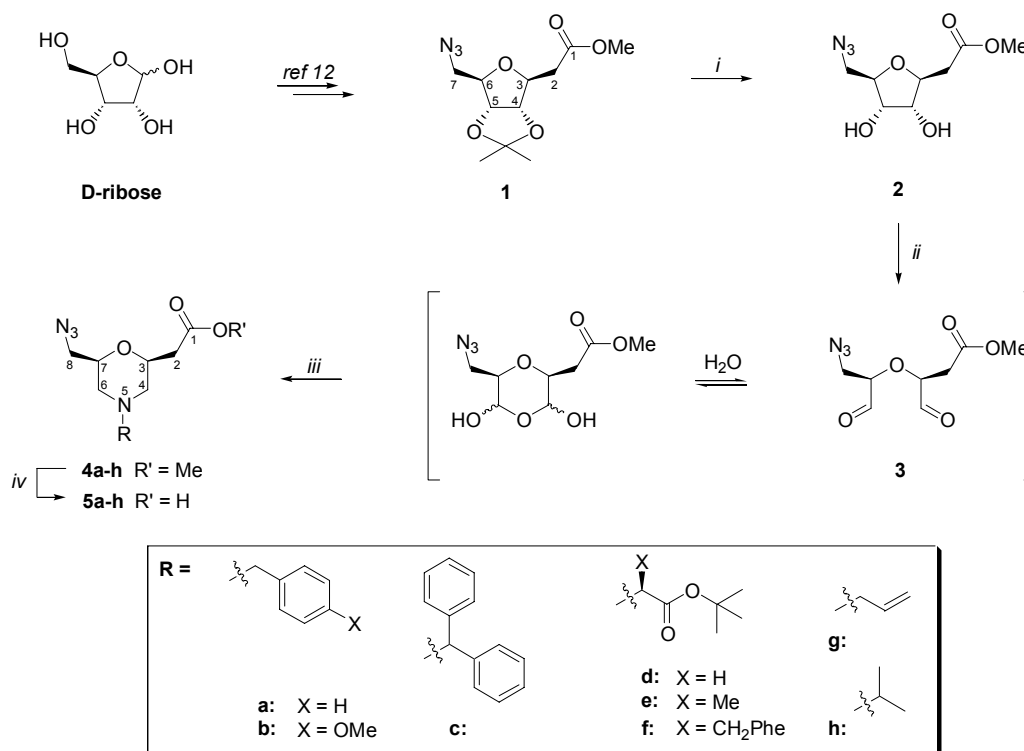
The design, synthesis and application of peptidomimetic compounds has been a focal point of research for many years. The generation of a plethora of peptide analogues and their incorporation in oligopeptides has led to the identification of pharmaceutically interesting compounds.² As secondary structure is a decisive factor in the functioning of peptides and proteins, scaffolds that restrict the conformational freedom have been applied to provide structural stabilization when incorporated in oligopeptides.³ Moreover, the incorporation of nonproteinogenic residues can have beneficial effects on metabolic stability, whereas additional functionalities on the molecular framework allow the attachment of potential pharmacophoric groups. Sugar amino acids (SAAs), carbohydrate scaffolds appended with an amine and carboxylic acid moiety, have been employed successfully as nonproteinogenic compounds.⁴ SAAs are a structurally and functionally diverse class of peptidomimetics and exist as furanoid, pyranoid, open chain⁵ and fused ring systems.⁶ Hydroxyl groups that originate from the parent sugar can participate in secondary structure formation. For example, in **Chapter 4** it was revealed that the incorporation of a furanoid SAA into the turn region of gramicidin S (GS) induces an unusual turn structure with a hydroxyl protruding into the turn region of GS causing a disruption in the H-bonding pattern as compared to the native peptide.⁷ The free hydroxyl functionalities in SAAs can also be equipped with

pharmacophores, thereby increasing their resemblance with native peptide sequences. This principle was elegantly demonstrated by Smith, III *et al.* in the synthesis of a potential mammalian ribonuclease reductase inhibitor.⁸ In this example, a tetrahydropyran scaffold was adorned with a methylene carboxylate and an isobutyl group that mimic the aspartic acid and leucine side chains, respectively. Next to the decoration of the hydroxyls, another type of derivatization can be envisaged based on oxidative glycol-cleavage of a 1,2-diol functionality on the furan or pyran core structure. The ring structure can subsequently be reinstalled by double reductive amination of the resulting dialdehyde, resulting in a substituted morpholine.⁹ Previously, Du *et al.* reported the synthesis of morpholino-glycopeptides starting from glycopyranosides.^{10a} Inositol-triphosphate analogues having substituted amines introduced into the carbacyclic core have been prepared by Malmberg *et al.*^{10b} Furthermore, the generation of morpholine derivatives from nucleoside building blocks and their incorporation in oligonucleotide analogues that possess favourable antisense properties has been described.¹¹

In this chapter, the synthesis of a series δ - and ϵ -morpholine amino acids (MAAs) is described that bear several different moieties on the endocyclic nitrogen, starting from SAA building blocks and following the aforementioned glycol-cleavage/reductive amination-strategy. To demonstrate the versatility of the approach, a single type II' β -turn of the model peptide GS has been replaced by an ϵ -MAA, both through direct incorporation of a MAA building block and by modification of a SAA-containing GS analogue after complete assembly of the cyclic oligopeptide.

Results and Discussion

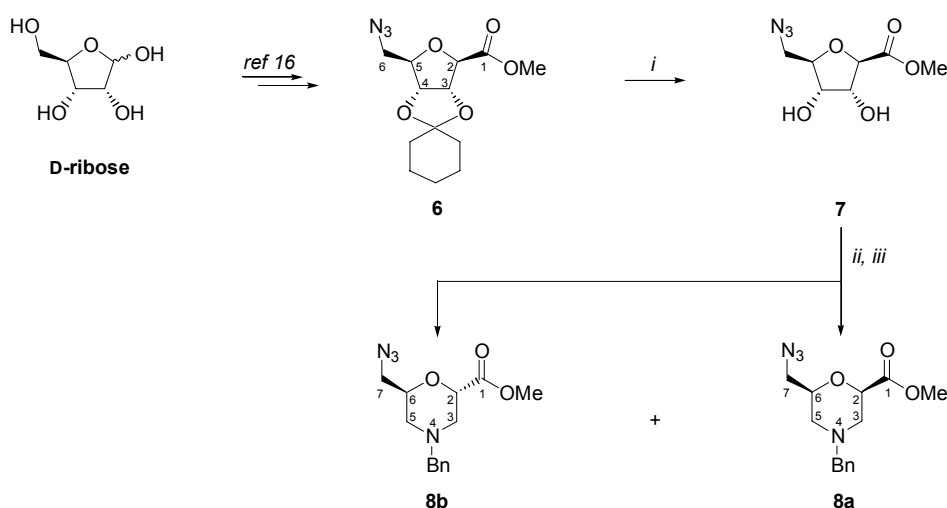
The synthesis of a set of ϵ -morpholine amino acids (**5a-h**) is outlined in Scheme 1. Starting from D-(+)-ribose, the protected SAA building block **1** was obtained following a high-yielding four-step procedure recently developed by van Well *et al.*¹² This route entails the installation of the acetonide at the 2,3-diol, Wittig olefination at the anomeric center, mesylation of the remaining hydroxyl functionality and subsequent introduction of the azide moiety. Removal of the isopropylidene protective group in SAA **1** by acidolysis exposed the *cis*-diol system to give **2** in 82% yield. Glycol cleavage was effected by treatment with periodic acid to afford dialdehyde **3**, together with its corresponding hydrates.¹³ The MAA-core structures **4a-h** were obtained after slow addition of a solution of the appropriate amine in MeOH, that had been acidified with AcOH to approximately pH = 5 in advance, to a mixture of **3** and NaCNBH₃.¹⁴ The yields of the double reductive aminations varied for the benzylic amines (**4a**; 54 %, **4b**; 36%, **4c**; 38%), the amino acid derivatives (**4d**; 59%, **4e**; 33%, **4f**; 45%) and for the aliphatic amines (**4g**; 41%, **4h**; 33%). Saponification of the methyl ester functionalities in **4a-h** produced the free ϵ -morpholine amino acids **5a-h**.



Scheme 1: Reagents and conditions: (i) 2 M HCl/MeOH (1/3 v/v), 16 h, 82%; (ii) H₅IO₆ (1.5 equiv), THF, 20 min, 94%; (iii) R-NH₂ (1.1 equiv), NaCNBH₃ (4.2 equiv), trimethylorthoformate/MeOH (1/3 v/v), AcOH, 3 Å mol. sieves, 16 h, **4a**, 54 %; **4b**, 36%; **4c**, 38%; **4d**, 59%; **4e**, 33%; **4f**, 45%; **4g**, 41% and **4h**, 33%; (iv) 1 M NaOH (2 equiv), THF, 4 h, then Amberlite IR-120 (H⁺), **5a**, quant; **5b**, 94%; **5c**, quant; **5d**, quant; **5e**, 78%; **5f**, 45%; **5g**, 78% and **5h**, quant.

In order to establish whether the above described approach to ϵ -MAAs is also amenable for δ -MAAs, SAA **7** (Scheme 2), having a similar *cis*-diol system as template **2**, was selected as next synthetic target. The appropriate precursor **6** was prepared in a five-step procedure, comprising Kiliani ascension¹⁵ of cyclohexylidene-protected D-(+)-ribose, followed by ditosylation, base-catalyzed ring contraction and introduction of the azide, according to the procedure developed by Fleet and co-workers.¹⁶ The *cis*-diol in **7** was unveiled by acidic release of the cyclohexylidene group in **6** (59%). Periodate oxidation followed directly by reductive amination of the crude dialdehyde furnished, after silica column chromatography, the diastereoisomeric morpholines **8a** and **8b**, both in 22%.

The 2,6-*cis* configuration and 2,6-*trans* configuration of **8a** and **8b**, respectively, were established by comparison of the ¹H spectra (see Figure 1). The large geminal (²*J*_{3ax,3eq} = 11.1 Hz) and vicinal (³*J*_{3ax,2} = 11.1 Hz) coupling constants confirmed a *anti*-periplanar relationship between H_{3ax} and H₂ in the case of **8a**. For **8b**, a large geminal (²*J*_{3ax,3eq} = 11.6 Hz) and moderate vicinal (³*J*_{3ax,2} = 4.1 Hz) coupling constant were observed, indicating a *gauche* relationship between H_{3ax} and H₂.



Scheme 2: Reagents and conditions: (i) 4 M HCl/MeOH (1/4 v/v), 50°C, 2 h, 59%; (ii) H_5IO_6 , THF, 30 min, 95%; (iii) R-NH₂ (1.1 equiv), NaCNBH₃ (4.2 equiv), trimethylorthoformate/MeOH (1/2 v/v), AcOH, 3 Å mol. sieves, 16 h, **8a**, 22% and **8b**, 22%.

The unsuccessful attempts to suppress or circumvent epimerisation during the glycol cleavage and ensuing reductive amination, together with the moderate yield and laborious separation, prompted us to select a sequence of reactions that excludes an intermediate β -keto ester. To this end, 2,5-anhydroglucitol **9** (Scheme 3) was prepared following a route described previously by Timmer *et al.*, which involves the acidic dehydration of D-(+)-mannitol, acetonation of the 1,3-*cis*-diol system and consecutive introduction of the primary azide.¹⁷ Acid-catalyzed methanolysis of the isopropylidene group produced triol **10**, which was subjected to glycol cleavage and reductive insertion of benzylamine to give morpholine **11** in 53%. Finally, oxidation of the primary hydroxyl in **11** was accomplished using the 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) / (bisacetoxyiodo)benzene (BAIB) system.¹⁸

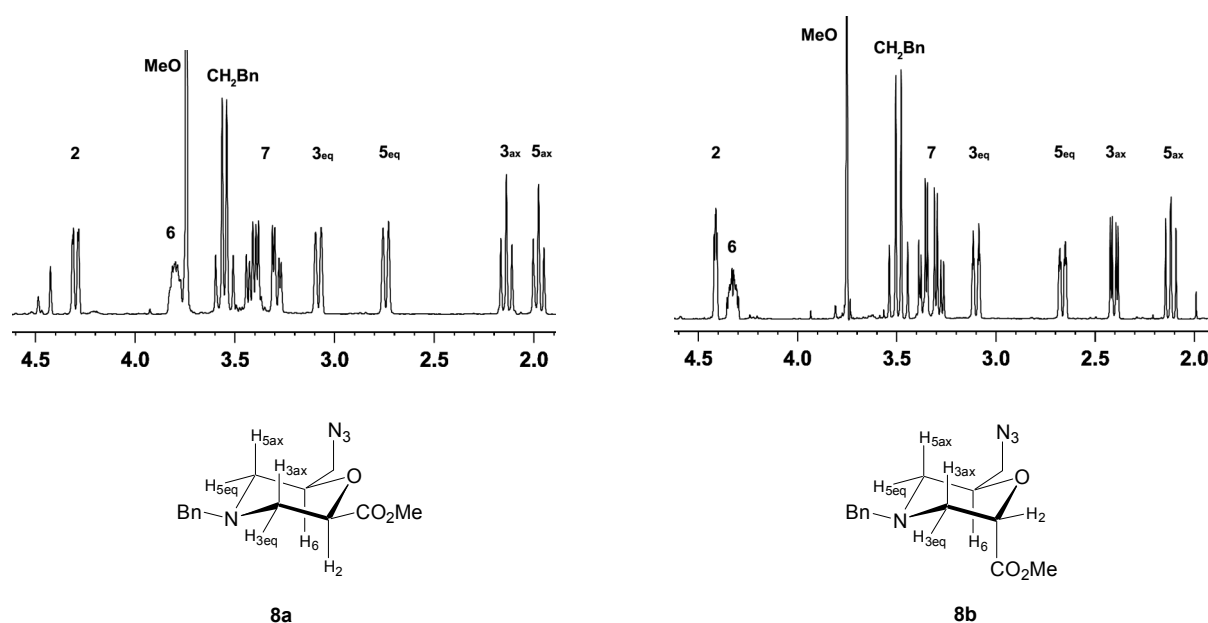
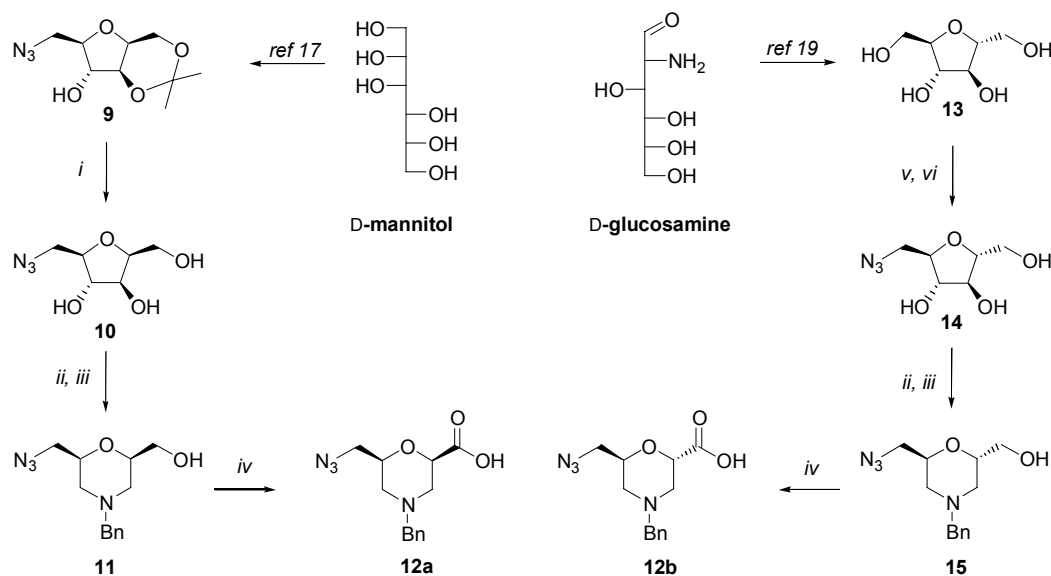


Figure 1: Parts of the ¹H NMR spectra of **8a** and **8b** (400 MHz, CDCl₃).

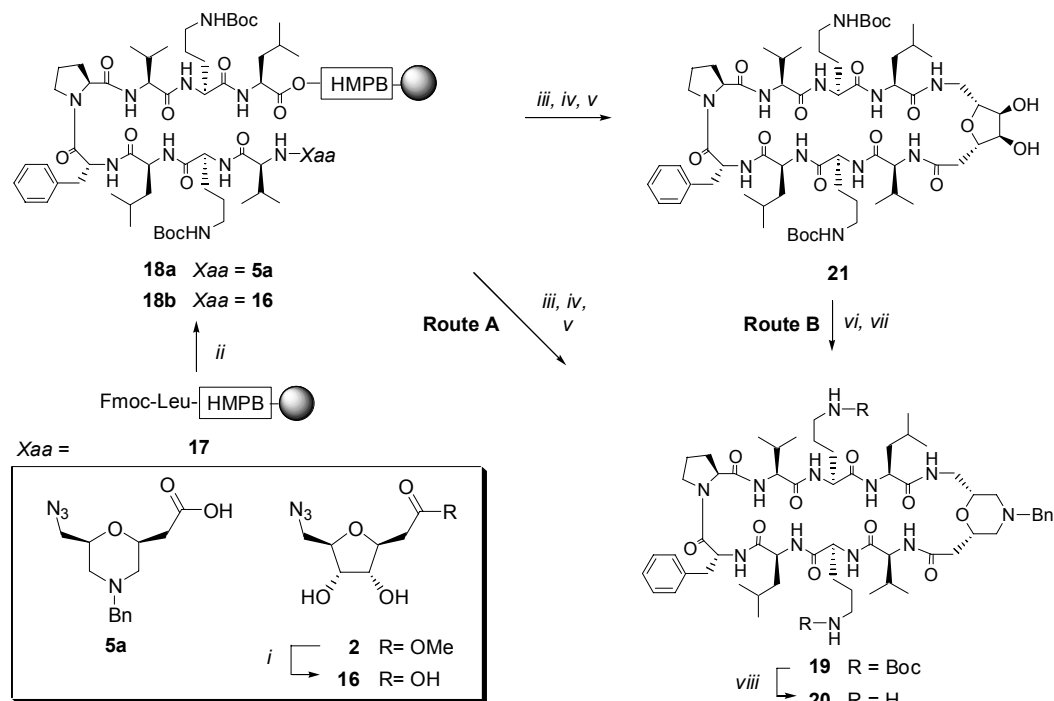
Gratifyingly, this mild procedure proved effective for the transformation of **11** into δ -MAA **12a**, without affecting the nitrogen of the morpholine, in 61% yield. The C₂-epimer of **12a** was constructed from 2,5-anhydromannitol **14**, that was obtained from D-(+)-glucosamine by nitrous deamination and NaBH₄-reduction, as described by Cassel *et al.*¹⁹ Ensuing selective mesylation of the resulting anhydromannitol **13**²⁰ and nucleophilic substitution with sodium azide, furnished **14** in 45% over 2 steps. Periodic acid-mediated ring-opening and reductive amination gave morpholine **15** (52%) that was subjected to TEMPO/BAIB-oxidation, to provide δ -MAA **12b** in 68%.



Scheme 3: Reagents and conditions: (i) TFA/MeOH (1/3 v/v), 1 h, quant; (ii) H₅IO₆, THF, 30 min; (iii) benzylamine (1.1 equiv), NaCNBH₃ (4.2 equiv), trimethylorthoformate/MeOH (1/3 v/v), AcOH, 3 Å mol. sieves, 16 h, **11**, 53% and **15**, 52% (2 steps); (iv) TEMPO (0.2 equiv), BAIB (2 equiv), DCM, 0°C, 6 h, **12a**, 61% and **12b**, 68%; (v) MsCl (1.0 equiv), pyridine, -40°C, 1 h to 0°C, 16 h; (vi) NaN₃ (2.5 equiv), DMF, 70°C, 48 h, 45% (two steps).

At this stage, the application of MAAs as peptidomimetic compounds was explored and ϵ -MAA **5a** was selected for incorporation in GS. Nonapeptide **18a** (Scheme 4) was assembled on HMPB-functionalized MBHA-resin **17** using standard Fmoc-based SPPS protocols. The terminal azide in **18a** was subjected to Staudinger reduction and the peptide was released from the solid support by acidolysis and subsequently cyclized under highly dilute conditions to give fully protected **19** in 71% (Route A).⁷ Liberation of the Boc protective groups, followed by HPLC purification produced peptide **20** in 77%, which was characterized by ¹H NMR to reveal that the peptide prevalently adopts a β -sheet secondary structure reminiscent of the native peptide.²¹ Encouraged by these results, it was decided to examine whether MAA-containing peptidic constructs are also accessible through the glycol cleavage/reductive amination-strategy when applied to SAAs that are already embedded in oligopeptide sequences. Thus, saponification of **2** gave SAA **16** in 98%. Following the sequence of

reactions as described above for compound **19**, resin-anchored nonapeptide **18b** was constructed through SPPS, from which GS analogue **21** was readily prepared in 63%.²² Treatment of the *cis*-diol-containing peptide **21** with NaIO₄ and reductive amination furnished **19** in 63% (Route B), which was deprotected to produce **20**. The MAA-containing GS analogue **20** obtained from both routes were spectroscopically and spectrometrically identical.



Scheme 4: Reagents and conditions: (i) 1 M NaOH/THF (1/2 v/v), 3 h, then Amberlite IR-120 (H⁺), 98%; (ii) Repetitive deprotection: piperidine/NMP (1/4 v/v); condensation: Fmoc-aa-OH (3 equiv) or N₃-Xaa-OH (2 equiv), BOP (3 equiv), HOBT (3 equiv), DiPEA (3.5 equiv), NMP; (iii) PMe₃ (16 equiv), 1,4-dioxane/H₂O (10/1 v/v); (iv) TFA/DCM (1/99 v/v), 4 × 10 min; (v) PyBOP (5 equiv), HOBT (5 equiv), DiPEA (15 equiv), DMF, 16 h, **19**, 71% and **21**, 63%; (vi) NaIO₄ (2 equiv), THF/DMF/H₂O (3/1/1 v/v/v), 16 h; (vii) benzylamine (1.5 equiv), NaCNBH₃ (5 equiv), trimethylorthoformate/MeOH (1/2 v/v), AcOH, 16 h, 63% (2 steps); (viii) TFA / DCM (1/1 v/v), 30 min, 77%.

Conclusion

ϵ -Morpholine amino acids, bearing different substituents on the nitrogen of the morpholine core structure, were synthesized from furanoid ϵ -SAAs *via* a two-step oxidative glycol cleavage/reductive amination approach. Diastereoisomeric mixtures of δ -MAAs were obtained when the corresponding furanoid δ -SAAs were subjected to the same sequence of events. In order to prevent epimerisation during oxidative ring-opening, an alternative route was developed, through which 2,5-anhydroglucitol and 2,5-anhydromannitol were readily transformed into their diastereoisomerically pure δ -MAA counterparts. It was further demonstrated that ϵ -MAA-containing GS analogue **19** could be obtained in two ways; by directly employing **5a** as building block or by first preparing GS analogue **21**, featuring ϵ -SAA **16**, which is subsequently subjected to our ring-opening / ring-closing approach.

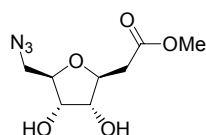
Experimental Section

General: Reactions were monitored by TLC-analysis using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by spraying with 20% H₂SO₄ in EtOH, (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid or by spraying with a solution of ninhydrin (3 g/L) in EtOH / AcOH (20/1 v/v), followed by charring at ~150°C. Column chromatography was performed on Fluka silicagel (0.04 – 0.063 mm) and size exclusion chromatography on Sephadex™ LH-20. For LC/MS analysis, a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical Alltima C₁₈ column (Alltech, 4.6 mmD × 250 mmL, 5 μ particle size) was used in combination with buffers A: H₂O, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI). For reversed-phase HPLC purification of the peptides, a BioCAD “Vision” automated HPLC system (PerSeptive Biosystems, inc.) equipped with a semi-preparative Alltima C₁₈ column (Alltech, 10.0 mmD × 250 mmL, 5 μ particle size) was used. The applied buffers were A: H₂O, B: MeCN and C: 1.0% aq. TFA.

Glycol Cleavage: Diol **2** (1.83 g, 7.90 mmol) was dissolved in THF (30 mL) and H₅IO₆ (2.74 g, 12 mmol, 1.5 equiv) was added. After continued stirring for 20 min, the solvents were removed *in vacuo* and the mixture partitioned between water and EtOAc. The organic layer was dried (MgSO₄), filtered and concentrated, to furnish dialdehyde **3** (1.84 g, 7.46 mmol, 94%) that was used without further purification.

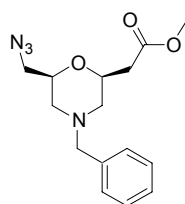
Reductive Amination: Crude **3** (988 mg, 4.0 mmol) was dissolved in MeOH (15 mL) and trimethylorthoformate (5 mL). To the stirred mixture were added activated molecular sieves (3 Å, ~0.5 g) and NaCNBH₃ (1.06 g, 16.8 mmol, 4.2 equiv). Subsequently, a mixture of benzylamine (481 μL, 4.4 mmol, 1.1 equiv) in MeOH (6 mL) and trimethylorthoformate (2 mL) that had been acidified to pH = 5 with AcOH, was added dropwise over 1 h. The reaction was stirred overnight, filtered through a pad of Celite and partitioned between water and EtOAc. The organic layer was dried (MgSO₄), filtered and concentrated. Silica gel column chromatography (15%→25% EtOAc in light PE) delivered morpholine **4a** as a clear oil (668 mg, 2.2 mmol, 54%). Compounds **4b-h** were obtained in a similar fashion from **3**, using a solution of the appropriate amine in MeOH and trimethylorthoformate that had been acidified to pH = 5 with AcOH.

Saponification: Methyl ester **4a** (207 mg, 0.68 mmol) was dissolved in THF (8 mL), before 1 M aq. NaOH (1.4 mL, 2 equiv) was added and the reaction was stirred 4 h. Subsequently, Amberlite IR-120 (H⁺) was added and the slightly acidic mixture was filtered and concentrated to quantitatively obtain carboxylic acid **5a** (102 mg, 0.35 mmol) as clear oil. Compounds **5b-h** were obtained in a similar fashion from their corresponding methyl esters.

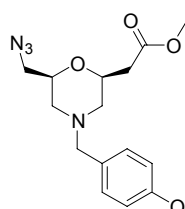


Methyl 3,6-anhydro-7-azido-2,7-dideoxy-D-allo-heptonate (2): To a mixture of methyl ester **1** (5.03 g, 18.56 mmol) in MeOH (75 mL) was added 2 M aq. HCl (25 mL) and the solution was stirred overnight. After neutralizing with 1 M aq. NaOH (50 mL), the mixture was partially concentrated and extracted with EtOAc (3×). The combined organic layers were dried (MgSO₄), filtered and concentrated. Silica gel column chromatography (50%→100% EtOAc in light PE) yielded the title compound as a clear oil (3.52 g, 15.25 mmol, 82%). ¹H NMR (400 MHz, CDCl₃): δ = 4.15 (ddd, 1H, H₃, J_{3,2a} = J_{3,2b} = 6.5 Hz, J_{3,4} = 6.3 Hz), 4.06 (dd, 1H, H₅, J_{5,6} = 5.4 Hz, J_{5,4} = 6.3 Hz), 4.00 (dd, 1H, H₆, J_{6,7} = 4.3 Hz, J_{6,5} = 5.4 Hz), 3.95 (dd, 1H, H₄, J_{4,5} = J_{4,3} = 6.3 Hz), 3.57 (dd, 1H, H_{7a}, J_{7a,6} = 3.4 Hz, J_{7a,7b} = 13.3 Hz), 3.31 (dd, 1H, H_{7b}, J_{7b,6} = 4.3 Hz, J_{7b,7a} = 13.3 Hz), 2.77 (dd, 1H, H_{2a}, J_{2a,3} = 6.5 Hz, J_{2a,2b} = 16.3 Hz), 2.69 (dd, 1H, H_{2b},

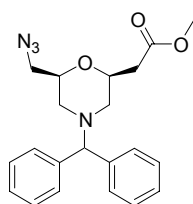
$J_{2b,3} = 6.5$ Hz, $J_{2b,2a} = 16.3$ Hz). ^{13}C NMR (100 MHz, CDCl_3): $\delta = 172.3$ (C_1), 82.8 (C_6), 79.1 (C_3), 74.6 (C_4), 72.1 (C_5), 52.1 (C_7), 52.0 (OMe), 37.9 (C_2). ATR-IR (thin film): 3396.4, 2956.2, 2098.4, 1728.1, 1438.8, 1400.2, 1274.9, 1172.6, 1097.4, 1037.6, 987.5, 910.3, 850.5, 829.3, 731.0 cm^{-1} . $[\alpha]_{\text{D}}^{23} = +80.4$ ($c = 1.0$, CH_2Cl_2). MS (ESI): $m/z = 232.1$ $[\text{M}+\text{H}]^+$, 253.8 $[\text{M}+\text{Na}]^+$, 463.0 $[2\text{M}+\text{H}]^+$.



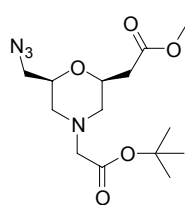
Methyl 3,7-anhydro-5-aza-8-azido-5-benzyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4a): Clear oil (668 mg, 2.2 mmol, 54%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.29$ (m, 5H, H_{ar}), 4.07 (m, 1H, H_3), 3.82 (m, 1H, H_7), 3.67 (s, 3H, OMe), 3.54 (d, 1H, CH_2Bn , $J_{\text{CHd,CHu}} = 13.1$ Hz), 3.47 (d, 1H, CH_2Bn , $J_{\text{CHu,CHd}} = 13.1$ Hz), 3.24 (dd, 1H, H_{8a} , $J_{8a,7} = 6.6$ Hz, $J_{8a,8b} = 12.9$ Hz), 3.10 (dd, 1H, H_{8b} , $J_{8b,7} = 3.9$ Hz, $J_{8b,8a} = 12.9$ Hz), 2.81 (ddd, 1H, $\text{H}_{4\text{eq}}$, $J_{4\text{eq},6\text{eq}} = J_{4\text{eq},3} = 1.9$ Hz, $J_{4\text{eq},4\text{ax}} = 10.9$ Hz), 2.67 (ddd, 1H, $\text{H}_{6\text{eq}}$, $J_{6\text{eq},4\text{eq}} = J_{6\text{eq},7} = 2.0$ Hz, $J_{6\text{eq},6\text{ax}} = 10.9$ Hz), 2.53 (dd, 1H, H_{2a} , $J_{2a,3} = 7.7$ Hz, $J_{2a,2b} = 15.3$ Hz), 2.38 (dd, 1H, H_{2b} , $J_{2b,3} = 5.3$ Hz, $J_{2a,2b} = 15.3$ Hz), 1.88 (dd, 1H, $\text{H}_{6\text{ax}}$, $J_{6\text{ax},6\text{eq}} = J_{6\text{ax},7} = 10.7$ Hz), 1.87 (dd, 1H, $\text{H}_{4\text{ax}}$, $J_{4\text{ax},4\text{eq}} = J_{4\text{ax},3} = 10.7$ Hz). ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.0$ (C_1), 137.3 (C_qBn), 129.0 128.3 128.1 127.2 (CH_{ar}), 75.2 (C_7), 72.5 (C_3), 62.8 (CH_2Bn), 57.2 (C_4), 54.6 (C_6), 52.7 (C_8), 51.7 (OMe), 38.6 (C_2). ATR-IR (thin film): 2094.6, 1735.8, 1454.2, 1436.9, 1348.1, 1330.8, 1288.4, 1251.7, 1213.1, 1168.8, 1149.5, 1110.9, 1056.9, 1028.0, 999.1, 956.6, 920.0, 742.5, 700.1 cm^{-1} . $[\alpha]_{\text{D}}^{23} = -7.4$ ($c = 1.0$, CH_2Cl_2). MS (ESI): $m/z = 305.0$ $[\text{M}+\text{H}]^+$, 327.1 $[\text{M}+\text{Na}]^+$.



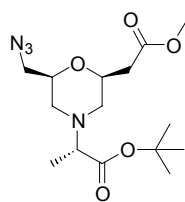
Methyl 3,7-anhydro-5-aza-8-azido-5-p-methoxybenzyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4b): Clear oil (358 mg, 1.12 mmol, 36%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.21$ (d, 2H, H_{ar} , $J = 8.6$ Hz), 6.85 (d, 2H, H_{ar} , $J = 8.6$ Hz), 4.06 (m, 1H, H_3), 3.79 (m, 4H, H_7 , Me PMB), 3.67 (s, 3H, COOMe), 3.50 (d, 1H, CH_2PMB , $J_{\text{CHd,CHu}} = 12.8$ Hz), 3.40 (d, 1H, CH_2PMB , $J_{\text{CHu,CHd}} = 12.8$ Hz), 3.25 (dd, 1H, H_{8a} , $J_{8a,7} = 6.8$ Hz, $J_{8a,8b} = 12.8$ Hz), 3.10 (dd, 1H, H_{8b} , $J_{8b,7} = 3.8$ Hz, $J_{8b,8a} = 12.8$ Hz), 2.80 (ddd, 1H, $\text{H}_{4\text{eq}}$, $J_{4\text{eq},6\text{eq}} = J_{4\text{eq},3} = 1.8$ Hz, $J_{4\text{eq},4\text{ax}} = 11.1$ Hz), 2.66 (ddd, 1H, $\text{H}_{6\text{eq}}$, $J_{6\text{eq},4\text{eq}} = J_{6\text{eq},7} = 1.8$ Hz, $J_{6\text{eq},6\text{ax}} = 11.1$ Hz), 2.53 (dd, 1H, H_{2a} , $J_{2a,3} = 7.8$ Hz, $J_{2a,2b} = 15.4$ Hz), 2.39 (dd, 1H, H_{2b} , $J_{2b,3} = 5.3$ Hz, $J_{2a,2b} = 15.4$ Hz), 1.85 (dd, 1H, $\text{H}_{6\text{ax}}$, $J_{6\text{ax},6\text{eq}} = J_{6\text{ax},7} = 10.8$ Hz), 1.84 (dd, 1H, $\text{H}_{4\text{ax}}$, $J_{4\text{ax},4\text{eq}} = J_{4\text{ax},3} = 10.8$ Hz). ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.0$ (C_1), 158.7 (C_qPMB), 130.1 (CH_{ar}), 129.2 (C_qPMB), 113.6 (CH_{ar}), 75.1 (C_7), 72.5 (C_3), 62.1 (CH_2PMB), 57.1 (C_4), 55.1 (Me PMB), 54.5 (C_6), 52.7 (C_8), 51.7 (OMe), 38.6 (C_2). ATR-IR (thin film): 2094.6, 1735.8, 1510.2, 1436.9, 1346.2, 1244.0, 1170.7, 1109.0, 1056.9, 1033.8, 817.8 cm^{-1} . $[\alpha]_{\text{D}}^{23} = -5.0$ ($c = 1.0$, CHCl_3). MS (ESI): $m/z = 355.2$ $[\text{M}+\text{H}]^+$, 357.0 $[\text{M}+\text{Na}]^+$.



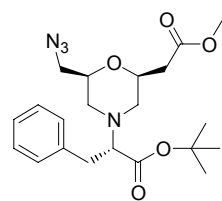
Methyl 3,7-anhydro-5-aza-8-azido-5-benzhydryl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4c): Clear oil (95 mg, 0.25 mmol, 38%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.27$ (m, 10H, H_{ar}), 4.22 (s, 1H, HCPH_2), 4.13 (m, 1H, H_3), 3.87 (m, 1H, H_7), 3.63 (s, 3H, OMe), 3.16 (dd, 1H, H_{8a} , $J_{8a,7} = 6.8$ Hz, $J_{8a,8b} = 13.0$ Hz), 3.01 (dd, 1H, H_{8b} , $J_{8b,7} = 3.5$ Hz, $J_{8b,8a} = 13.0$ Hz), 2.79 (ddd, 1H, $\text{H}_{4\text{eq}}$, $J_{4\text{eq},6\text{eq}} = J_{4\text{eq},3} = 2.0$ Hz, $J_{4\text{eq},4\text{ax}} = 11.3$ Hz), 2.66 (ddd, 1H, $\text{H}_{6\text{eq}}$, $J_{6\text{eq},4\text{eq}} = J_{6\text{eq},7} = 2.0$ Hz, $J_{6\text{eq},6\text{ax}} = 11.3$ Hz), 2.46 (dd, 1H, H_{2a} , $J_{2a,3} = 8.4$ Hz, $J_{2a,2b} = 15.1$ Hz), 2.30 (dd, 1H, H_{2b} , $J_{2b,3} = 4.8$ Hz, $J_{2a,2b} = 15.1$ Hz), 1.79 (dd, 1H, $\text{H}_{6\text{ax}}$, $J_{6\text{ax},6\text{eq}} = J_{6\text{ax},7} = 11.1$ Hz), 1.74 (dd, 1H, $\text{H}_{4\text{ax}}$, $J_{4\text{ax},3} = 10.5$ Hz, $J_{4\text{ax},4\text{eq}} = 11.1$ Hz). ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.0$ (C_1), 141.7 (C_qPh), 128.6, 127.8, 127.1 (CH_{ar}), 76.0 (CHPh_2), 75.4 (C_7), 72.7 (C_3), 55.9 (C_4), 53.5 (C_6), 52.7 (C_8), 51.7 (OMe), 38.6 (C_2). ATR-IR (thin film): 2094.6, 1735.8, 1490.9, 1450.4, 1436.9, 1282.6, 1251.7, 1168.8, 1110.9, 1055.0, 1028.0, 763.8, 746.4, 705.9 cm^{-1} . $[\alpha]_{\text{D}}^{23} = -2.4$ ($c = 1.0$, CHCl_3). MS (ESI): $m/z = 381.1$ $[\text{M}+\text{H}]^+$, 403.1 $[\text{M}+\text{Na}]^+$.



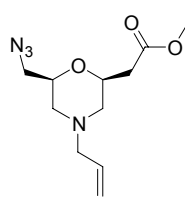
Methyl 3,7-anhydro-5-aza-8-azido-5-(*tert*butylglyciny)-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4d): Clear oil (87 mg, 0.266 mmol, 59%). ^1H NMR (400 MHz, CDCl_3): δ = 4.11 (m, 1H, H_3), 3.87 (m, 1H, H_7), 3.69 (s, 3H, OMe), 3.28 (dd, 1H, H_{8a} , $J_{7,8a}$ = 6.5 Hz, $J_{8a,8b}$ = 12.9 Hz), 3.17 (dd, 1H, H_{8b} , $J_{7,8b}$ = 4.1 Hz, $J_{8a,8b}$ = 12.9 Hz), 3.14 (m, 2H, H_α Gly), 2.88 (ddd, 1H, H_{4eq} , $J_{4eq,6eq}$ = $J_{4eq,3}$ = 1.7 Hz, $J_{4eq,4ax}$ = 10.9 Hz), 2.80 (ddd, 1H, H_{6eq} , $J_{6eq,7}$ = $J_{6eq,4eq}$ = 1.7 Hz, $J_{6eq,6ax}$ = 10.9 Hz), 2.56 (dd, 1H, H_{2a} , $J_{2a,3}$ = 7.5 Hz, $J_{2a,2b}$ = 15.3 Hz), 2.43 (dd, 1H, H_{2b} , $J_{2b,3}$ = 5.6 Hz, $J_{2a,2b}$ = 15.3 Hz), 2.15 (dd, 1H, H_{6ax} , $J_{6ax,6eq}$ = $J_{6ax,7}$ = 10.9 Hz), 2.12 (dd, 1H, H_{4ax} , $J_{4ax,4eq}$ = $J_{4ax,3}$ = 10.9 Hz), 1.47 (s, 9H, *t*Bu). ^{13}C NMR (100 MHz, CDCl_3): δ = 170.8, 169.1 (C_1 , $\text{COO}t\text{Bu}$), 81.3 (C_q *t*Bu), 75.0 (C_7), 72.4 (C_3), 59.5 (C_α Gly), 56.5 (C_4), 54.1 (C_6), 52.6 (C_8), 51.7 (OMe), 38.5 (C_2), 28.0 (CH_3 *t*Bu). ATR-IR (thin film): 2098.4, 1735.8, 1442.7, 1365.5, 1218.9, 1149.5, 1064.6 cm^{-1} . $[\alpha]_D^{23}$ = -4.4 (c = 1.0, CH_2Cl_2). HRMS: calcd for $\text{C}_{14}\text{H}_{24}\text{N}_4\text{O}_5\text{H}$ 329.18195, found 329.18140.



Methyl 3,7-anhydro-5-aza-8-azido-5-(*tert*butyl-L-alaniny)-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4e): Clear oil (110 mg, 0.43 mmol, 33%). ^1H NMR (400 MHz, CDCl_3): δ = 4.01 (m, 1H, H_3), 3.81 (m, 1H, H_7), 3.69 (s, 3H, OMe), 3.29 (dd, 1H, H_{8a} , $J_{7,8a}$ = 6.6 Hz, $J_{8a,8b}$ = 12.9 Hz), 3.19 (q, 1H, H_α Ala, $J_{\alpha,\beta}$ = 7.1 Hz), 3.14 (dd, 1H, H_{8b} , $J_{7,8b}$ = 4.2 Hz, $J_{8a,8b}$ = 12.9 Hz), 2.85 (ddd, 1H, H_{4eq} , $J_{4eq,6eq}$ = $J_{4eq,3}$ = 2.0 Hz, $J_{4eq,4ax}$ = 10.9 Hz), 2.73 (ddd, 1H, H_{6eq} , $J_{6eq,7}$ = $J_{6eq,4eq}$ = 1.9 Hz, $J_{6eq,6ax}$ = 10.9 Hz), 2.56 (dd, 1H, H_{2a} , $J_{2a,3}$ = 7.6 Hz, $J_{2a,2b}$ = 15.4 Hz), 2.42 (dd, 1H, H_{2b} , $J_{2b,3}$ = 5.6 Hz, $J_{2a,2b}$ = 15.4 Hz), 2.33 (dd, 1H, H_{6ax} , $J_{6ax,6eq}$ = $J_{6ax,7}$ = 10.7 Hz), 2.21 (dd, 1H, H_{4ax} , $J_{4ax,4eq}$ = $J_{4ax,3}$ = 10.7 Hz), 1.47 (s, 9H, *t*Bu), 1.26 (d, 3H, H_β Ala, $J_{\alpha,\beta}$ = 7.1 Hz). ^{13}C NMR (100 MHz, CDCl_3): δ = 171.9, 171.0 (C_1 , $\text{COO}t\text{Bu}$), 81.1 (C_q *t*Bu), 75.3 (C_7), 72.9 (C_3), 62.7 (C_α Ala), 52.7 (C_6 , C_8), 51.7 (OMe), 51.6 (C_4), 38.5 (C_2), 28.0 (CH_3 *t*Bu), 14.5 (C_β Ala). ATR-IR (thin film): 2098.4, 1722.3, 1436.9, 1367.4, 1350.1, 1255.6, 1318.0, 1145.6, 1114.8, 1091.6, 1062.7, 1049.2, 993.3, 952.8, 881.4, 846.7 cm^{-1} . $[\alpha]_D^{23}$ = -23.2 (c = 1.0, CH_2Cl_2). MS (ESI): m/z = 343.1 [$\text{M}+\text{H}$] $^+$, 365.2 [$\text{M}+\text{Na}$] $^+$.

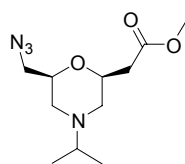


Methyl 3,7-anhydro-5-aza-8-azido-5-(*tert*butyl-L-phenylalaniny)-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4f): Clear oil (103 mg, 0.25 mmol, 45%). ^1H NMR (400 MHz, CDCl_3): δ = 7.23 (m, 5H, CH_{ar}), 3.98 (m, 1H, H_3), 3.76 (m, 1H, H_7), 3.70 (s, 3H, OMe), 3.33 (dd, 1H, H_α , $J_{\alpha,\beta}$ = 6.6 Hz, $J_{\alpha,\beta a}$ = 9.1 Hz), 3.27 (dd, 1H, H_{8a} , $J_{8a,7}$ = 6.8 Hz, $J_{8a,8b}$ = 13.0 Hz), 3.10 (dd, 1H, H_{8b} , $J_{8b,7}$ = 4.1 Hz, $J_{8b,8a}$ = 13.0 Hz), 3.00 (m, 2H, H_{4eq} , $\text{H}_{\beta d}$), 2.89 (dd, 1H, $\text{H}_{\beta u}$, $J_{\beta u,\alpha}$ = 6.6 Hz, $J_{\beta u,\beta d}$ = 13.4 Hz), 2.69 (ddd, 1H, H_{6eq} , $J_{6eq,4eq}$ = $J_{6eq,7}$ = 2.0 Hz, $J_{6eq,6ax}$ = 11.1 Hz), 2.57 (dd, 1H, H_{2a} , $J_{2a,3}$ = 7.7 Hz, $J_{2a,2b}$ = 15.4 Hz), 2.45 (dd, 1H, H_{2b} , $J_{2b,3}$ = 5.5 Hz, $J_{2b,2a}$ = 15.4 Hz), 2.40 (dd, 1H, H_{6ax} , $J_{6ax,6eq}$ = $J_{6ax,7}$ = 10.7 Hz), 1.81 (dd, 1H, H_{4ax} , $J_{4ax,4eq}$ = $J_{4ax,3}$ = 10.8 Hz), 1.35 (s, 9H, CH_3 *t*Bu). ^{13}C NMR (100 MHz, CDCl_3): δ = 171.0, 170.2 (C_1 , $\text{COO}t\text{Bu}$), 137.8 (C_q Ph), 129.6, 129.4, 129.2, 128.2, 126.3 (CH_{ar}), 81.4 (CH_q *t*Bu), 75.4 (C_7), 72.9 (C_3), 69.6 (CH_α), 53.9 (C_6), 52.5 (C_8), 51.7 (OMe), 51.2 (C_4), 38.4 (C_2), 35.3 (C_β), 28.1 (CH_3 *t*Bu). ATR-IR (thin film): 2098.4, 1422.3, 1367.4, 1288.4, 1253.6, 1145.6, 1112.9, 1064.6, 844.8, 742.5, 700.1 cm^{-1} . $[\alpha]_D^{23}$ = -13.6 (c = 1.0, CHCl_3). MS (ESI): m/z = 419.2 [$\text{M}+\text{H}$] $^+$, 441.2 [$\text{M}+\text{Na}$] $^+$. HRMS: calcd for $\text{C}_{21}\text{H}_{30}\text{N}_4\text{O}_5\text{H}$ 419.22890, found 419.22794.



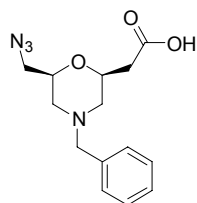
Methyl 3,7-anhydro-5-aza-8-azido-5-allyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4g): Clear oil (121 mg, 0.48 mmol, 41%). ^1H NMR (400 MHz, CDCl_3): δ = 5.82 (m, 1H, CH All), 5.19 (m, 2H, CH_2 All), 4.05 (m, 1H, H_3), 3.82 (m, 1H, H_7), 3.69 (s, 3H, OMe), 3.23 (dd, 1H, H_{8a} , $J_{8a,7}$ = 6.6 Hz, $J_{8a,8b}$ = 13.0 Hz), 3.11 (dd, 1H, H_{8b} , $J_{7,8b}$ = 4.1 Hz, $J_{8b,8a}$ = 13.0 Hz), 3.00 (m, 2H, CH_2 All), 2.86 (ddd, 1H, H_{4eq} , $J_{4eq,6eq}$ = $J_{4eq,3}$ = 1.9 Hz, $J_{4eq,4ax}$ = 11.2 Hz), 2.75 (ddd, 1H, H_{6eq} , $J_{6eq,4eq}$ = $J_{6eq,7}$ = 1.9 Hz, $J_{6eq,6ax}$ = 11.2 Hz), 2.56 (dd, 1H, H_{2a} , $J_{2a,3}$ = 7.7 Hz, $J_{2a,2b}$ = 15.4 Hz), 2.43 (dd, 1H, H_{2b} , $J_{2b,3}$ = 5.8 Hz, $J_{2b,2a}$ = 15.4 Hz), 1.85 (dd, 1H, H_{6ax} , $J_{6ax,6eq}$ = $J_{6ax,7}$ = 10.9 Hz), 1.81 (dd, 1H, H_{4ax} , $J_{4ax,4eq}$

= $J_{4ax,3}$ = 10.8 Hz). ^{13}C NMR (100 MHz, CDCl_3): δ = 171.0 (C_1), 134.2 (CH All), 118.6 (CH_2 All), 75.1 (C_7), 72.5 (C_3), 61.7 (CH_2 All), 57.2 (C_4), 54.7 (C_6), 52.8 (C_8), 51.8 (OMe), 38.7 (C_2). ATR-IR (thin film): 2098.4, 1735.8, 1434.9, 1342.4, 1288.4, 1172.6, 1110.9, 1064.6, 995.2, 925.8 cm^{-1} . $[\alpha]_{\text{D}}^{23}$ = -12.8 (c = 1.0, CH_2Cl_2). MS (ESI): m/z = 255.0 $[\text{M}+\text{H}]^+$. HRMS: calcd for $\text{C}_{11}\text{H}_{18}\text{N}_4\text{O}_3\text{H}$ 255.14517, found 255.14462.



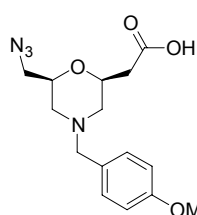
Methyl 3,7-anhydro-5-aza-8-azido-5-isopropyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonate (4h):

Clear oil (110 mg, 0.43 mmol, 33%). ^1H NMR (400 MHz, CDCl_3): δ = 3.98 (m, 1H, H_3), 3.75 (m, 1H, H_7), 3.63 (s, 3H, OMe), 3.23 (dd, 1H, H_{8a} , $J_{8a,7}$ = 6.4 Hz, $J_{8a,8b}$ = 12.9 Hz), 3.11 (dd, 1H, H_{8b} , $J_{7,8b}$ = 4.2 Hz, $J_{8a,8b}$ = 12.9 Hz), 2.75 (ddd, 1H, H_{4eq} , $J_{4eq,6eq}$ = $J_{4eq,3}$ = 1.9 Hz, $J_{4eq,4ax}$ = 11.1 Hz), 2.63 (m, 2H, H_{6eq} , CH *iPr*), 2.51 (dd, 1H, H_{2a} , $J_{2a,3}$ = 7.4 Hz, $J_{2a,2b}$ = 15.3 Hz), 2.37 (dd, 1H, H_{2b} , $J_{2b,3}$ = 5.7 Hz, $J_{2b,2a}$ = 15.3 Hz), 1.99 (dd, 1H, H_{6ax} , $J_{6ax,6eq}$ = $J_{6ax,7}$ = 10.7 Hz), 1.95 (dd, 1H, H_{4ax} , $J_{4ax,4eq}$ = $J_{4ax,3}$ = 10.7 Hz), 0.99 (s, 3H, CH_3 *iPr*), 0.97 (s, 3H, CH_3 *iPr*). ^{13}C NMR (100 MHz, CDCl_3): δ = 171.0 (C_1), 75.2 (C_7), 73.9 (C_3), 54.5 (CH *iPr*), 52.9 (C_4 , C_8), 51.8 (OMe), 50.4 (C_6), 38.7 (C_2), 18.1 (CH_3 *iPr*), 18.0 (CH_3 *iPr*). ATR-IR (thin film): 2098.4, 1737.7, 1436.9, 1350.1, 1257.5, 1168.8, 1109.0, 1060.8, 999.1 cm^{-1} . $[\alpha]_{\text{D}}^{23}$ = -14.6 (c = 1.0, CH_2Cl_2). MS (ESI): m/z 257.2 $[\text{M}+\text{H}]^+$, 279.0 $[\text{M}+\text{Na}]^+$.



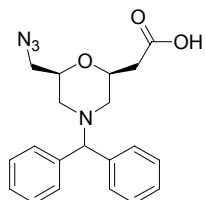
3,7-Anhydro-5-aza-8-azido-5-benzyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5a):

Clear oil (102 mg, 0.35 mmol, quant). ^1H NMR (400 MHz, MeOD): δ = 7.22 (m, 5H, H_{ar}), 3.93 (m, 1H, H_3), 3.67 (m, 1H, H_7), 3.45 (d, 1H, CH_2 Bn, $J_{\text{CHd},\text{CHu}}$ = 12.8 Hz), 3.39 (d, 1H, CH_2 Bn, $J_{\text{CHu},\text{CHd}}$ = 12.8 Hz), 3.13 (m, 2H, H_8), 2.84 (ddd, 1H, H_{4eq} , $J_{4eq,6eq}$ = $J_{4eq,3}$ = 2.0 Hz, $J_{4eq,4ax}$ = 11.3 Hz), 2.62 (ddd, 1H, H_{6eq} , $J_{6eq,4eq}$ = $J_{6eq,7}$ = 2.0 Hz, $J_{6eq,6ax}$ = 11.1 Hz), 2.35 (dd, 1H, H_{2a} , $J_{2a,3}$ = 6.3 Hz, $J_{2a,2b}$ = 14.4 Hz), 2.13 (dd, 1H, H_{2b} , $J_{2b,3}$ = 7.3 Hz, $J_{2a,2b}$ = 14.4 Hz), 1.79 (dd, 1H, H_{6ax} , $J_{6ax,6eq}$ = $J_{6ax,7}$ = 11.0 Hz), 1.74 (dd, 1H, H_{4ax} , $J_{4ax,3}$ = 10.3 Hz, $J_{4ax,4eq}$ = 11.3 Hz). ^{13}C NMR (100 MHz, MeOD): δ = 177.4 (C_1), 136.8 (C_q Bn), 129.0, 127.8, 126.9 (CH_{ar}), 74.4 (C_7), 73.7 (C_3), 62.6 (CH_2 Bn), 57.5 (C_4), 54.5 (C_6), 52.6 (C_8), 42.2 (C_2). ATR-IR (thin film): 3033.8, 2786.9, 2933.5, 2380.0, 2100.3, 1569.9, 1494.7, 1398.3, 1361.7, 1296.1, 1191.9, 1107.1, 1051.1, 1028.0, 925.8, 742.5, 698.2 cm^{-1} . $[\alpha]_{\text{D}}^{23}$ = +16.0 (c = 1.0, MeOH). MS (ESI): m/z = 290.8 $[\text{M}+\text{H}]^+$, 313.0 $[\text{M}+\text{Na}]^+$.



3,7-Anhydro-5-aza-8-azido-5-p-methoxybenzyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5b):

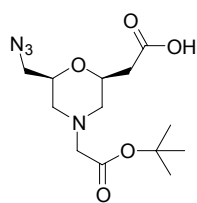
Clear oil (117g, 0.37 mmol, 94%). ^1H NMR (400 MHz, MeOD): δ = 7.14 (d, 2H, H_{ar} , J = 8.5 Hz), 6.76 (d, 2H, H_{ar} , J = 8.5 Hz), 3.92 (m, 1H, H_3), 3.67 (m, 1H, H_7), 3.64 (s, 3H, OMe), 3.48 (m, 2H, CH_2 PMB), 3.15 (m, 2H, H_8), 2.86 (ddd, 1H, H_{4eq} , $J_{4eq,3}$ = 1.5 Hz, $J_{4eq,6eq}$ = 2.0 Hz, $J_{4eq,4ax}$ = 11.4 Hz), 2.69 (ddd, 1H, H_{6eq} , $J_{6eq,4eq}$ = $J_{6eq,7}$ = 2.0 Hz, $J_{6eq,6ax}$ = 11.4 Hz), 2.33 (dd, 1H, H_{2a} , $J_{2a,3}$ = 7.0 Hz, $J_{2a,2b}$ = 15.0 Hz), 2.21 (dd, 1H, H_{2b} , $J_{2b,3}$ = 6.3 Hz, $J_{2a,2b}$ = 15.0 Hz), 1.93 (dd, 1H, H_{6ax} , $J_{6ax,6eq}$ = $J_{6ax,7}$ = 11.4 Hz), 1.87 (dd, 1H, H_{4ax} , $J_{4ax,4eq}$ = $J_{4ax,3}$ = 11.4 Hz). ^{13}C NMR (100 MHz, MeOD): δ = 176.3 (C_1), 160.9 (C_q PMB), 132.2 (CH_{ar}), 128.5 (C_q PMB), 114.9 (CH_{ar}), 75.7 (C_7), 74.0 (C_3), 63.0 (CH_2 PMB), 57.8 (C_4), 55.7 (Me PMB), 55.2 (C_6), 53.9 (C_8), 41.4 (C_2). ATR-IR (thin film): 2098.4, 1705.0, 1612.4, 1512.1, 1404.1, 1242.1, 1180.4, 1110.9, 1033.8, 817.8, 732.9 cm^{-1} . $[\alpha]_{\text{D}}^{23}$ = +20.0 (c = 1.0, CHCl_3). MS (ESI): m/z = 321.1 $[\text{M}+\text{H}]^+$, 343.0 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{15}\text{H}_{20}\text{N}_4\text{O}_4\text{H}$ 321.15573, found 321.15512.



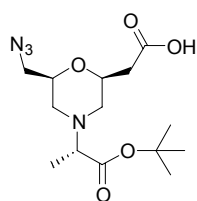
3,7-Anhydro-5-aza-8-azido-5-benzhydryl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5c):

White solid (109 mg, 0.30 mmol, quant). ^1H NMR (400 MHz, MeOD): δ = 7.17 (m, 10H, H_{ar}), 4.08 (s, 1H, HCPH_2), 3.95 (m, 1H, H_3), 3.64 (m, 1H, H_7), 2.92 (m, 2H, H_8), 2.72 (ddd, 1H, H_{4eq} , $J_{4eq,6eq}$ = $J_{4eq,3}$ = 1.9 Hz, $J_{4eq,4ax}$ = 11.2 Hz), 2.54 (ddd, 1H, H_{6eq} , $J_{6eq,4eq}$ = $J_{6eq,7}$ = 1.9 Hz, $J_{6eq,6ax}$ = 11.0 Hz), 2.23

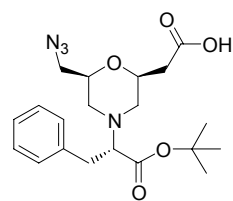
(dd, 1H, H_{2a} , $J_{2a,3} = 7.2$ Hz, $J_{2a,2b} = 15.2$ Hz), 2.04 (dd, 1H, H_{2b} , $J_{2b,3} = 5.8$ Hz, $J_{2a,2b} = 15.2$ Hz), 1.61 (dd, 1H, H_{6ax} , $J_{6ax,6eq} = J_{6ax,7} = 11.0$ Hz), 1.56 (dd, 1H, H_{4ax} , $J_{4ax,3} = 10.5$ Hz, $J_{4ax,4eq} = 11.2$ Hz). ^{13}C NMR (100 MHz, MeOD): $\delta = 177.5$ (C_1), 143.5 (C_q Ph), 129.6, 129.0, 128.1 (CH_{ar}), 77.6 (CH Ph_2), 76.2 (C_7), 74.8 (C_3), 57.7 (C_4), 55.1 (C_6), 54.0 (C_8), 42.0 (C_2). ATR-IR (thin film): 2098.4, 1712.7, 1581.5, 1450.4, 1265.2, 1110.9, 1056.9, 933.5, 732.9, 702.0 cm^{-1} . $[\alpha]_D^{23} = +23.8$ ($c = 1.0$, CHCl_3). MS (ESI): $m/z = 367.2$ $[\text{M}+\text{H}]^+$, 389.3 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3\text{H}$ 367.17647, found 367.17575.



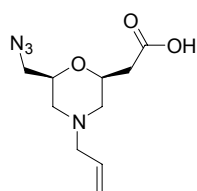
3,7-Anhydro-5-aza-8-azido-5-(tertbutyl-glyciny)-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5d): Clear oil (61 mg, 0.19 mmol, quant). ^1H NMR (400 MHz, CDCl_3): $\delta = 4.04$ (m, 1H, H_3), 3.83 (m, 1H, H_7), 3.22 (dd, 1H, H_{8a} , $J_{8,7} = 6.1$ Hz, $J_{8a,8b} = 12.9$ Hz), 3.16 (dd, 1H, H_{8b} , $J_{8b,7} = 4.3$ Hz, $J_{8b,8a} = 12.9$ Hz), 3.15 (d, 1H, H_α Gly, $J_{\alpha a, \alpha b} = 16.7$ Hz), 3.09 (d, 1H, H_α Gly, $J_{\alpha b, \alpha a} = 16.7$ Hz), 2.90 (ddd, 1H, H_{4eq} , $J_{4eq,6eq} = J_{4eq,3} = 1.8$ Hz, $J_{4eq,4ax} = 11.0$ Hz), 2.82 (ddd, 1H, H_{6eq} , $J_{6eq,7} = J_{6eq,4eq} = 1.8$ Hz, $J_{6eq,6ax} = 11.0$ Hz), 2.50 (dd, 1H, H_{2a} , $J_{2a,3} = 7.1$ Hz, $J_{2a,2b} = 15.5$ Hz), 2.36 (dd, 1H, H_{2b} , $J_{2b,3} = 6.0$ Hz, $J_{2a,2b} = 15.5$ Hz), 2.17 (dd, 1H, H_{6ax} , $J_{6ax,6eq} = J_{6ax,7} = 11.0$ Hz), 2.12 (dd, 1H, H_{4ax} , $J_{4ax,3} = J_{4ax,4eq} = 11.0$ Hz), 1.39 (s, 9H, $t\text{Bu}$). ^{13}C NMR (100 MHz, CDCl_3): $\delta = 174.4$ (C_1), 168.7 ($\text{COO}t\text{Bu}$), 81.7 (C_q $t\text{Bu}$), 74.5 (C_7), 72.0 (C_3), 59.0 (C_α Gly), 56.0 (C_4), 53.7 (C_6), 52.6 (C_8), 38.7 (C_2), 28.0 (CH_3 $t\text{Bu}$). ATR-IR (thin film): 2977.9, 2098.4, 1728.1, 1367.4, 1288.4, 1222.8, 1149.5, 1110.9, 1058.8, 914.2, 842.8, 731.0 cm^{-1} . $[\alpha]_D^{23} = +7.8$ ($c = 1.0$, CHCl_3). MS (ESI): $m/z = 315.0$ $[\text{M}+\text{H}]^+$, 337.3 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{13}\text{H}_{23}\text{N}_4\text{O}_5\text{H}$ 315.16630, found 315.15637.



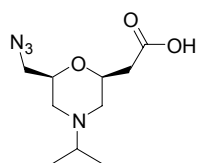
3,7-Anhydro-5-aza-8-azido-5-(tertbutyl-L-alaniny)-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5e): White solid (57 mg, 0.17 mmol, 78%). ^1H NMR (400 MHz, MeOD): $\delta = 3.86$ (m, 1H, H_3), 3.67 (m, 1H, H_7), 3.17 (d, 2H, H_8 , $J_{8,7} = 5.0$ Hz), 3.19 (q, 1H, H_α Ala, $J_{\alpha\beta} = 7.1$ Hz), 2.84 (ddd, 1H, H_{4eq} , $J_{4eq,6eq} = J_{4eq,3} = 2.0$ Hz, $J_{4eq,4ax} = 11.3$ Hz), 2.68 (ddd, 1H, H_{6eq} , $J_{6eq,7} = J_{6eq,4eq} = 2.0$ Hz, $J_{6eq,6ax} = 11.0$ Hz), 2.36 (dd, 1H, H_{2a} , $J_{2a,3} = 6.8$ Hz, $J_{2a,2b} = 15.1$ Hz), 2.24 (dd, 1H, H_{2b} , $J_{2b,3} = 6.6$ Hz, $J_{2a,2b} = 15.1$ Hz), 2.21 (dd, 1H, H_{6ax} , $J_{6ax,6eq} = J_{6ax,7} = 10.9$ Hz), 2.04 (dd, 1H, H_{4ax} , $J_{4ax,3} = 10.3$ Hz, $J_{4ax,4eq} = 11.3$ Hz), 1.41 (s, 9H, $t\text{Bu}$), 1.26 (d, 3H, H_β Ala, $J_{\beta\alpha} = 7.1$ Hz). ^{13}C NMR (100 MHz, MeOD): $\delta = 175.2$ (C_1), 172.1 ($\text{COO}t\text{Bu}$), 80.9 (C_q $t\text{Bu}$), 74.8 (C_7), 73.4 (C_3), 62.9 (C_α Ala), 52.5, 52.2, 52.1 (C_4 , C_6 , C_8), 40.1 (C_2), 26.9 (CH_3 $t\text{Bu}$), 13.4 (C_β Ala). ATR-IR (thin film): 2098.4, 1722.3, 1581.5, 1367.4, 1255.6, 1218.9, 1145.6, 1089.7, 1031.8, 991.3, 846.7 cm^{-1} . $[\alpha]_D^{23} = -3.6$ ($c = 1.0$, MeOH). MS (ESI): $m/z = 243.0$ $[\text{M}+\text{H}]^+$.



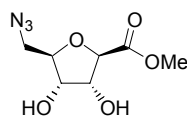
3,7-Anhydro-5-aza-8-azido-5-(tertbutyl-L-phenylalaniny)-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5f): White solid (55 mg, 0.14 mmol, 45%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.20$ (m, 5H, CH_{ar}), 4.00 (m, 1H, H_3), 3.79 (m, 1H, H_7), 3.38 (dd, 1H, H_α , $J_{\alpha\beta} = 6.1$ Hz, $J_{\alpha,\beta a} = 9.4$ Hz), 3.28 (dd, 1H, H_{8a} , $J_{8a,7} = 6.3$ Hz, $J_{8a,8b} = 12.9$ Hz), 3.15 (dd, 1H, H_{8b} , $J_{8b,7} = 4.3$ Hz, $J_{8b,8a} = 12.9$ Hz), 3.01 (m, 2H, H_{4eq} , $H_{\beta a}$), 2.91 (dd, 1H, $H_{\beta u}$, $J_{\beta u, \alpha} = 6.1$ Hz, $J_{\beta u, \beta d} = 13.3$ Hz), 2.75 (ddd, 1H, H_{6eq} , $J_{6eq,4eq} = J_{6eq,7} = 1.8$ Hz, $J_{6eq,6ax} = 11.1$ Hz), 2.60 (dd, 1H, H_{2a} , $J_{2a,3} = 7.7$ Hz, $J_{2a,2b} = 15.7$ Hz), 2.50 (dd, 1H, H_{2b} , $J_{2b,3} = 5.7$ Hz, $J_{2b,2a} = 15.7$ Hz), 2.41 (dd, 1H, H_{6ax} , $J_{6ax,6eq} = J_{6ax,7} = 10.9$ Hz), 2.33 (dd, 1H, H_{4ax} , $J_{4ax,4eq} = J_{4ax,3} = 10.9$ Hz), 1.34 (s, 9H, CH_3 $t\text{Bu}$). ^{13}C NMR (100 MHz, CDCl_3): $\delta = 175.6$ (C_1), 170.2 ($\text{COO}t\text{Bu}$), 137.6 (C_q Ph), 129.3, 128.2, 126.4 (CH_{ar}), 81.6 (CH_q $t\text{Bu}$), 75.2 (C_7), 72.6 (C_3), 69.5 (CH_α), 53.6 (C_6), 52.6 (C_8), 51.1 (C_4), 38.5 (C_2), 35.3 (C_β), 28.0 (CH_3 $t\text{Bu}$). ATR-IR (thin film): 2098.4, 1720.4, 1450.4, 1365.5, 1257.5, 1149.5, 840.9 cm^{-1} . $[\alpha]_D^{23} = -8.8$ ($c = 1.0$, CH_2Cl_2). HRMS: calcd for $\text{C}_{20}\text{H}_{29}\text{N}_4\text{O}_5\text{H}$ 405.21325, found 405.21136.



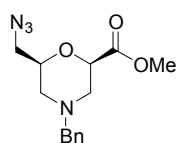
3,7-Anhydro-5-aza-8-azido-5-allyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5g): White solid (45 mg, 0.19 mmol, 78%). ^1H NMR (400 MHz, MeOD): δ = 5.81 (m, 1H, CH All), 5.24 (m, 2H, CH₂ All), 4.01 (m, 1H, H₃), 3.77 (m, 1H, H₇), 3.22 (m, 2H, H₈), 3.18 (m, 2H, CH₂ All), 3.05 (ddd, 1H, H_{4eq}, $J_{4eq,6eq}$ = $J_{4eq,3}$ = 1.7 Hz, $J_{4eq,4ax}$ = 11.6 Hz), 2.91 (ddd, 1H, H_{6eq}, $J_{6eq,4eq}$ = $J_{6eq,7}$ = 1.7 Hz, $J_{6eq,6ax}$ = 11.6 Hz), 2.41 (dd, 1H, H_{2a}, $J_{2a,3}$ = 7.0 Hz, $J_{2a,2b}$ = 15.5 Hz), 2.34 (dd, 1H, H_{2b}, $J_{2b,3}$ = 6.1 Hz, $J_{2b,2a}$ = 15.5 Hz), 2.10 (dd, 1H, H_{6ax}, $J_{6ax,6eq}$ = $J_{6ax,7}$ = 11.2 Hz), 2.03 (dd, 1H, H_{4ax}, $J_{4ax,4eq}$ = $J_{4ax,3}$ = 11.1 Hz). ^{13}C NMR (100 MHz, MeOD): δ = 175.5 (C₁), 132.6 (CH All), 121.9 (CH₂ All), 75.4 (C₇), 73.4 (C₃), 61.9 (CH₂ All), 57.2 (C₄), 54.7 (C₆), 53.7 (C₈), 40.5 (C₂). ATR-IR (thin film): 2094.6, 1705.0, 1573.8, 1423.4, 1296.1, 1188.1, 1110.9, 1051.1, 999.1, 927.7, 819.7 cm⁻¹. $[\alpha]_{\text{D}}^{23}$ = +12.0 (c = 1.0, MeOH). HRMS: calcd for C₁₀H₁₆N₄O₃H 241.12952, found 241.12821.



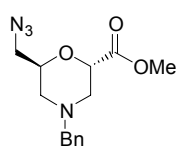
3,7-Anhydro-5-aza-8-azido-5-isopropyl-2,4,5,6,8-pentadeoxy-D-glycero-D-allo-octonic acid (5h): White solid (53 mg, 0.22 mmol, quant). ^1H NMR (400 MHz, MeOD): δ = 4.04 (m, 1H, H₃), 3.81 (m, 1H, H₇), 3.27 (m, 2H, H₈), 3.15 (ddd, 1H, H_{4eq}, $J_{4eq,6eq}$ = $J_{4eq,3}$ = 1.7 Hz, $J_{4eq,4ax}$ = 11.6 Hz), 2.99 (m, 2H, H_{6eq}, CH *iPr*), 2.42 (dd, 1H, H_{2a}, $J_{2a,3}$ = 6.3 Hz, $J_{2a,2b}$ = 15.2 Hz), 2.36 (dd, 1H, H_{6ax}, $J_{6ax,6eq}$ = $J_{6ax,7}$ = 11.2 Hz), 2.30 (m, 2H, H_{4ax}, H_{2b}), 1.14 (s, 3H, CH₃ *iPr*), 1.11 (s, 3H, CH₃ *iPr*). ^{13}C NMR (100 MHz, CDCl₃): δ = 176.0 (C₁), 75.1 (C₇), 73.9 (C₃), 58.0 (CH *iPr*), 53.7, 53.5 (C₄, C₈), 51.0 (C₆), 42.2 (C₂), 17.8 (CH₃ *iPr*). ATR-IR (thin film): 3375.2, 1211.9, 1733.9, 1635.5, 1575.7, 1398.3, 1107.1, 1020.3 cm⁻¹. $[\alpha]_{\text{D}}^{23}$ = +11.2 (c = 1.0, MeOH). MS (ESI): m/z = 243.0 [M+H]⁺. HRMS: calcd for C₁₀H₁₈N₄O₃H 243.14517, found 243.14368.



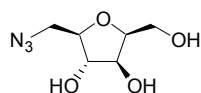
Methyl 2,5-anhydro-6-azido-6-deoxy-D-allonate (7): Cyclohexylidene-protected **6** (1.45 g, 4.88 mmol) was dissolved in MeOH (20 mL) and treated with 4 M aq. HCl (5 mL). The solution was stirred 2 h at 50°C and poured into sat. aq. NaHCO₃. The aqueous layer was extracted with EtOAc (3×) and the combined organic layers were dried (MgSO₄) and concentrated. Purification by silica column chromatography (50%→100% EtOAc in light PE) gave diol **7** as a clear oil (628 mg, 2.88 mmol, 59%). ^1H NMR (200 MHz, CDCl₃): δ = 4.44 (d, 1H, H₂, $J_{2,3}$ = 4.4 Hz), 4.43 (dd, 1H, H₃, $J_{3,4}$ = $J_{3,2}$ = 4.4 Hz), 4.09 (m, 2H, H₄, H₅), 3.80 (s, 3H, OMe), 3.60 (dd, 1H, H_{6a}, $J_{6a,5}$ = 3.3 Hz, $J_{6a,6b}$ = 13.5 Hz), 3.45 (dd, 1H, H_{7b}, $J_{7b,6}$ = 4.7 Hz, $J_{7b,7a}$ = 13.5 Hz). ^{13}C NMR (50 MHz, CDCl₃): δ = 171.3 (C₁), 82.4, 81.9 (C₂, C₅), 74.0, 72.2 (C₄, C₃), 52.6 (OMe), 52.1 (C₆). MS (ESI): m/z = 218.1 [M+H]⁺, 239.9 [M+Na]⁺.



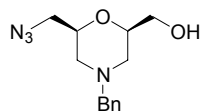
Methyl 2,6-anhydro-4-aza-7-azido-4-benzyl-3,4,5,7-tetradecoxy-D-glycero-D-ribo-heptonate (8a): Diol **7** (109 mg, 0.47 mmol) was treated as described in the general procedure for glycol cleavage and reductive amination. Silica gel column chromatography of the resulting mixture (10% EtOAc in light PE) first gave **8b** (32 mg, 0.11 mmol, 22%). Upon further eluting of the column (10%→15% EtOAc in light PE), the lower running title compound **8a** (31 mg, 0.11 mmol, 22%) was obtained as a clear oil. ^1H NMR (400 MHz, CDCl₃): δ = 7.31 (m, 5H, H_{ar}), 4.30 (dd, 1H, H₂, $J_{2,3eq}$ = 2.7 Hz, $J_{2,3ax}$ = 10.9 Hz), 3.80 (m, 1H, H₆), 3.58 (d, 1H, CH₂ Bn, $J_{\text{CHd},\text{CHu}}$ = 12.9 Hz), 3.52 (d, 1H, CH₂ Bn, $J_{\text{CHu},\text{CHd}}$ = 12.9 Hz), 3.42 (dd, 1H, H_{7a}, $J_{7a,6}$ = 6.3 Hz, $J_{7a,7b}$ = 12.9 Hz), 3.29 (dd, 1H, H_{7b}, $J_{7b,6}$ = 4.8 Hz, $J_{7b,7a}$ = 12.9 Hz), 3.08 (ddd, 1H, H_{3eq}, $J_{3eq,5eq}$ = 1.7 Hz, $J_{3eq,2}$ = 2.7 Hz, $J_{3eq,3ax}$ = 11.0 Hz), 2.74 (ddd, 1H, H_{5eq}, $J_{5eq,3eq}$ = $J_{5eq,6}$ = 1.7 Hz, $J_{5eq,5ax}$ = 11.1 Hz), 2.14 (dd, 1H, H_{3ax}, $J_{3ax,3eq}$ = $J_{3ax,2}$ = 11.0 Hz), 1.97 (dd, 1H, H_{5ax}, $J_{5ax,5eq}$ = $J_{5ax,6}$ = 11.1 Hz). ^{13}C NMR (100 MHz, CDCl₃): δ = 169.6 (C₁), 136.9 (C_q Bn), 129.0, 128.4, 127.8, 127.4 (CH_{ar}), 75.1 (C₂), 74.9 (C₆), 62.8 (CH₂ Bn), 54.5 (C₅), 54.2 (C₃), 52.7 (C₇), 52.2 (OMe). ATR-IR (thin film): 2098.4, 1759.0, 1288.4, 1203.5, 1118.6, 1064.6, 740.6, 702.0 cm⁻¹. $[\alpha]_{\text{D}}^{23}$ = -7.6 (c = 1.0, CH₂Cl₂). HRMS: calcd for C₁₄H₁₈N₄O₃H 291.14517, found 291.14508.



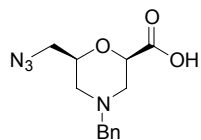
Methyl 2,6-anhydro-4-aza-7-azido-4-benzyl-3,4,5,7-tetradeoxy-D-glycero-D-arabino-heptonate (8b): Clear oil (32 mg, 0.11 mmol, 22%). ^1H NMR (400 MHz, CDCl_3): δ = 7.31 (m, 5H, H_{ar}), 4.42 (dd, 1H, H_2 , $J_{2,3\text{eq}} = 2.2$ Hz, $J_{2,3\text{ax}} = 4.1$ Hz), 4.33 (m, 1H, H_6), 3.52 (d, 1H, CH_2 Bn, $J_{\text{CHd,CHu}} = 13.3$ Hz), 3.46 (d, 1H, CH_2 Bn, $J_{\text{CHu,CHd}} = 13.3$ Hz), 3.37 (dd, 1H, $\text{H}_{7\text{a}}$, $J_{7\text{a},6} = 4.5$ Hz, $J_{7\text{a},7\text{b}} = 13.0$ Hz), 3.29 (dd, 1H, $\text{H}_{7\text{b}}$, $J_{7\text{b},6} = 5.8$ Hz, $J_{7\text{b},7\text{a}} = 13.0$ Hz), 3.10 (ddd, 1H, $\text{H}_{3\text{eq}}$, $J_{3\text{eq},5\text{eq}} = J_{3\text{eq},2} = 2.2$ Hz, $J_{3\text{eq},3\text{ax}} = 11.6$ Hz), 2.74 (ddd, 1H, $\text{H}_{5\text{eq}}$, $J_{5\text{eq},6} = 1.7$ Hz, $J_{5\text{eq},3\text{eq}} = 2.2$ Hz, $J_{5\text{eq},5\text{ax}} = 11.2$ Hz), 2.41 (dd, 1H, $\text{H}_{3\text{ax}}$, $J_{3\text{ax},2} = 4.1$ Hz, $J_{3\text{ax},\text{eq}} = 11.6$ Hz), 1.97 (dd, 1H, $\text{H}_{5\text{ax}}$, $J_{5\text{ax},6} = 9.9$ Hz, $J_{5\text{ax},5\text{eq}} = 11.2$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ = 171.3 (C_1), 137.2 (C_{q} Bn), 128.7, 128.2, 127.3 (CH_{ar}), 72.6 (C_2), 70.9 (C_6), 62.6 (CH_2 Bn), 54.9 (C_5), 53.5 (C_3), 52.7 (C_7), 51.9 (OMe). ATR-IR (thin film): 2098.4, 1743.5, 1272.9, 1203.5, 1126.4, 1026.1, 740.6, 702.0 cm^{-1} . $[\alpha]_{\text{D}}^{23} = +49.6$ ($c = 1.0$, CH_2Cl_2). HRMS: calcd for $\text{C}_{14}\text{H}_{18}\text{N}_4\text{O}_3$ 291.14517, found 291.14456.



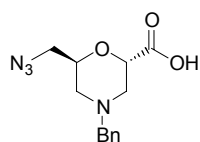
2,5-Anhydro-6-azido-6-deoxy-D-glucitol (10): Azide **9** (98 mg, 0.43 mmol) was dissolved in MeOH (3 mL) and TFA (1 mL) was added. The mixture was stirred for 1 h and all solvents were removed *in vacuo*. Residual traces of acid were removed by repeated coevaporation with toluene, to furnish the title compound **10** (83 mg, 0.43 mmol) quantitatively as a clear oil. ^1H NMR (400 MHz, MeOD): δ = 3.96 (m, 2H, H_2 , H_3), 3.80 (dd, 1H, H_4 , $J_{4,3} = 1.9$ Hz, $J_{4,5} = 3.6$ Hz), 3.71 (m, 2H, H_5 , $\text{H}_{1\text{a}}$), 3.64 (dd, 1H, $\text{H}_{1\text{b}}$, $J_{1\text{b},2} = 5.7$ Hz, $J_{1\text{b},1\text{a}} = 11.7$ Hz), 3.36 (dd, 1H, $\text{H}_{6\text{a}}$, $J_{6\text{a},5} = 6.5$ Hz, $J_{6\text{a},6\text{b}} = 12.8$ Hz), 3.32 (dd, 1H, $\text{H}_{6\text{b}}$, $J_{6\text{b},5} = 5.1$ Hz, $J_{6\text{b},6\text{a}} = 12.8$ Hz). ^{13}C NMR (100 MHz, MeOD): δ = 85.2 (C_5), 82.9 (C_2), 80.7 (C_4), 78.8 (C_3), 61.8 (C_1), 53.8 (C_6). ATR-IR (thin film): 3357.4, 3103.3, 2924.5, 2104.2, 1635.5, 1338.5, 1280.6, 1045.3, 974.0, 923.8 cm^{-1} . $[\alpha]_{\text{D}}^{23} = +49.0$ ($c = 1.0$, MeOH). MS (ESI): $m/z = 190.0$ [$\text{M}+\text{H}$] $^+$, 212.0 [$\text{M}+\text{Na}$] $^+$.



2,6-Anhydro-4-aza-7-azido-4-benzyl-3,4,5,7-tetradeoxy-D-glycero-D-ribo-heptitol (11): Triol **10** (693 mg, 3.5 mmol) was subjected to glycol cleavage and reductive amination, as described in the general procedure, to deliver **11** (486 mg, 1.85 mmol, 53%) as a white solid. ^1H NMR (400 MHz, CDCl_3): δ = 7.30 (m, 5H, H_{ar}), 3.87 (m, 1H, H_6), 3.76 (m, 1H, H_2), 3.65 (dd, 1H, $\text{H}_{1\text{a}}$, $J_{1\text{a},2} = 3.6$ Hz, $J_{1\text{a},1\text{b}} = 11.6$ Hz), 3.55 (dd, 1H, $\text{H}_{1\text{b}}$, $J_{1\text{b},2} = 6.3$ Hz, $J_{1\text{b},1\text{a}} = 11.6$ Hz), 3.53 (s, 2H, CH_2 Bn), 3.28 (dd, 1H, $\text{H}_{7\text{a}}$, $J_{7\text{a},6} = 6.3$ Hz, $J_{7\text{a},7\text{b}} = 12.9$ Hz), 3.22 (dd, 1H, $\text{H}_{7\text{b}}$, $J_{7\text{b},6} = 4.1$ Hz, $J_{7\text{b},7\text{a}} = 12.9$ Hz), 2.72 (ddd, 2H, $\text{H}_{3\text{eq}}$, $\text{H}_{5\text{eq}}$, $J_{3\text{eq},5\text{eq}} = J_{3\text{eq},2} = J_{5\text{eq},3\text{eq}} = J_{5\text{eq},6} = 1.7$ Hz, $J_{3\text{eq},3\text{ax}} = J_{5\text{eq},5\text{ax}} = 10.6$ Hz), 1.98 (dd, 1H, $\text{H}_{3\text{ax}}$, $J_{3\text{ax},3\text{eq}} = J_{3\text{ax},2} = 11.0$ Hz), 1.95 (dd, 1H, $\text{H}_{5\text{ax}}$, $J_{5\text{ax},5\text{eq}} = J_{5\text{ax},6} = 11.0$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ = 130.7 (C_{q} Bn), 129.2, 128.4, 127.4 (CH_{ar}), 76.3 (C_2), 75.2 (C_6), 64.0 (C_1), 63.1 (CH_2 Bn), 55.0 (C_5), 53.8 (C_3), 53.0 (C_7). ATR-IR (thin film): 3386.8, 2923.9, 2877.6, 2098.4, 1450.4, 1288.4, 1118.6, 1064.6, 918.1 cm^{-1} . $[\alpha]_{\text{D}}^{23} = +2.0$ ($c = 1.0$, CH_2Cl_2). HRMS: calcd for $\text{C}_{13}\text{H}_{18}\text{N}_4\text{O}_2$ 263.15025, found 263.14951.

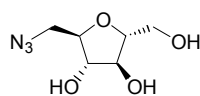


2,6-Anhydro-4-aza-7-azido-4-benzyl-3,4,5,7-tetradeoxy-D-glycero-D-ribo-heptonic acid (12a): To a vigorously stirred solution of morpholine **11** (79 mg, 0.30 mmol) in DCM (1 mL) and water (0.5 mL), that was cooled to 0°C , were added TEMPO (9.4 mg, 0.06 mmol, 0.2 equiv) and BAIB (193 mg, 0.6 mmol, 2 equiv). After 6 h, the reaction was quenched with MeOH and the mixture was evaporated to dryness. Silica gel column chromatography (0%→10% of a mixture of *n*-BuOH / AcOH / water (1/1/1 v/v/v) in EtOAc) provided **12a** as a clear oil (50 mg, 0.18 mmol, 61%). ^1H NMR (400 MHz, MeOD): δ = 7.38 (m, 5H, H_{ar}), 4.24 (m, 1H, H_2), 3.96 (m, 2H, CH_2 Bn), 3.87 (m, 1H, H_6), 3.41 (m, 3H, H_7 , $\text{H}_{3\text{eq}}$), 3.06 (d, 1H, $\text{H}_{5\text{eq}}$, $J_{5\text{eq},5\text{ax}} = 11.4$ Hz), 2.51 (dd, 1H, $\text{H}_{3\text{ax}}$, $J_{3\text{ax},3\text{eq}} = J_{3\text{ax},2} = 11.4$ Hz), 2.43 (dd, 1H, $\text{H}_{5\text{ax}}$, $J_{5\text{ax},5\text{eq}} = J_{5\text{ax},6} = 11.2$ Hz). ^{13}C NMR (100 MHz, MeOD): δ = 173.1 (C_1), 134.3 (C_{q} Bn), 131.4, 129.8 (CH_{ar}), 75.7 (C_2), 74.7 (C_6), 62.9 (CH_2 Bn), 54.7, 54.4 (C_3 , C_5), 53.4 (C_7). ATR-IR (thin film): 2100.3, 1608.5, 1456.2, 1373.2, 1274.9, 1120.6, 1053.1, 999.1, 862.1, 754.1, 698.2 cm^{-1} . $[\alpha]_{\text{D}}^{23} = +4.8$ ($c = 1.0$, MeOH). HRMS: calcd for $\text{C}_{13}\text{H}_{17}\text{N}_4\text{O}_3$ 277.12952, found 277.12817.



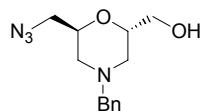
2,6-Anhydro-4-aza-7-azido-4-benzyl-3,4,5,7-tetra-deoxy-D-glycero-D-arabino-heptonic acid (12b):

Compound **15** (95 mg, 0.36 mmol) was treated following the same procedure as for **11**, to deliver MAA **12b** (68 mg, 0.24 mmol, 68%) as a clear oil. ^1H NMR (400 MHz, MeOD): δ = 7.36 (m, 5H, H_{ar}), 4.30 (1H, H_2 , $J_{2,3\text{eq}}$ = 3.2 Hz, $J_{2,3\text{ax}}$ = 4.1 Hz), 4.24 (m, 1H, H_6), 3.69 (d, 1H, CH_2 Bn, $J_{\text{CHd,CHu}}$ = 13.1 Hz), 3.65 (d, 1H, CH_2 Bn, $J_{\text{CHu,CHd}}$ = 13.1 Hz), 3.37 (dd, 1H, $\text{H}_{7\text{a}}$, $J_{7\text{a},6}$ = 4.7 Hz, $J_{7\text{a},7\text{b}}$ = 13.0 Hz), 3.30 (dd, 1H, $\text{H}_{7\text{b}}$, $J_{7\text{b},6}$ = 5.5 Hz, $J_{7\text{b},7\text{a}}$ = 13.0 Hz), 3.16 (ddd, 1H, $\text{H}_{3\text{eq}}$, $J_{3\text{eq},5\text{eq}}$ = 1.7 Hz, $J_{3\text{eq},2}$ = 3.2 Hz, $J_{3\text{eq},3\text{ax}}$ = 11.8 Hz), 2.75 (ddd, 1H, $\text{H}_{5\text{eq}}$, $J_{5\text{eq},3\text{eq}}$ = 1.7 Hz, $J_{5\text{eq},6}$ = 2.6 Hz, $J_{5\text{eq},5\text{ax}}$ = 11.6 Hz), 2.61 (dd, 1H, $\text{H}_{3\text{ax}}$, $J_{3\text{ax},2}$ = 4.3 Hz, $J_{3\text{ax},3\text{eq}}$ = 11.8 Hz), 2.35 (dd, 1H, $\text{H}_{5\text{ax}}$, $J_{5\text{ax},6}$ = 9.3 Hz, $J_{5\text{ax},5\text{eq}}$ = 11.6 Hz). ^{13}C NMR (100 MHz, MeOD): δ = 175.7 (C_1) 136.2 (C_{q} Bn), 130.8, 129.6, 129.1 (CH_{ar}), 73.7, (C_2), 71.6 (C_6), 63.3 (CH_2 Bn), 55.2 (C_5), 54.5 (C_3), 53.4 (C_7). ATR-IR (thin film): 2098.4, 1716.5, 1602.7, 1456.2, 1396.4, 1274.9, 1213.1, 1120.6, 752.2, 700.1 cm^{-1} . $[\alpha]_{\text{D}}^{23}$ = +12.8 (c = 0.1, MeOH). MS (ESI): m/z = 277.0 $[\text{M}+\text{H}]^+$, 298.9 $[\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{13}\text{H}_{17}\text{N}_4\text{O}_3\text{H}$ 277.12952, found 277.12799.



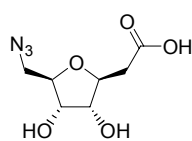
2,5-Anhydro-6-azido-6-deoxy-D-mannitol (14):

Anhydromannitol **13** (6.65 g, 20 mmol) was mesylated as described by Guthrie *et al.*²⁰ The intermediate mesylate (2.20 g, 9.1 mmol) was subsequently dissolved in DMF (50 mL), NaN_3 (1.47 g, 22.7 mmol, 2.5 equiv) was added and the mixture was stirred at 70°C for 48 h. Evaporation of the volatiles and silica column chromatography (0%→10% of MeOH in EtOAc) produced **14** (1.73 g, 9.1 mmol, 45% over two steps) as a clear oil. ^1H NMR (400 MHz, MeOD): δ = 3.94 (dd, 1H, H_3 , $J_{3,2}$ = $J_{3,4}$ = 6.1 Hz), 3.89 (dd, 1H, H_4 , $J_{4,3}$ = $J_{4,5}$ = 6.1 Hz), 3.84 (m, 1H, H_5), 3.76 (m, 1H, H_2), 3.65 (dd, 1H, $\text{H}_{1\text{a}}$, $J_{1\text{a},2}$ = 3.4 Hz, $J_{1\text{a},1\text{b}}$ = 11.9 Hz), 3.55 (dd, 1H, $\text{H}_{1\text{b}}$, $J_{1\text{b},2}$ = 4.9 Hz, $J_{1\text{b},1\text{a}}$ = 11.9 Hz), 3.42 (dd, 1H, $\text{H}_{6\text{a}}$, $J_{6\text{a},5}$ = 3.6 Hz, $J_{6\text{a},6\text{b}}$ = 13.1 Hz), 3.28 (dd, 1H, $\text{H}_{6\text{b}}$, $J_{6\text{b},5}$ = 5.6 Hz, $J_{6\text{b},6\text{a}}$ = 13.1 Hz). ^{13}C NMR (100 MHz, MeOD): δ = 84.9 (C_2), 83.4 (C_5), 79.2 (C_4), 78.2 (C_3), 63.0 (C_1), 53.3 (C_6). ATR-IR (thin film): 3357.8, 2923.9, 2104.2, 1645.2, 1440.7, 1280.6, 1109.0, 1045.3, 933.5 cm^{-1} . $[\alpha]_{\text{D}}^{23}$ = +73.4 (c = 1.0, MeOH). MS (ESI): m/z = 190.0 $[\text{M}+\text{H}]^+$, 212.1 $[\text{M}+\text{Na}]^+$.



2,6-Anhydro-4-aza-7-azido-4-benzyl-3,4,5,7-tetra-deoxy-D-glycero-D-arabino-heptitol (15):

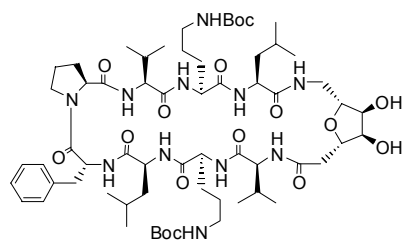
Triol **14** (280 mg, 1.4 mmol) was subjected to glycol cleavage and reductive amination, as described in the general procedure, to obtain title compound **15** (191 mg, 0.73 mmol, 52%) as a clear oil. ^1H NMR (400 MHz, CDCl_3): δ = 7.30 (m, 5H, H_{ar}), 4.16 (m, 1H, H_6), 3.92 (m, 1H, H_2), 3.84 (dd, 1H, $\text{H}_{1\text{a}}$, $J_{1\text{a},2}$ = 5.8 Hz, $J_{1\text{a},1\text{b}}$ = 11.6 Hz), 3.74 (dd, 1H, $\text{H}_{1\text{b}}$, $J_{1\text{b},2}$ = 3.4 Hz, $J_{1\text{b},1\text{a}}$ = 11.6 Hz), 3.61 (dd, 1H, $\text{H}_{7\text{a}}$, $J_{7\text{a},6}$ = 7.3 Hz, $J_{7\text{a},7\text{b}}$ = 12.9 Hz), 3.47 (s, 2H, CH_2 Bn), 3.27 (dd, 1H, $\text{H}_{7\text{b}}$, $J_{7\text{b},6}$ = 4.8 Hz, $J_{7\text{b},7\text{a}}$ = 12.9 Hz), 2.58 (dd, 1H, $\text{H}_{3\text{a}}$, $J_{3\text{a},2}$ = 3.4 Hz, $J_{3\text{a},3\text{b}}$ = 11.4 Hz), 2.53 (dd, 1H, $\text{H}_{5\text{a}}$, $J_{5\text{a},6}$ = 3.4 Hz, $J_{5\text{a},5\text{b}}$ = 11.6 Hz), 2.47 (dd, 1H, $\text{H}_{3\text{b}}$, $J_{3\text{b},2}$ = 5.6 Hz, $J_{3\text{b},3\text{a}}$ = 11.4 Hz), 2.32 (dd, 1H, $\text{H}_{5\text{b}}$, $J_{5\text{b},6}$ = 6.1 Hz, $J_{5\text{b},5\text{a}}$ = 11.6 Hz). ^{13}C NMR (100 MHz, CDCl_3): δ = 137.1 (C_{q} Bn), 128.9, 128.5, 127.4 (CH_{ar}), 71.3, 71.2 (C_2 , C_6), 64.8 (C_1), 63.1 (CH_2 Bn), 54.4 (C_3), 54.2 (C_5), 51.8 (C_7). ATR-IR (thin film): 3384.5, 2936.2, 2094.6, 1454.2, 1265.2, 1149.6, 1112.9, 1053.1, 912.3, 742.5, 698.2 cm^{-1} . $[\alpha]_{\text{D}}^{23}$ = +3.8 (c = 1.0, CH_2Cl_2). HRMS: calcd for $\text{C}_{13}\text{H}_{18}\text{N}_4\text{O}_2\text{H}$ 263.15025, found 263.15015.



3,6-Anhydro-7-azido-2,7-dideoxy-D-allo-heptonic acid (16):

To a solution of ester **2** (350 mg, 1.52 mmol) in THF (4 mL) was added 1 M aq. NaOH (2 mL). The mixture was neutralized after 3 h with Amberlite IR-120 (H^+), filtered and concentrated. Purification by silica column chromatography (0%→2% AcOH in EtOAc) furnished **16**, as a clear oil (323 mg, 1.49 mmol, 98%). ^1H NMR (400 MHz, CD_3OD): δ = 4.16 (ddd, 1H, H_3 , $J_{3,2\text{a}}$ = 4.9 Hz, $J_{3,4}$ = 5.3 Hz, $J_{3,2\text{b}}$ = 8.4 Hz), 3.96 (m, 2H, H_5 , H_6), 3.84 (dd, 1H, H_4 , $J_{4,3}$ = 5.3 Hz, $J_{4,5}$ = 5.4 Hz), 3.51 (dd, 1H, $\text{H}_{7\text{a}}$, $J_{7\text{a},6}$ = 3.1 Hz, $J_{7\text{a},7\text{b}}$ = 13.2 Hz), 3.29 (dd, 1H, $\text{H}_{7\text{b}}$, $J_{7\text{b},6}$ = 4.4 Hz, $J_{7\text{b},7\text{a}}$ = 13.2 Hz), 2.67 (dd, 1H, $\text{H}_{2\text{a}}$, $J_{2\text{a},3}$ = 4.9 Hz, $J_{2\text{a},2\text{b}}$ = 15.7 Hz), 2.50 (dd, 1H, $\text{H}_{2\text{b}}$, $J_{2\text{b},3}$ = 8.4 Hz, $J_{2\text{b},2\text{a}}$ = 15.7 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ = 174.6 (C_1), 83.9 (C_6), 81.2 (C_3), 75.7 (C_4),

73.0 (C₅), 53.5 (C₇), 39.4 (C₂). ATR-IR (thin film): 3434.6, 2927.7, 2100.3, 1706.9, 1406.0, 1272.9, 1180.4, 1097.4, 1033.8, 977.8, 912.3, 827.4, 748.3 cm⁻¹. [α]_D²³ +54.4 (*c* = 1.0, MeOH). MS (ESI): *m/z* 217.9 [M+H]⁺, 241.0 [M+Na]⁺, 435.1 [2M+H]⁺, 457.1 [2M+Na]⁺. HRMS: calcd for C₇H₁₁N₃O₅H 218.07715, found 218.07724.

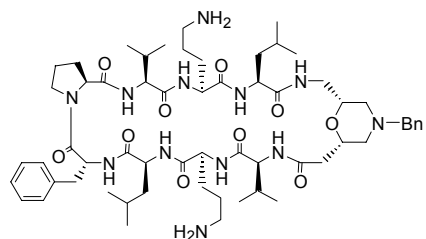


cyclo-(MAA-Val-Orn(Boc)-Leu-^DPhe-Pro-Val-Orn-Leu) (19):

Route A: Fmoc-based solid phase peptide synthesis was performed as described previously,⁷ starting with preloaded resin **17** (100 μ mol). The final coupling of **5a** (64 mg, 0.2 mmol, 2 equiv) with BOP (132 mg, 0.3 mmol, 3 equiv), HOBT (41 mg, 0.3 mmol, 3 equiv) and DIPEA (58 μ L, 0.35 mmol, 3.5 equiv) in NMP (2 mL) furnished the title compound **19** (96 mg, 71 μ mol,

71%) as a white amorphous solid.

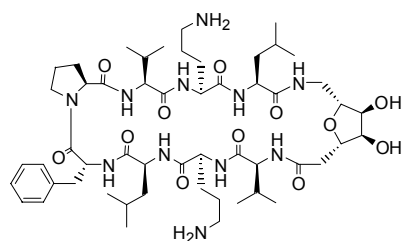
Route B: Boc-protected GS analogue **21** (50 mg, 40 μ mol) was dissolved in THF (7.5 mL) and DMF (2.5 mL) and a solution of sodium periodate (17 mg, 80 μ mol, 2 equiv) in water (2.5 mL) was added. Stirring was continued overnight, after which the milky suspension was concentrated and partitioned between water and chloroform. The water layer was extracted with chloroform (2 $\times$) and the combined organic layers were dried (MgSO₄), filtered and concentrated to quantitatively produce the crude dialdehyde (50 mg, 40 μ mol). Subsequently, the dialdehyde (25 mg, 20 μ mol) was dissolved in MeOH (4 mL) and trimethylorthoformate (2 mL) and NaCNBH₃ (7 mg, 100 μ mol, 5 equiv) was added. To this mixture was added a solution of benzylamine (3.2 μ L, 30 μ mol, 1.5 equiv) in MeOH (0.5 mL), trimethylorthoformate (0.2 mL) and DMF (0.2 mL) that had been acidified to pH = 5 with AcOH in advance. After stirring overnight, all solvents were evaporated and the mixture was applied to a size exclusion column that was eluted with MeOH, to yield the title compound **19** (17 mg, 12 μ mol, 63%) as a white amorphous solid.



cyclo-(MAA-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu) (20):

A mixture of MAA-containing **19** (17 mg, 12 μ mol) in DCM (2 mL) was cooled to 0°C, treated with TFA (2 mL) and stirred for 30 min. The solvents were evaporated and the crude mixture was analyzed by LC/MS (*R*_t 12.74 min (linear gradient 5 $\rightarrow$ 90% B in 20 min.; *m/z* = 1144.0 [M+H]⁺, 572.7 [M+H]²⁺) followed by semi-preparative RP-HPLC purification (linear gradient of

3.0 CV; 35 $\rightarrow$ 55% B; *R*_t 2.2 CV), to produce **20** (10.6 mg, 9.3 μ mol) in 77% after lyophilization of the pooled fractions. ¹H NMR (600 MHz, CD₃OH): δ = 8.86 (d, 1H, NH ^DPhe₅, *J*_{NH,H α} = 3.8 Hz), 8.65 (d, 1H, NH α Orn₈, *J*_{NH,H α} = 8.2 Hz), 8.56 (d, 1H, NH α Orn₃, *J*_{NH,H α} = 8.9 Hz), 8.48 (d, 1H, NH Leu₄, *J*_{NH,H α} = 8.7 Hz), 8.21 (d, 1H, NH Leu₉, *J*_{NH,H α} = 8.4 Hz), 7.89 (d, 1H, NH Val₇, *J*_{NH,H α} = 7.1 Hz), 7.83 (t, 1H, NH MAA₁, *J*_{NH,8} = 5.8 Hz), 7.47 – 7.45 (m, 5H, H_{ar}), 7.39 (d, 1H, NH Val₂, *J*_{NH,H α} = 8.3 Hz), 7.31 – 7.23 (m, 5H, H_{ar}), 4.98 (m, 1H, H α Orn₃), 4.63 (m, 1H, H α Leu₄), 4.53 (m, 1H, H α ^DPhe₅), 4.43 (m, 2H, H α Leu₉, H α Orn₈), 4.35 (m, 2H, H α Pro₆, H α Val₂), 4.23 (m, 2H, CH₂ Bn), 4.11 (m, 1H, H₃ MAA₁), 3.93 (m, 1H, H₇ MAA₁), 3.88 (m, 1H, H α Val₇), 3.71 (m, 1H, H δ d Pro₆), 3.36 (m, 1H, H δ d MAA₁), 3.32 (m, 2H, H δ d, H δ d MAA₁), 3.07 (m, 2H, H β d ^DPhe₅, H δ u MAA₁), 2.98 (m, 5H, H δ Orn₃, H δ Orn₈, H β u ^DPhe₅), 2.75 (m, 1H, H δ u MAA₁), 2.67 (m, 1H, H δ u MAA₁), 2.53 (m, 2H, H δ d MAA₁, H δ u Pro₆), 2.30 (m, 1H, H β Val₇), 1.95 (m, 3H, H β Val₂, H β d Pro₆, H β d Orn₈), 1.88 (m, 1H, H β d Orn₃), 1.75 (m, 5H, H β u, γ Orn₃, H γ Orn₈), 1.72 (m, 2H, H β u, γ d Pro₆), 1.67 (m, 1H, H β u Orn₃), 1.66 (m, 1H, H β u Orn₈), 1.65 (m, 1H, H γ u Pro₆), 1.64 (m, 3H, H β , γ Leu₉), 1.56 (m, 2H, H β d, γ Leu₄), 1.41 (m, 1H, H β u Leu₄), 0.97 (m, 3H, H γ d Val₇), 0.93 (m, 6H, H γ Val₂), 0.90 (m, 9H, H δ Leu₄, H γ u Val₇), 0.84 (m, 6H, H δ Leu₉). HRMS: calcd for C₆₀H₉₄N₁₂O₁₀H 1143.72886, found 1143.72632.



cyclo-(SAA-Val-Orn(Boc)-Leu-^DPhe-Pro-Val-Orn-Leu) (21):

In a similar scheme described in Route A, preloaded resin **17** (100 μmol) was elongated in a stepwise fashion, with a final condensation of SAA **16** (44 mg, 0.2 mmol, 2 equiv), BOP (132 mg, 0.3 mmol, 3 equiv), HOBt (41 mg, 0.3 mmol, 3 equiv) and DiPEA (58 μL, 0.35 mmol, 3.5 equiv) in NMP (3 mL), to ultimately obtain the title peptide **21** (80 mg, 63 μmol, 63%) as

off-white amorphous solid. An aliquot of **21** (14 mg, 11.0 μmol) was then dissolved in DCM (2 mL), cooled to 0°C and TFA (2 mL) was added slowly. After stirring the mixture for 30 min, the volatiles were removed *in vacuo* and the crude peptide was analyzed by LC/MS (R_t 14.71 min (linear gradient 10→90% B in 20 min.; m/z = 1070.8 $[M+H]^+$, 536.1 $[M+H]^{2+}$), purified by RP-HPLC (linear gradient of 3.0 CV; 40→50% B; R_t = 1.9 CV) and the combined fractions were lyophilized to furnish the unprotected peptide (8.1 mg, 7.6 μmol, 69%) as amorphous white powder. ^1H NMR (600 MHz, CD_3OH): δ = 8.90 (d, 1H, NH ^DPhe₅, $J_{NH,H\alpha}$ = 3.5 Hz), 8.68 (d, 1H, NH _{α} Orn₃, $J_{NH,H\alpha}$ = 8.1 Hz), 8.62 (d, 1H, NH Leu₄, $J_{NH,H\alpha}$ = 9.4 Hz), 8.61 (d, 1H, NH _{α} Orn₈, $J_{NH,H\alpha}$ = 8.9 Hz), 8.56 (d, 1H, NH Leu₉, $J_{NH,H\alpha}$ = 8.9 Hz), 8.07 (t, 1H, NH SAA₁, $J_{NH,H\alpha}$ = 6.1 Hz), 7.86 (bs, 2H, NH _{δ} Orn_{3,8}), 7.74 (d, 1H, NH Val₇, $J_{NH,H\alpha}$ = 8.6 Hz), 7.55 (d, 1H, NH Val₂, $J_{NH,H\alpha}$ = 8.5 Hz), 7.38 – 7.21 (m, 5H, H_{ar}), 4.98 (m, 1H, H _{α} Orn₃), 4.71 (m, 1H, H _{α} Orn₈), 4.65 (m, 1H, H _{α} Leu₄), 4.56 (m, 1H, H _{α} Leu₉), 4.51 (m, 1H, H _{α} ^DPhe₅), 4.34 (m, 1H, H _{α} Pro₆), 4.24 (m, 1H, H _{α} Val₂), 4.06 (m, 1H, H _{α} Val₇), 3.95 (m, 2H, H₃, H₆ SAA₁), 3.86 (dd, 1H, H₅ SAA₁, $J_{5,4}$ = 5.2 Hz, $J_{5,6}$ = 3.0 Hz), 3.78 (dd, 1H, H₄ SAA₁, $J_{4,5}$ = 5.2 Hz, $J_{4,3}$ = 6.5 Hz), 3.72 (m, 1H, H _{δd} Pro₆), 3.36 (m, 1H, H_{7d} SAA₁), 3.31 (m, 1H, H_{7u} SAA₁), 3.07 (dd, 1H, H _{βd} ^DPhe₅, $J_{\beta d,\beta u}$ = 12.6 Hz, $J_{\beta d,\alpha}$ = 5.0 Hz), 3.02 (m, 1H, H _{δd} Orn₃), 2.98 (m, 1H, H _{δd} Orn₈), 2.96 (m, 3H, H _{δu} Orn₃, H _{δu} Orn₈, H _{βu} ^DPhe₅), 2.50 (m, 3H, H₂ SAA₁, H _{δu} Pro₆), 2.28 (m, 1H, H _{β} Val₇), 1.99 (m, 3H, H _{βd} Pro₆, H _{βd} Orn₃, H _{β} Val₂), 1.83 (m, 1H, H _{βd} Orn₈), 1.74 (m, 2H, H _{γ} Orn₃), 1.71 (m, 2H, H _{βu} , γd Pro₆), 1.67 (m, 1H, H _{βu} Orn₃), 1.66 (m, 2H, H _{γ} Orn₈), 1.64 (m, 3H, H _{β} , γ Leu₉), 1.59 (m, 1H, H _{γu} Pro₆), 1.56 (m, 2H, H _{βd} , γ Leu₄), 1.39 (m, 1H, H _{βu} Leu₄), 0.95 (m, 3H, H _{γd} Val₇), 0.94 (m, 3H, H _{γd} Val₂), 0.92 (m, 3H, H _{γu} Val₂), 0.90 (m, 6H, H _{δ} Leu₄), 0.88 (m, 3H, H _{γu} Val₇), 0.86 (m, 6H, H _{δ} Leu₉). ATR-IR (thin film): 3278.1, 3071.9, 2959.2, 2935.6, 2873.4, 1669.8, 1636.5, 1539.2, 1464.7, 1456.7, 1437.0, 1203.7, 1182.7, 1135.0, 1033.3, 1020.8, 837.1, 800.1, 722.6, 702.5 cm^{-1} . HRMS: calcd for $C_{53}H_{87}N_{11}O_{12}H$ 1079.6608, found 1070.6521.

References and Notes

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13. Glycol cleavage could similarly be effected by sodium periodate although the reaction proceeded sluggishly.
14. The azide functionality proved to be stable under these reducing conditions as no deterioration of **2** was observed when subjected to the same reaction conditions.
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21. Upon perusal of the acquired data reported in the experimental section, it was found that the coupling constants ($^3J_{\text{NH,H}\alpha}$) and chemical shift perturbation ($\Delta\delta\text{H}_\alpha$) for the proteinogenic residues in peptide **20** follow a similar trend compared to native GS. These distinctive features validate a β -sheet conformation in GS analogues, as we have previously observed: Grotenbreg, G. M.; Kronemeijer, M.; Timmer, M. S. M.; El Oualid, F.; van Well, R. M.; Verdoes, M.; Spalburg, E.; van Hooft, P. A. V.; de Neeling, A. J.; Noort, D.; van Boom, J. H.; van der Marel, G. A.; Overkleeft H. S.; Overhand, M. *J. Org. Chem.*, in press.
22. To facilitate the characterization of GS analogue **21**, a small aliquot was deprotected and purified by reversed-phase HPLC, to produce the unprotected peptide in 69% yield.

Chapter 7

Gramicidin S Analogues Containing Decorated Sugar Amino Acids

Abstract: *The design and synthesis of a series of sugar amino acids (SAAs) that were functionalized with aromatic groups is described. Ensuing incorporation of the SAAs in the cationic antimicrobial peptide gramicidin S (GS), replacing a single ^DPhe-Pro reverse turn, resulted in GS analogues **2a-c**. ¹H NMR analysis revealed that the peptides adopt a β -sheet conformation featuring an unusual reverse turn induced by the SAAs. The GS analogues **2a-c** proved to be as effective as GS itself in lysing both bacteria and erythrocytes, thus underscoring the potential of decorated SAAs as replacement of selected peptide sequences.¹*

Introduction

The characteristic β -sheet structure of gramicidin S (GS) is thought to be an essential element contributing to its antimicrobial and hemolytic activity. However, the mechanism by which GS induces membrane-permeability has not yet been conclusively established. In **Chapter 4**, the synthesis and structural evaluation of GS analogue **1**^{2a} is described in which a furanoid sugar amino acid (SAA)³ replaces a single ^DPhe-Pro dipeptide sequence in GS (depicted in Figure 1 **A** and **B**). Upon comparison of the two-dimensional ¹H NMR and single crystal X-ray diffraction data, an unusual reverse turn structure was identified that was adopted by the peptide in both the solution phase and the crystalline state. The C₃-hydroxyl stemming from the parent sugar was implicated in the distortion of the hydrogen bonding pattern normally found in GS-like peptides. The overall β -sheet structure of GS analogue **B**, however, did not drastically differ from that observed in native GS. In spite of this, the antimicrobial and hemolytic assays of GS analogue **1** (see **Chapter 5**) revealed that its capacity to inflict a loss of barrier function of cellular membranes, had largely dissipated in comparison with GS.^{2b}

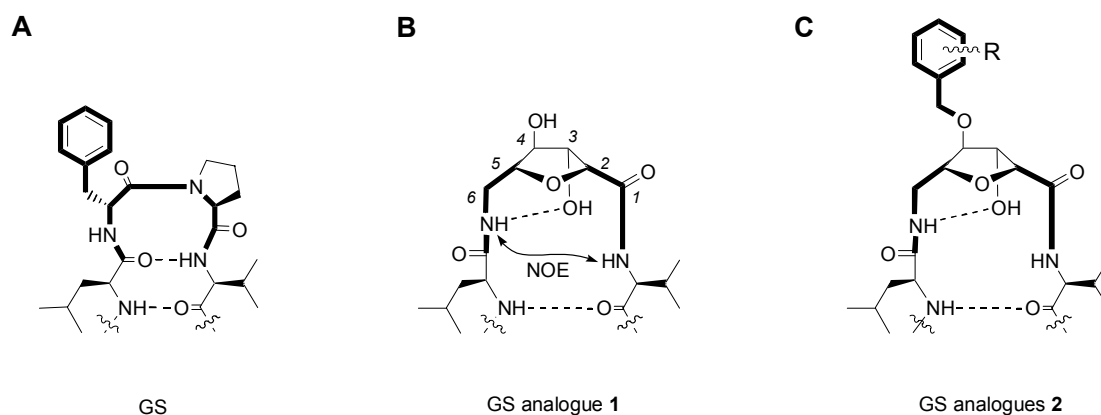


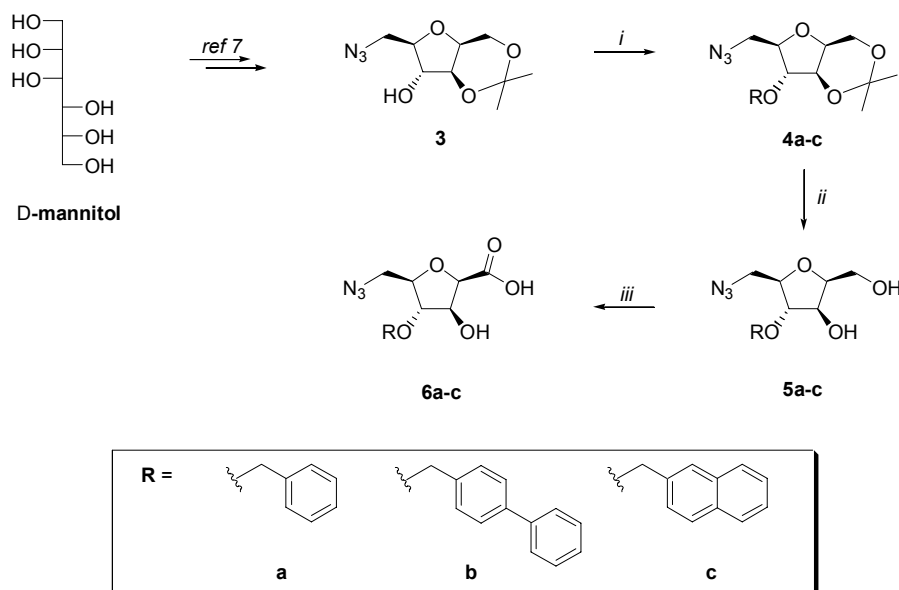
Figure 1: Reverse turn structures of (A) GS, (B) GS analogue 1 described in chapter 4 and (C) GS analogues containing SAAs with aromatic moieties described in this chapter.

Perusal of the reverse turn structures in native GS and analogue 1 reveals two distinct differences. First, the ^{D} Phe-Pro dipeptide sequence in GS adopts a type II' β -turn, whereas the furanoid SAA dipeptide isostere induces an altered peptide backbone geometry. Second, the native peptide contains an aromatic amino acid residue (*i.e.* ^{D} Phe), whereas 1 does not contain an aromatic functionality in the SAA-containing turn. To enhance the mimicry of GS analogue 1 towards the original reverse turn, the installation of an aromatic moiety on the C4-hydroxyl function was envisaged, as is depicted for GS analogue 2 (Figure 1). The fact that the introduction of additional aromaticity at this position can have a beneficial effect on the biological activity of GS-based peptides, has been demonstrated in various studies among which those described in **Chapter 2**.^{4,5,6}

This chapter describes the design and synthesis of peptidomimetic SAA **6a** (Scheme 1), that has been functionalized with a benzyl group, and its subsequent incorporation into GS analogue **2a**. To probe the extent of aromaticity required in the reverse turn of GS, the SAAs **6b** and **c** were also incorporated in their respective analogues **2b** and **c**. To enable correlation of the structural and functional data, the ^1H NMR experiments on GS analogues **2a-c** were closely examined to confirm the presence of a β -sheet secondary structure and to probe the reverse turn structure adopted by the furanoid SAAs. Both antimicrobial and hemolytic properties of GS analogues **2a-c** were determined and compared with those of GS and 1.

Results and Discussion

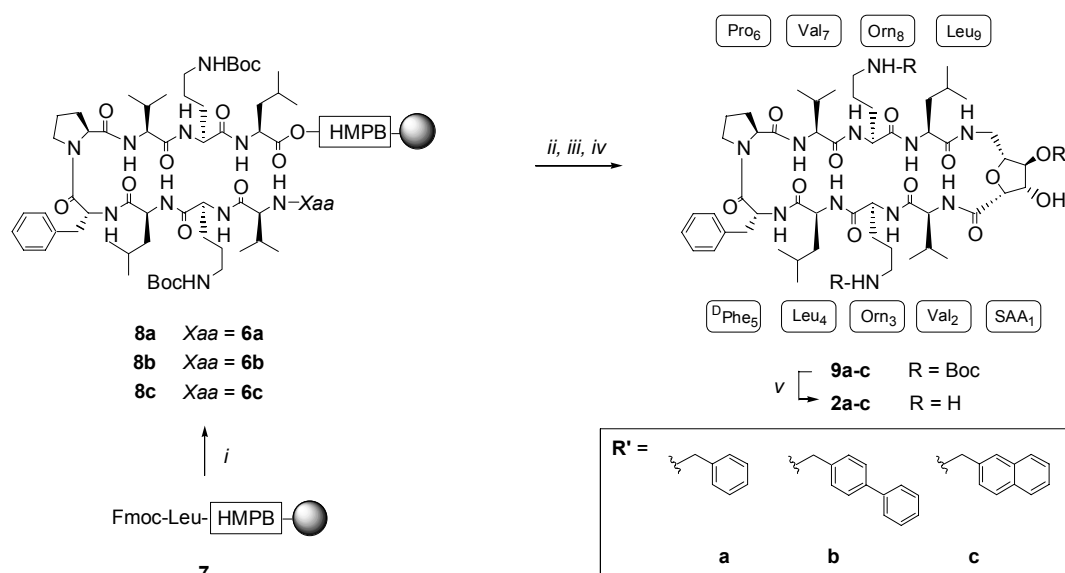
The construction of the decorated SAAs **6a-c** was accomplished as follows. The synthesis of 2,5-anhydroglucitol **3** (Scheme 1) employed a synthetic route that was recently developed by Timmer *et al.*⁷ Acidic dehydration of D-(+)-mannitol, followed by acetonation of the 1,3-*cis*-diol system and successive introduction of the primary azide gave **3**. Ensuing alkylation of the remaining hydroxyl in **3** with benzyl bromide resulted in the formation of fully protected anhydroglucitol **4a** in 93% yield. The other aromatic bromides required for alkylation were



Scheme 1: Reagents and conditions: (i) NaH (1.1 equiv), RBr (1 equiv), DMF, 0 °C, 16 h, **4a**, 93%; **4b**, 89%; **4c**, 77%; (ii) PPTS (cat), MeOH, 50 °C, 16 h, **5a**, 68%; **5b**, 58%; **5c**, 69%; (iii) TEMPO (cat), NaOCl, NaHCO₃ (aq), MeCN, 0°C, **6a**, quant; **6b**, 52%; **6c**, 72%.

prepared from their corresponding alcohols according to a procedure described by Brun *et al.*⁸ 4-Biphenylmethanol and 1-naphthalenemethanol were treated with PBr₃ and used without further purification in the ensuing alkylation step to provide biphenyl derivative **4b** (89%) and naphthalene derivative **4c** (77%). Mild methanolytic cleavage of the isopropylidene group using a catalytic amount of pyridinium *p*-toluenesulfonate (PPTS) gave diols **5a-c** in their respective yields of 68%, 58%, and 50%. Finally, the carboxylic acids were installed by selective oxidation of the primary alcohol in **5a-c**, employing a catalytic amount of TEMPO (2,2,6,6-tetramethyl-piperidinyl-1-oxyl) and NaOCl as co-oxidant, to provide the SAAs **6a** (quant.), **6b** (52%), and **6c** (72%), respectively.

Next, the assembly of the GS analogues **2a-c** having SAAs **6a-c** incorporated was undertaken as is depicted in Scheme 2. MBHA-resin **7**, functionalized with the acid-labile HMPB-linker system and loaded with Fmoc-Leu-OH, was elongated using standard Fmoc-based SPPS protocols.^{2b} The *N*-terminal azides of the immobilized nonapeptides **8a-c** were subjected to Staudinger reduction. The resulting peptides were released from the resin (50% TFA in DCM) and cyclized under dilute conditions (*i.e.* dropwise addition to a vigorously stirred solution of PyBOP (5 equiv), HOBT (5 equiv) and DiPEA (15 equiv)) followed by size exclusion chromatography to furnish the fully protected **9a** (86%), **9b** (56%) and **9c** (98%), respectively. Finally, removal of the Boc protective groups and HPLC purification furnished homogeneous GS analogues **2a** (87%), **2b** (34%)⁹ and **2c** (57%), respectively.¹⁰



Scheme 2: Reagents and conditions: (i) Fmoc deprotection: piperidine / NMP (1/4 v/v), condensation: Fmoc-aa-OH (3 equiv) or SAA **6a**, **6b**, and **6c** (2 equiv), BOP (3 equiv), HOBT (3 equiv), DiPEA (3.3 equiv), NMP, 90 min; (ii) PMe_3 (16 equiv), 1,4-dioxane / H_2O (10/1 v/v); (iii) TFA / DCM (1/99 v/v), 4×10 min; (iv) PyBOP (5 equiv), HOBT (5 equiv), DiPEA (15 equiv), DMF, 16 h, **9a**, 86%; **9b**, 56%; **9c**, 98%; (v) TFA / DCM (1/1 v/v), 30 min, **2a**, 87%; **2b**, 34%; **2c**, 57%.

Having the peptides in hand, attention was focussed on the evaluation of their structural properties. The unambiguous ^1H NMR resonance assignment of each peptide was performed using COSY, TOCSY and NOESY or ROESY experiments. The large resonance distribution, already indicative of secondary structure formation, allowed complete assignment of the data sets of peptides **2a-c**. To aid in identifying the presence of secondary structure elements, the vicinal spin-spin coupling constants of the amide bonds ($^3J_{\text{HN}\alpha}$, Figure 2A)¹¹ and the chemical shift perturbation of the H_α of individual amino acid residues ($\Delta\delta\text{H}_\alpha$, Figure 2B)¹² were compared to those found in native GS, as previously described in **Chapter 2** and **Chapter 5**. The distinctive trends found in these data were largely comparable to native GS and consistent with β -sheet formation in peptides **2a-c**.

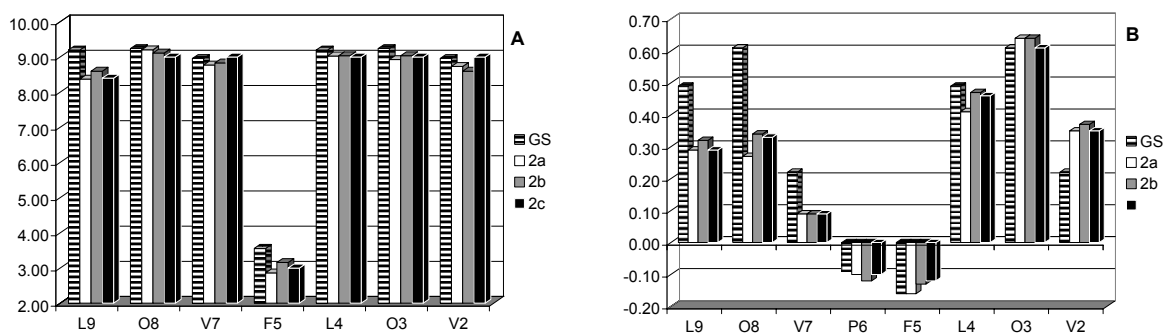


Figure 2: Coupling constants ($^3J_{\text{HN}\alpha}$) and the chemical shift perturbations ($\Delta\delta\text{H}_\alpha = \text{observed } \delta\text{H}_\alpha - \text{random coil } \delta\text{H}_\alpha$)¹³ found in **2a-c**.

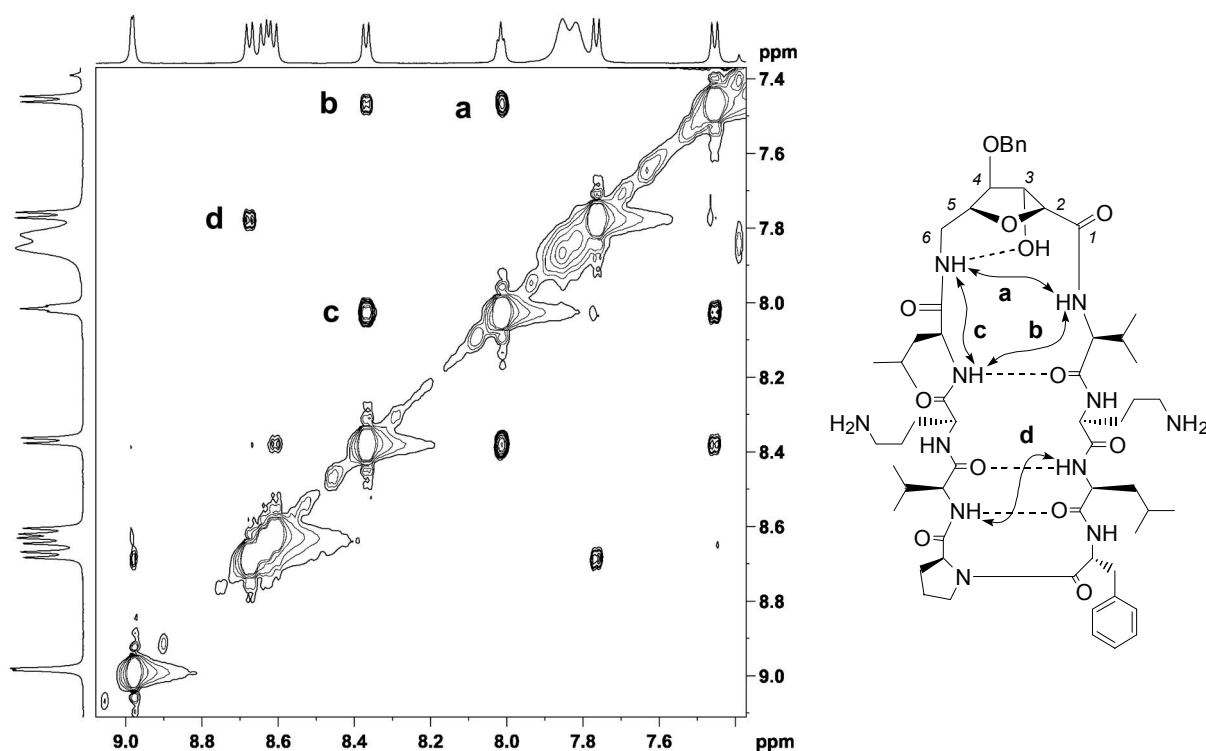


Figure 3: Amide region of the ROESY spectrum (600 MHz, CD₃OH) of peptide **2a**.

The preservation of the β -sheet structure in benzyl-derivatized **2a** was further corroborated by the observation of interstrand NH-NH NOEs, such as Val₂-Leu₉ (NOE **b**, Figure 3) and Val₇-Leu₄ (NOE **d**), as well as the sequential NH-NH crosspeak of SAA₁-Leu₉ (NOE **c**). Moreover, the characteristic NOE-contact between SAA₁-NH and its neighbouring Val₂-NH was discerned (NOE **a**, Figure 3). This provided evidence that the structure adopted by **1**, as observed through ¹H NMR and single crystal X-ray analysis (see **Chapter 4**),^{2a} was again assumed by GS analogue **2a**. Similar observations through spectroscopic comparison were made for biphenyl- and naphthalene-functionalized **2b** and **2c**, respectively. Thus, the 2,5-anhydroglucitol-based scaffold of SAAs **6a-c** appears to induce the same reverse turn conformation in GS analogues **2a-c** as the unfunctionalized SAA does in GS analogue **1** (see **Chapter 4**).

Table 1: Antimicrobial activity (MIC in μ g/mL).

Peptide	<i>S. aureus</i> ^a		<i>S. epidermidis</i> ^a		<i>E. faecalis</i> ^a		<i>B. cereus</i> ^a		<i>E. coli</i> ^b		<i>P. aeruginosa</i> ^b	
	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d	25W ^c	MT ^d
GS	4	4	2	2	8	8	4	4	>64	64	>64	>64
2a	8	2	2	2	16-32	16	4	4	64	64	>64	>64
2b	8	8	2	4	16	8-16	8	8	>64	>64	>64	>64
2c	4-8	4	2	4	8	8	4	4	>64	>64	>64	>64

Measurements were executed using standard agar two-fold dilution techniques.

^a Gram-positive ^b Gram-negative ^c 3 mL / 25 well plates ^d 100 μ L / 96 microtiter plates.

Having established the structure of GS analogues **2a-c**, the bactericidal activity of these analogues against a number of Gram-positive and -negative strains was assayed (see Table 1). The Gram-negative *E. coli* and *P. aeruginosa* strains proved resistant to the action of these GS-based antibiotics. However, the assays also indicated that Gram-positive strains such as *S. aureus*, *S. epidermidis*, *E. faecalis* and *B. cereus*, were once again susceptible to treatment with these antimicrobial peptides, with MIC-values comparable to those observed for native GS. The nature of the aromatic appendage from the SAA does not influence the biological profile, as the benzyl-, biphenyl- and naphthalene-derivatives proved to be equally active.

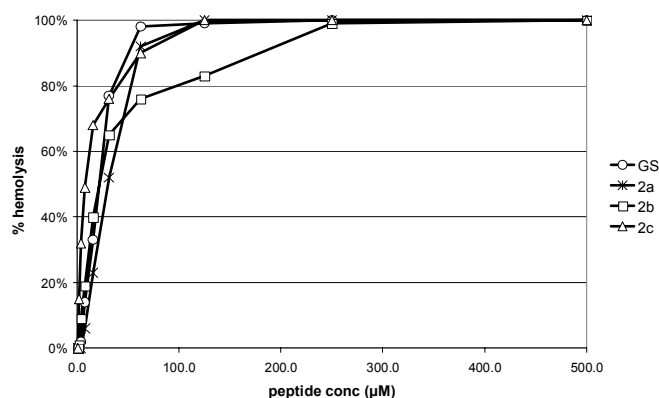


Figure 4: Hemolytic activity of GS analogues **2a-c**.

The hemolytic properties of peptides **2a-c** towards human erythrocytes was similarly assessed and compared to native GS, as is shown in Figure 4. The peptides **2a-c** show an increased toxicity compared to **1** (see **Chapter 5**) that is not influenced by the difference in aromatic moieties in GS analogues **2a-c**, as the benzyl-, biphenyl- and naphthalene-derivatives display hemolytic profiles comparable to GS. These data demonstrate that the advantageous effect of aromatic decoration of the SAAs on the capacity of peptides **2a-c** to arrest proliferation of various strains of bacteria, concurrently increases the toxicity towards human erythrocytes.

Conclusion

GS analogue **1**, of which the intriguing structure was reported in **Chapter 4** and that was later shown to exhibit a reduced bactericidal ability, formed the basis for the design of peptidomimetic SAAs from a 2,5-anhydroglucitol scaffold that were suitably decorated with aromaticity. The incorporation of the three SAAs **6a-c**, bearing a benzyl, biphenyl and naphthylene moiety, respectively, was successfully accomplished. Structural analysis, through perusal of the ^1H NMR data of the SAA-containing GS analogues **2a-c**, revealed that all three peptides adopt a β -sheet structure with the SAAs occupying a distinctive reverse turn analogous to peptide **1**, irrespective of the aromatic group appended. Examination of the biological activity showed that the bactericidal properties of the SAA-containing GS

analogues was completely restored to the level of the parent compound GS, albeit with concomitant increase in hemolytic activity. While these results do not bear on the potential clinical utility of GS analogues, they do suggest a valuable role for SAA-based compounds as peptidomimetics and encourage further efforts to design and synthesize alternate substrates to further our understanding of the structural requirements for biological activity of GS-based peptides.

Experimental Section

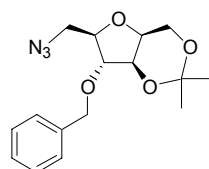
General: All reactions were performed under an inert atmosphere and at ambient temperature unless stated otherwise. Reactions were monitored by TLC-analysis using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by spraying with 20% H₂SO₄ in ethanol followed by charring at ~150°C or by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid followed by charring at ~150°C. Column chromatography was performed on Fluka silicagel (0.040 – 0.063 nm) and size exclusion chromatography on Sephadex™ LH-20. Mass spectra were recorded on a PE/Sciex API 165 instrument with a custom-build Electrospray Ionisation (ESI) interface and HRMS (SIM mode) were recorded on a TSQ Quantum (Thermo Finnigan) fitted with an accurate mass option, interpolating between PEG-calibration peaks. For LC/MS analysis, a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical Alltima C₁₈ column (Alltech, 4.6 mmD × 250 mmL, 5 μ particle size) in combination with buffers A: H₂O, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI) was used. For RP-HPLC purification of the peptides, a BioCAD “Vision” automated HPLC system (PerSeptiveBiosystems, inc.) equipped with a semi-preparative Alltima C₁₈ column (Alltech, 10.0 mmD × 250 mmL, 5 μm particle size) was used. The applied buffers were A: H₂O, B: MeCN and C: 1.0 % aq. TFA. ¹H- and ¹³C NMR spectra were recorded on a Bruker AV-400 (400/100 MHz) and the peptides were analyzed using a Bruker DMX-600 (600 MHz) spectrometer equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard (¹H NMR) or CDCl₃ (¹³C NMR). Coupling constants are given in Hz. All presented ¹³C-APT spectra are proton decoupled. Optical rotations were measured on a Propol automatic polarimeter (Sodium D line, λ = 589 nm) and ATR-IR spectra were recorded on a Shimadzu FTIR-8300 fitted with a single bounce DurasamplIR diamond crystal ATR-element.

General Alkylation Procedure: Alcohol **3** (1.96 g, 8.55 mmol) was dissolved in DMF (40 mL) and cooled to 0°C. To the solution were added benzylbromide (1.124 mL, 9.4 mmol, 1.1 equiv) and sodium hydride (376 mg, 9.4 mmol, 1.1 equiv) and the mixture was stirred overnight. The reaction was quenched with methanol (5 mL) and concentrated *in vacuo*. The crude mixture was partitioned between sat. aq. NaHCO₃ and EtOAc and the aqueous layer was subsequently extracted with EtOAc twice. The combined organic layers were dried (MgSO₄), filtered and concentrated. Silica gel column chromatography (20%→30% EtOAc in light PE) yielded the azide **4a** (2.54 g, 7.95 mmol) in 93% as a transparant oil.

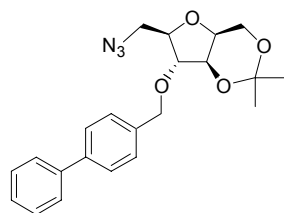
General Methanolysis Procedure: Isopropylidene-protected **4a** (1.6 g, 5.0 mmol) was dissolved in methanol (10 mL) and PPTS (~50 mg, cat) was added. The mixture was heated to 50°C and stirred overnight. The reaction was diluted with EtOAc, extracted with sat. aq. NaHCO₃, dried (MgSO₄), filtered and evaporated to dryness. Silica gel column chromatography (50%→70% EtOAc in light PE) produced diol **5a** (0.945 g, 3.39 mmol) in 68% as a white amorphous solid.

General Oxidation Procedure: Diol **5a** (474 mg, 1.70 mmol) was dissolved in acetonitrile (10 mL) after which sat. aq. NaHCO_3 (4 mL) containing KBr (~20 mg, cat) was added and the mixture was cooled to 0°C. Subsequently, TEMPO catalyst (5 mg) in acetonitrile (3 mL) was added and a premixed solution of 15% NaOCl (7.5 mL, 1.80 mmol) in sat. aq. NaHCO_3 (4.5 mL) and sat. aq. NaCl (8.8 mL) was added dropwise to the reaction mixture, keeping it oscillating between yellow and colorless. The reaction was then quenched with MeOH, acidified to pH = 4 with HCl (1 M) and extracted with DCM thrice. The combined organic layers were dried (MgSO_4), filtered and evaporated. Silica gel column chromatography (20%→30% EtOAc in light PE) quantitatively furnished SAA **6a** (507 mg, 1.70 mmol) as a transparent oil.

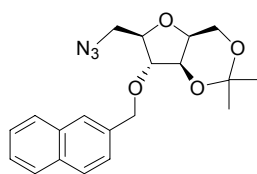
General Peptide Synthesis Procedure: Fmoc-based solid phase peptide synthesis was performed as described in Chapter 4 and Chapter 5, starting with preloaded resin **7** (100 μmol , 200 mg) and a final coupling of **6a**, **6b** or **6c** (0.2 mmol, 2 equiv), BOP (132 mg, 0.3 mmol, 3 equiv), HOBT (41 mg, 0.3 mmol, 3 equiv) and DiPEA (58 μL , 0.35 mmol, 3.5 equiv) in NMP (2 mL), to ultimately furnish the title compound **9a** (116 mg, 86 μmol , 86%), **9b** (80 mg, 56 μmol , 56%) and **9c** (137 mg, 98 μmol , 98%), respectively as white amorphous solids. The Boc-protected peptides **9a** (58 mg, 43 μmol), **9b** (20 mg, 14 μmol) and **9c** (11.6 mg, 8.3 μmol) were individually dissolved in DCM (2 mL) and cooled to 0°C. These mixtures were subsequently treated with TFA (2 mL), stirred for 30 min and diluted with toluene (10 mL) after which the volatiles were removed *in vacuo*.



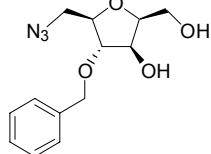
2,5-Anhydro-6-azido-4-O-benzyl-6-deoxy-1,3-O-isopropylidene-D-glucitol (4a): Starting from alcohol **3** (1.96 g, 8.55 mmol), the title compound (2.54 g, 7.95 mmol, 93%) was prepared as described in the general procedure, as a transparent oil. ^1H NMR (400 MHz, CDCl_3): δ = 7.29-7.24 (m, 5H, H_{ar}), 4.57 (s, 2H, CH_2 Bn), 4.27 (d, 1H, H_3 , $J_{3,2}$ = 2.9 Hz), 4.08 (ddd, 1H, H_5 , $J_{5,4}$ = 2.4 Hz, $J_{5,6b}$ = 6.1 Hz, $J_{5,6a}$ = 6.8 Hz), 4.04 (dd, 1H, H_{1a} , $J_{1a,2}$ = 2.9 Hz, $J_{1a,1b}$ = 13.3 Hz), 3.97 (dd, 1H, H_{1b} , $J_{1b,2}$ = 2.0 Hz, $J_{1b,1a}$ = 13.3 Hz), 3.90 (ddd, 1H, H_2 , $J_{2,1b}$ = 2.0 Hz, $J_{2,1a}$ = 2.9 Hz, $J_{2,3}$ = 2.9 Hz), 3.81 (d, 1H, H_4 , $J_{4,5}$ = 2.4 Hz), 3.54 (dd, 1H, H_{6a} , $J_{6a,5}$ = 6.8 Hz, $J_{6a,6b}$ = 12.4 Hz), 3.38 (dd, 1H, H_{6b} , $J_{6b,5}$ = 6.1 Hz, $J_{6b,6a}$ = 12.4 Hz), 1.41 (s, 3H, CH_3 *i*Pr), 1.37 (s, 3H, CH_3 *i*Pr). ^{13}C NMR (100 MHz, CDCl_3): δ = 137.3 (C_q Bn), 128.4-127.6 (C_{ar} Bn), 97.5 (C_q *i*Pr), 86.2 (C_4), 82.8 (C_5), 73.8 (C_3), 73.2 (C_2), 71.8 (CH_2 Bn), 60.3 (C_1), 52.3 (C_6), 28.6 (CH_3 *i*Pr), 19.0 (CH_3 *i*Pr). ATR-IR (thin film): 2877.6, 2096.5, 1454.2, 1375.2, 1276.8, 1197.7, 1130.2, 1089.7, 1028.0, 970.1, 925.8, 846.7, 736.8, 698.2 cm^{-1} . $[\alpha]_D^{23}$ +34.0 (c = 1.00, CHCl_3). MS (ESI): m/z = 320.0 $[\text{M}+\text{H}]^+$, 342.0 $[\text{M}+\text{Na}]^+$, 661.3 $[2\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{16}\text{H}_{21}\text{N}_3\text{O}_4\text{NH}_4$ 337.18758, found 337.18790.



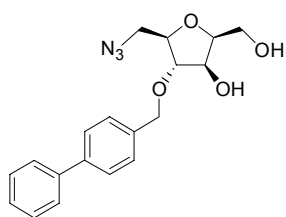
2,5-Anhydro-6-azido-4-O-(biphenyl-4-ylmethyl)-6-deoxy-1,3-O-isopropylidene-D-glucitol (4b): Starting from alcohol **3** (0.92 g, 4.0 mmol), the title compound was prepared (1.41 g, 3.56 mmol, 89%) as described in the general procedure, as a transparent oil. ^1H NMR (400 MHz, CDCl_3): δ = 7.59-7.33 (m, 9H, H_{ar}), 4.61 (s, 2H, CH_2 Biph), 4.30 (d, 1H, H_3 , $J_{3,2}$ = 2.8 Hz), 4.11 (ddd, 1H, H_5 , $J_{5,4}$ = 2.2 Hz, $J_{5,6b}$ = 6.1 Hz, $J_{5,6a}$ = 6.8 Hz), 4.06 (dd, 1H, H_{1a} , $J_{1a,2}$ = 2.9 Hz, $J_{1a,1b}$ = 13.3 Hz), 3.99 (dd, 1H, H_{1b} , $J_{1b,2}$ = 1.9 Hz, $J_{1b,1a}$ = 13.3 Hz), 3.92 (ddd, 1H, H_2 , $J_{2,1b}$ = 1.9 Hz, $J_{2,1a}$ = 1.9 Hz, $J_{2,3}$ = 2.8 Hz), 3.85 (d, 1H, H_4 , $J_{4,5}$ = 2.2 Hz), 3.57 (dd, 1H, H_{6a} , $J_{6a,5}$ = 6.8 Hz, $J_{6a,6b}$ = 12.4 Hz), 3.42 (dd, 1H, H_{6b} , $J_{6b,5}$ = 6.1 Hz, $J_{6b,6a}$ = 12.4 Hz), 1.42 (s, 3H, CH_3 *i*Pr), 1.38 (s, 3H, CH_3 *i*Pr). ^{13}C NMR (100 MHz, CDCl_3): δ = 141.0 (C_q Biph), 140.7 (C_q Biph), 136.3 (C_q Biph), 128.6-127.1 (C_{ar} Biph), 97.6 (C_q *i*Pr), 86.3 (C_4), 82.8 (C_5), 73.9 (C_3), 73.3 (C_2), 71.7 (CH_2 Biph), 60.4 (C_1), 52.8 (C_6), 28.7 (CH_3 *i*Pr), 19.1 (CH_3 *i*Pr). ATR-IR (thin film): 2993.3, 2916.2, 2360.7, 2098.4, 1488.9, 1450.4, 1373.2, 1280.6, 1195.8, 1087.8, 1010.6, 972.1, 925.8, 840.9, 763.8 cm^{-1} . $[\alpha]_D^{23}$ +26.4 (c = 1.00, CHCl_3). MS (ESI): m/z = 418.3 $[\text{M}+\text{Na}]^+$, 813.5 $[2\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_4\text{Na}$ 418.17373, found 418.17349.



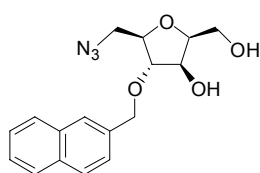
2,5-Anhydro-6-azido-6-deoxy-1,3-O-isopropylidene-4-O-(naphthalen-2-ylmethyl)-D-glucitol (4c): Starting from alcohol **3** (0.86 g, 3.75 mmol), the title compound was prepared (1.06 g, 2.88 mmol, 77%) as described in the general procedure, as a transparent oil. ^1H NMR (400 MHz, CDCl_3): δ = 7.86–7.42 (m, 7H, H_{ar}), 5.05 (d, 1H, CH_2 Naph, $J_{\text{Ha,Hb}}$ = 11.9 Hz), 4.98 (d, 1H, CH_2 Naph, $J_{\text{Hb,Hb}} = 11.9$ Hz), 4.26 (d, 1H, H_3 , $J_{3,2} = 2.9$ Hz), 4.10 (ddd, 1H, H_5 , $J_{5,4} = 2.2$ Hz, $J_{5,6b} = 6.2$ Hz, $J_{5,6a} = 6.7$ Hz), 4.03 (dd, 1H, H_{1a} , $J_{1a,2} = 2.9$ Hz, $J_{1a,1b} = 13.3$ Hz), 3.96 (dd, 1H, H_{1b} , $J_{1b,2} = 1.9$ Hz, $J_{1b,1a} = 13.3$ Hz), 3.90 (m, 2H, H_2 , H_4), 3.53 (dd, 1H, H_{6a} , $J_{6a,5} = 6.7$ Hz, $J_{6a,6b} = 12.4$ Hz), 3.36 (dd, 1H, H_{6b} , $J_{6b,5} = 6.2$ Hz, $J_{6b,6a} = 12.4$ Hz), 1.38 (s, 3H, CH_3 *i*Pr), 1.37 (s, 3H, CH_3 *i*Pr). ^{13}C NMR (100 MHz, CDCl_3): δ = 133.8 (C_q Naph), 132.8 (C_q Naph), 131.5 (C_q Naph), 129.0–123.7 (C_{ar} Naph), 97.6 (C_q *i*Pr), 86.2 (C_4), 82.7 (C_5), 74.0 (C_3), 73.3 (C_2), 70.6 (CH_2 Naph), 60.4 (C_1), 52.8 (C_6), 28.6 (CH_3 *i*Pr), 19.1 (CH_3 *i*Pr). ATR-IR (thin film): 2993.3, 2916.2, 2360.7, 2098.4, 1512.1, 1450.4, 1373.2, 1280.6, 1195.8, 1087.8, 1010.6, 972.1, 925.8, 840.6, 771.5 cm^{-1} . $[\alpha]_{\text{D}}^{23} +21.2$ ($c = 1.00$, CHCl_3). MS (ESI): m/z = 370.1 $[\text{M}+\text{H}]^+$, 392.0 $[\text{M}+\text{Na}]^+$, 761.3 $[2\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{20}\text{H}_{23}\text{N}_3\text{O}_4\text{Na}$ 392.15808, found 392.15881.



2,5-Anhydro-6-azido-4-O-benzyl-6-deoxy-D-glucitol (5a): Starting from acetonide **4a** (1.6 g, 5 mmol), the title compound was prepared (0.945 g, 3.39 mmol, 68%) as described in the general procedure, and obtained as a white amorphous solid. ^1H NMR (400 MHz, CDCl_3): δ = 7.35–7.22 (m, 5H, H_{ar}), 4.62 (d, 1H, CH_2 Bn, $J_{\text{Ha,Hb}}$ = 11.7 Hz), 4.50 (d, 1H, CH_2 Bn, $J_{\text{Hb,Hb}} = 11.7$ Hz), 4.37 (dd, 1H, H_3 , $J_{3,2} = 1.7$ Hz, $J_{3,4} = 3.8$ Hz), 4.0 (m, 4H, H_{1a} , H_{1b} , H_2 , H_5), 3.84 (dd, 1H, H_4 , $J_{4,5} = 2.1$ Hz, $J_{4,3} = 3.8$ Hz), 3.63 (dd, 1H, H_{6a} , $J_{6a,5} = 3.8$ Hz, $J_{6a,6b} = 12.9$ Hz), 3.41 (dd, 1H, H_{6b} , $J_{6b,5} = 4.9$ Hz, $J_{6b,6a} = 12.9$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ = 137.1 (C_q Bn), 128.4–127.6 (C_{ar} Bn), 86.5 (C_4), 81.7 (C_5), 80.2 (C_2), 76.2 (C_3), 71.0 (CH_2 Bn), 60.6 (C_1), 52.3 (C_6). ATR-IR (thin film): 2877.6, 2360.7, 2102.3, 1784.0, 1670.2, 1558.4, 1456.2, 1436.9, 1278.7, 1203.5, 1141.8, 1066.6, 1028.0, 908.4, 839.0, 800.4, 727.1 cm^{-1} . $[\alpha]_{\text{D}}^{23} +76.6$ ($c = 1.00$, CHCl_3). MS (ESI): m/z 280.2 $[\text{M}+\text{H}]^+$, 301.9 $[\text{M}+\text{Na}]^+$, 581.4 $[2\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{13}\text{H}_{17}\text{N}_3\text{O}_4\text{NH}_4$ 297.15628, found 297.15866.

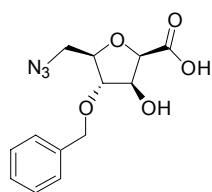


2,5-Anhydro-6-azido-4-O-(biphenyl-4-ylmethyl)-6-deoxy-D-glucitol (5b): Starting from acetonide **4b** (0.671 g, 1.7 mmol), the title compound was prepared (0.348 g, 0.98 mmol, 58%) as described in the general procedure, and obtained as a white amorphous solid. ^1H NMR (400 MHz, CDCl_3): δ = 7.56–7.32 (m, 9H, H_{ar}), 4.70 (d, 1H, CH_2 Biph, $J_{\text{Ha,Hb}}$ = 11.8 Hz), 4.59 (d, 1H, CH_2 Biph, $J_{\text{Hb,Hb}} = 11.8$ Hz), 4.36 (dd, 1H, H_3 , $J_{3,2} = 2.0$ Hz, $J_{3,4} = 3.9$ Hz), 4.05 (ddd, 1H, H_5 , $J_{5,4} = 3.8$ Hz, $J_{5,6a} = 3.9$ Hz, $J_{5,6b} = 4.0$ Hz), 4.03 (dd, 1H, H_2 , $J_{2,3} = 3.9$ Hz, $J_{2,1a} = 3.9$ Hz), 4.0 (dd, 1H, H_{1a} , $J_{1a,2} = 3.9$ Hz, $J_{1a,1b} = 12.3$ Hz), 3.94 (m, 1H, H_{1b}), 3.84 (dd, 1H, H_4 , $J_{4,3} = 2.0$ Hz, $J_{4,5} = 3.8$ Hz), 3.56 (dd, 1H, H_{6a} , $J_{6a,5} = 3.9$ Hz, $J_{6a,6b} = 12.9$ Hz), 3.45 (dd, 1H, H_{6b} , $J_{6b,5} = 5.5$ Hz, $J_{6b,6a} = 12.9$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ = 140.9 (C_q Biph), 140.6 (C_q Biph), 136.4 (C_q Biph), 128.7–127.0 (C_{ar} Biph), 86.8 (C_4), 81.6 (C_5), 80.3 (C_2), 77.1 (C_3), 71.7 (CH_2 Biph), 61.4 (C_1), 52.6 (C_6). ATR-IR (thin film): 3402.2, 3031.9, 2923.9, 2098.4, 1720.4, 1488.9, 1450.4, 1404.1, 1365.5, 1280.6, 1072.3, 918.1, 825.5, 763.8, 694.3 cm^{-1} . $[\alpha]_{\text{D}}^{23} +68.0$ ($c = 1.00$, CHCl_3). MS (ESI): m/z 378.2 $[\text{M}+\text{Na}]^+$, 733.3 $[2\text{M}+\text{Na}]^+$. HRMS: calcd for $\text{C}_{19}\text{H}_{21}\text{N}_3\text{O}_4\text{Na}$ 378.14243, found 378.14264.

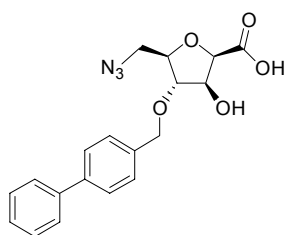


2,5-Anhydro-6-azido-6-deoxy-4-O-(naphthalen-2-ylmethyl)-D-glucitol (5c): Starting from acetonide **4c** (0.517 g, 1.4 mmol), the title compound was prepared (0.232 g, 0.704 mmol, 50%) as described in the general procedure, and obtained as a white amorphous solid. ^1H NMR (400 MHz, CDCl_3): δ = 7.82–7.38 (m, 7H, H_{ar}), 5.08 (d, 1H, CH_2 Naph, $J_{\text{Ha,Hb}}$ = 11.9 Hz), 4.95 (d, 1H, CH_2 Naph, $J_{\text{Hb,Hb}} = 11.9$ Hz), 4.32 (bs, 1H, H_3), 4.00 (dd, 1H, H_2 , $J_{2,3} = 3.9$ Hz, $J_{2,1a} = 4.0$ Hz), 3.9 (m,

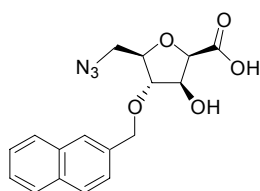
3H, H_{1a}, H_{1b}, H₅), 3.84 (dd, 1H, H₄, $J_{4,3} = 2.0$ Hz, $J_{4,5} = 3.9$ Hz), 3.41 (dd, 1H, H_{6a}, $J_{6a,5} = 4.1$ Hz, $J_{6a,6b} = 12.8$ Hz), 3.28 (dd, 1H, H_{6b}, $J_{6b,5} = 5.5$ Hz, $J_{6b,6a} = 12.8$ Hz). ¹³C NMR (100 MHz, CDCl₃): $\delta = 133.6$ (C_q Naph), 132.8 (C_q Naph), 131.4 (C_q Naph), 128.9-123.6 (C_{ar} Naph), 86.6 (C₄), 81.4 (C₅), 80.3 (C₂), 76.9 (C₃), 70.5 (CH₂ Naph), 61.2 (C₁), 52.5 (C₆). ATR-IR (thin film): 3394.5, 3055.0, 2923.9, 2877.6, 2360.7, 2098.4, 1720.7, 1596.9, 1512.1, 1442.7, 1272.9, 1041.5, 925.8, 756.0 cm⁻¹. $[\alpha]_D^{23} +75.6$ (c = 1.00, CHCl₃). MS (ESI): m/z 352.0 [M+Na]⁺, 659.7 [2M+H]⁺, 681.4 [2M+Na]⁺. HRMS: calcd for C₁₇H₁₉N₃O₄Na 352.12678, found 352.12714.



2,5-Anhydro-6-azido-4-O-benzyl-6-deoxy-D-gluconic acid (6a): Starting from diol **5a** (0.474 g, 1.70 mmol), the title compound was prepared (0.507 g, 1.70 mmol, quant.) as described in the general procedure, and obtained as a transparent oil. ¹H NMR (400 MHz, CDCl₃): $\delta = 7.38$ -7.30 (m, 5H, H_{ar}), 4.69 (d, 1H, H₂, $J_{2,3} = 3.9$ Hz), 4.66 (d, 1H, CH₂ Bn, $J_{Ha-Hb} = 11.8$ Hz), 4.55 (d, 1H, CH₂ Bn, $J_{Hb-Ha} = 11.8$ Hz), 4.52 (dd, 1H, H₃, $J_{3,4} = 1.2$ Hz, $J_{3,2} = 3.9$ Hz), 4.14 (ddd, 1H, H₅, $J_{5,4} = 2.7$ Hz, $J_{5,6b} = 4.9$ Hz, $J_{5,6a} = 4.95$ Hz), 3.89 (dd, 1H, H₄, $J_{4,3} = 1.2$ Hz, $J_{4,5} = 2.7$ Hz), 3.58 (dd, 1H, H_{6a}, $J_{6a,5} = 4.9$ Hz, $J_{6a,6b} = 12.8$ Hz), 3.57 (dd, 1H, H_{6b}, $J_{6b,5} = 4.9$ Hz, $J_{6b,6a} = 12.8$ Hz). ¹³C NMR (100 MHz, CDCl₃): $\delta = 172.2$ (C₁), 136.9 (C_q Bn), 128.6-127.8 (C_{ar} Bn), 85.5 (C₄), 83.1 (C₅), 81.4 (C₂), 75.9 (C₃), 72.1 (CH₂ Bn), 52.2 (C₆). ATR-IR (thin film): 3853.5, 3649.1, 2875.7, 2360.7, 2339.5, 2104.2, 1733.9, 1652.9, 1624.0, 1558.4, 1496.7, 1454.2, 1436.9, 1282.6, 1207.4, 1095.5, 1070.4, 1028.0, 912.3, 881.4, 819.7, 740.6, 700.1 cm⁻¹. $[\alpha]_D^{23} +90.0$ (c 1.00, CHCl₃). MS (ESI): m/z 316.1 [M+Na]⁺, 609.1 [2M+Na]⁺, 902.3 [3M+Na]⁺.

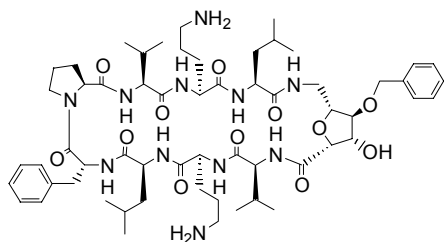


2,5-Anhydro-6-azido-4-O-(biphenyl-4-ylmethyl)-6-deoxy-D-gluconic acid (6b): Starting from diol **5b** (107 mg, 0.30 mmol), the title compound was prepared (56 mg, 0.15 mmol, 52%) as described in the general procedure, and obtained as a transparent oil. ¹H NMR (400 MHz, CDCl₃): $\delta = 7.60$ -7.33 (m, 9H, H_{ar}), 4.70 (d, 1H, CH₂ Biph, $J_{Ha-Hb} = 11.8$ Hz), 4.66 (d, 1H, H₂, $J_{2,3} = 4.1$ Hz), 4.62 (d, 1H, CH₂ Biph, $J_{Hb-Ha} = 11.8$ Hz), 4.51 (dd, 1H, H₃, $J_{3,4} = 1.0$ Hz, $J_{3,2} = 4.1$ Hz), 4.14 (ddd, 1H, H₅, $J_{5,4} = 2.6$ Hz, $J_{5,6b} = 5.5$ Hz, $J_{5,6a} = 5.8$ Hz), 3.91 (dd, 1H, H₄, $J_{4,3} = 1.0$ Hz, $J_{4,5} = 2.6$ Hz), 3.64 (dd, 1H, H_{6a}, $J_{6a,5} = 5.8$ Hz, $J_{6a,6b} = 12.6$ Hz), 3.55 (dd, 1H, H_{6b}, $J_{6b,5} = 5.5$ Hz, $J_{6b,6a} = 12.6$ Hz). ¹³C NMR (100 MHz, CDCl₃): $\delta = 170.9$ (C₁), 140.9 (C_q Biph), 140.5 (C_q Biph), 136.0 (C_q Biph), 128.9-126.9 (C_{ar} Biph), 85.8 (C₄), 82.8 (C₅), 81.4 (C₂), 75.6 (C₃), 71.5 (CH₂ Biph), 52.3 (C₆). ATR-IR (thin film): 3309.6, 3031.9, 2923.9, 2360.7, 2098.4, 1728.1, 1604.7, 1450.4, 1396.4, 1280.6, 1218.9, 1080.1, 972.1, 910.3, 825.5, 756.0 cm⁻¹. $[\alpha]_D^{23} +50.8$ (c 1.00, CHCl₃). MS (ESI): m/z 392.1 [M+Na]⁺, 739.4 [2M+H]⁺, 761.4 [2M+Na]⁺. HRMS: calcd for C₁₉H₁₉N₃O₅Na 392.12169, found 392.12253.



2,5-Anhydro-6-azido-6-deoxy-4-O-(naphthalen-2-ylmethyl)-D-gluconic acid (6c): Starting from diol **5a** (132 mg, 0.4 mmol), the title compound was prepared (100 mg, 0.29 mmol, 72%) as described in the general procedure, and obtained as a transparent oil. ¹H NMR (400 MHz, CDCl₃): $\delta = 8.02$ -7.39 (m, 7H, H_{ar}), 5.08 (d, 1H, CH₂ Naph, $J_{Ha-Hb} = 11.9$ Hz), 4.94 (d, 1H, CH₂ Naph, $J_{Hb-Ha} = 11.9$ Hz), 4.67 (d, 1H, H₂, $J_{2,3} = 3.9$ Hz), 4.54 (dd, 1H, H₃, $J_{3,4} = 1.2$ Hz, $J_{3,2} = 3.9$ Hz), 4.07 (ddd, 1H, H₅, $J_{5,4} = 2.5$ Hz, $J_{5,6a} = 4.6$ Hz, $J_{5,6b} = 5.8$ Hz), 3.92 (dd, 1H, H₄, $J_{4,3} = 1.2$ Hz, $J_{4,5} = 2.5$ Hz), 3.46 (dd, 1H, H_{6a}, $J_{6a,5} = 4.6$ Hz, $J_{6a,6b} = 12.8$ Hz), 3.41 (dd, 1H, H_{6b}, $J_{6b,5} = 5.8$ Hz, $J_{6b,6a} = 12.8$ Hz). ¹³C NMR (100 MHz, CDCl₃): $\delta = 172.2$ (C₁), 133.7 (C_q Naph), 132.4 (C_q Naph), 131.5 (C_q Naph), 129.2-123.7 (C_{ar} Naph), 85.2 (C₄), 83.0 (C₅), 81.4 (C₂), 76.0 (C₃), 70.7 (CH₂ Naph), 52.1 (C₆). ATR-IR (thin film): 3409.9, 3209.3, 3055.0, 2923.9, 2646.2, 2360.7, 2106.1, 1735.8, 1596.9, 1512.1, 1434.9, 1350.1, 1272.9, 1218.9, 1095.5, 910.3, 879.5, 779.2 cm⁻¹. $[\alpha]_D^{23} +84.2$ (c 1.00,

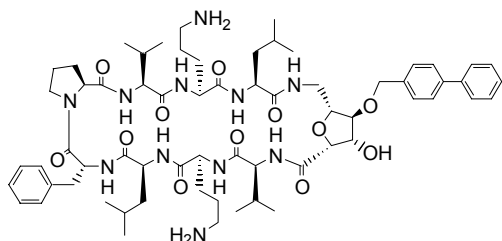
CHCl₃). MS (ESI): m/z 344.0 [M+H]⁺, 366.0 [M+Na]⁺, 687.4 [2M+H]⁺, 709.4 [2M+Na]⁺. HRMS: calcd for C₁₇H₁₇N₃O₅ Na 366.10604, found 366.10657.



cyclo-[SAA_{6a}-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu] (2a):

The obtained peptide (see General Procedure) was analyzed by LC/MS (R_t 14.64 min, linear gradient 05→90% B in 20 min; m/z = 1146.8 [M+H]⁺, 574.2 [M+H]²⁺) and purified by semi-preparative RP-HPLC (linear gradient of 3.0 CV; 40→50% B; R_t 2.9 CV). Lyophilization of the combined

fractions furnished peptide **2a** (43.2 mg, 37.6 μ mol, 87%) as white amorphous powder. ¹H NMR (600 MHz, CD₃OH): δ 8.98 (d, 1H, NH ^DPhe₅, $J_{\text{NH,H}\alpha}$ = 2.9 Hz), 8.67 (d, 1H, NH Leu₄, $J_{\text{NH,H}\alpha}$ = 9.0 Hz), 8.64 (d, 1H, NH _{α} Orn₃, $J_{\text{NH,H}\alpha}$ = 8.9 Hz), 8.63 (d, 1H, NH _{α} Orn₈, $J_{\text{NH,H}\alpha}$ = 9.2 Hz), 8.37 (d, 1H, NH Leu₉, $J_{\text{NH,H}\alpha}$ = 8.4 Hz), 8.02 (t, 1H, NH SAA₁, $J_{\text{NH,H}\alpha}$ = 4.4 Hz), 7.85 (bs, 2H, NH _{δ} Orn₃), 7.82 (bs, 2H, NH _{δ} Orn₈), 7.77 (d, 1H, NH Val₇, $J_{\text{NH,H}\alpha}$ = 8.8 Hz), 7.46 (d, 1H, NH Val₂, $J_{\text{NH,H}\alpha}$ = 8.7 Hz), 7.33 – 7.22 (m, 10H, H_{ar} ^DPhe₅, H_{ar} SAA₁), 5.00 (m, 1H, H _{α} Orn₃), 4.63 (m, 1H, H _{α} Orn₈), 4.64 (m, 1H, H _{α} Leu₄), 4.62 (d, 1H, CH_d Bn SAA₁, $J_{\text{CHd,CHu}}$ = 11.7 Hz), 4.57 (d, 1H, CH_u Bn SAA₁, $J_{\text{CHu,CHd}}$ = 11.7 Hz), 4.50 (m, 1H, H _{α} ^DPhe₅), 4.49 (m, 2H, H₂, H₃ SAA₁), 4.46 (m, 1H, H _{α} Leu₉), 4.34 (m, 1H, H _{α} Pro₆), 4.30 (m, 1H, H _{α} Val₂), 4.27 (m, 1H, H₅ SAA₁), 4.04 (m, 1H, H _{α} Val₇), 3.85 (d, 1H, H₄ SAA₁, $J_{\text{H4,H5}}$ = 1.7 Hz), 3.72 (m, 1H, H _{δ d} Pro₆), 3.63 (ddd, 1H, H_{6d} SAA₁, $J_{6d,5}$ = $J_{6d,\text{NH}}$ = 4.4 Hz, $J_{6d,6u}$ = 14.7 Hz), 3.38 (ddd, 1H, H_{6u} SAA₁, $J_{6u,5}$ = $J_{6u,\text{NH}}$ = 4.4 Hz, $J_{6u,6d}$ = 14.7 Hz), 3.08 (dd, 1H, H _{β d} ^DPhe₅, $J_{\beta d,\alpha}$ = 5.0 Hz, $J_{\beta d,\beta u}$ = 12.6 Hz), 3.00 (m, 1H, H _{δ d} Orn₃), 2.93 (m, 4H, H _{δ} Orn₈, H _{δu} Orn₃, H _{βu} ^DPhe₅), 2.46 (m, 1H, H _{δu} Pro₆), 2.29 (m, 1H, H _{β} Val₇), 2.06 (m, 1H, H _{β} Val₂), 1.97 (m, 2H, H _{β d} Pro₆, H _{β d} Orn₃), 1.83 (m, 1H, H _{β d} Orn₈), 1.76 (m, 3H, H _{βu} , γ Orn₃), 1.68 (m, 2H, H _{βu} , γ d Pro₆), 1.67 (m, 2H, H _{γ} Orn₈), 1.65 (m, 3H, H _{β} , γ Leu₉), 1.63 (m, 1H, H _{βu} Orn₈), 1.57 (m, 1H, H _{γu} Pro₆), 1.54 (m, 2H, H _{β d}, γ Leu₄), 1.40 (m, 1H, H _{βu} Leu₄), 0.96 (m, 9H, H _{γ d} Val₇, H _{γ} Val₂), 0.89 (m, 6H, H _{δ} Leu₄), 0.85 (m, 9H, H _{δ} Leu₉, H _{γu} Val₇).

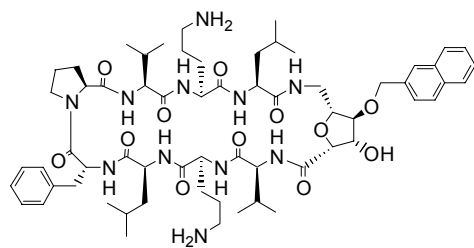


cyclo-[SAA_{6b}-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu] (2b):

The obtained peptide (see General Procedure) was analyzed by LC/MS (R_t 16.51 min, linear gradient 10→90% B in 20 min; m/z = 1223.0 [M+H]⁺, 612.1 [M+H]²⁺) and purified by semi-preparative RP-HPLC (linear gradient of 3.0 CV; 45→65% B; R_t 2.8 CV).

Lyophilization of the combined fractions furnished

peptide **2b** (5.7 mg, 4.7 μ mol, 34%) as white amorphous powder. ¹H NMR (600 MHz, CD₃OH): δ = 8.94 (d, 1H, NH ^DPhe₅, $J_{\text{NH,H}\alpha}$ = 3.2 Hz), 8.65 (d, 1H, NH Leu₄, $J_{\text{NH,H}\alpha}$ = 9.0 Hz), 8.61 (d, 1H, NH _{α} Orn₃, $J_{\text{NH,H}\alpha}$ = 9.0 Hz), 8.60 (d, 1H, NH _{α} Orn₈, $J_{\text{NH,H}\alpha}$ = 9.1 Hz), 8.29 (d, 1H, NH Leu₉, $J_{\text{NH,H}\alpha}$ = 8.6 Hz), 8.03 (t, 1H, NH SAA₁, $J_{\text{NH,H}\alpha}$ = 4.5 Hz), 7.78 (d, 1H, NH Val₇, $J_{\text{NH,H}\alpha}$ = 8.8 Hz), 7.60 (m, 4H, H_{ar} SAA₁), 7.49 (d, 1H, NH Val₂, $J_{\text{NH,H}\alpha}$ = 8.6 Hz), 7.42 (m, 4H, H_{ar} SAA₁), 7.33 – 7.23 (m, 6H, H_{ar} SAA₁, H_{ar} ^DPhe₅), 5.00 (m, 1H, H _{α} Orn₃), 4.70 (m, 1H, H _{α} Orn₈), 4.67 (m, 1H, H _{α} Leu₄), 4.64 (d, 1H, CH_d SAA₁, $J_{\text{CHd,CHu}}$ = 11.1 Hz), 4.62 (d, 1H, CH_u SAA₁, $J_{\text{CHu,CHd}}$ = 11.1 Hz), 4.53 (m, 1H, H _{α} ^DPhe₅), 4.49 (m, 2H, H₂, H₃ SAA₁), 4.47 (m, 1H, H _{α} Leu₉), 4.32 (m, 1H, H _{α} Pro₆), 4.32 (m, 1H, H _{α} Val₂), 4.28 (m, 1H, H₅ SAA₁), 4.04 (m, 1H, H _{α} Val₇), 3.86 (d, 1H, H₄ SAA₁, $J_{\text{H4,H5}}$ = 1.6 Hz), 3.73 (m, 1H, H _{δ d} Pro₆), 3.57 (ddd, 1H, H_{6d} SAA₁, $J_{6d,5}$ = $J_{6d,\text{NH}}$ = 3.8 Hz, $J_{6d,6u}$ = 14.5 Hz), 3.38 (ddd, 1H, H_{6u} SAA₁, $J_{6u,5}$ = $J_{6u,\text{NH}}$ = 3.8 Hz, $J_{6u,6d}$ = 14.5 Hz), 3.08 (dd, 1H, H _{β d} ^DPhe₅, $J_{\beta d,\alpha}$ = 4.9 Hz, $J_{\beta d,\beta u}$ = 12.6 Hz), 2.99 (m, 1H, H _{δ d} Orn₃), 2.91 (m, 4H, H _{δ} Orn₈, H _{δu} Orn₃, H _{βu} ^DPhe₅), 2.48 (m, 1H, H _{δu} Pro₆), 2.28 (m, 1H, H _{β} Val₇), 2.08 (m, 1H, H _{β} Val₂), 1.97 (m, 2H, H _{β d} Pro₆, H _{β d} Orn₃), 1.83 (m, 1H, H _{β d} Orn₈), 1.76 (m, 3H, H _{βu} , γ Orn₃), 1.68 (m, 2H, H _{βu} , γ d Pro₆), 1.67 (m, 2H, H _{γ} Orn₈), 1.65 (m, 3H, H _{β} , γ Leu₉), 1.63 (m, 1H, H _{βu} Orn₈), 1.57 (m, 1H, H _{γu} Pro₆), 1.54 (m, 2H, H _{β d}, γ Leu₄), 1.42 (m, 1H, H _{βu} Leu₄), 0.95 (m, 9H, H _{γ d} Val₇, H _{γ} Val₂), 0.91 (m, 6H, H _{δ} Leu₄), 0.87 (m, 9H, H _{δ} Leu₉, H _{γu} Val₇).



cyclo-[SAA_{6c}-Val-Orn-Leu-^DPhe-Pro-Val-Orn-Leu]

(2c): The obtained peptide (see General Procedure) was analyzed by LC/MS (R_t 15.59 min, linear gradient 10→90% B in 20 min; m/z = 1197.0 $[M+H]^+$, 599.2 $[M+H]^{2+}$) and purified by semi-preparative RP-HPLC (linear gradient of 3.0 CV; 45→55% B; R_t 2.6 CV). Lyophilization of the combined fractions furnished peptide **2c** (5.6 mg, 4.7 μ mol,

57%) as white amorphous powder. ^1H NMR (600 MHz, CD_3OH): δ = 8.96 (d, 1H, NH ^DPhe₅, $J_{NH,H\alpha}$ = 3.0 Hz), 8.66 (d, 1H, NH Leu₄, $J_{NH,H\alpha}$ = 9.0 Hz), 8.61 (d, 1H, NH α Orn₃, $J_{NH,H\alpha}$ = 9.0 Hz), 8.60 (d, 1H, NH α Orn₈, $J_{NH,H\alpha}$ = 9.0 Hz), 8.32 (d, 1H, NH Leu₉, $J_{NH,H\alpha}$ = 8.4 Hz), 8.10 (m, 1H, H_{ar} SAA₁), 8.01 (t, 1H, NH SAA₁, $J_{NH,6}$ = 4.6 Hz), 7.88 (m, 1H, H_{ar} SAA₁), 7.84 (m, 1H, H_{ar} SAA₁), 7.77 (d, 1H, NH Val₇, $J_{NH,H\alpha}$ = 9.0 Hz), 7.47 (d, 1H, NH Val₂, $J_{NH,H\alpha}$ = 9.0 Hz), 7.55 – 7.43 (m, 4H, H_{ar} SAA₁), 7.32 – 7.23 (m, 5H, H_{ar} ^DPhe₅), 5.09 (d, 1H, CH_a SAA₁ $J_{CHd,CHu}$ = 12.0 Hz), 5.05 (d, 1H, CH_b SAA₁ $J_{CHd,CHu}$ = 12.0 Hz), 4.97 (m, 1H, H α Orn₃), 4.69 (m, 1H, H α Orn₈), 4.63 (m, 1H, H α Leu₄), 4.54 (m, 1H, H α ^DPhe₅), 4.48 (m, 2H, H₂, H₃ SAA₁), 4.46 (m, 1H, H α Leu₉), 4.34 (m, 1H, H α Pro₆), 4.30 (m, 1H, H α Val₂), 4.25 (m, 1H, H₅ SAA₁), 4.04 (m, 1H, H α Val₇), 3.92 (d, 1H, H₄ SAA₁, $J_{H4,H5}$ = 1.5 Hz), 3.72 (m, 1H, H δ _d Pro₆), 3.54 (ddd, 1H, H_{6d} SAA₁, $J_{6d,5}$ = $J_{6d,NH}$ = 4.6 Hz, $J_{6d,6u}$ = 14.4 Hz), 3.40 (ddd, 1H, H_{6u} SAA₁, $J_{6u,5}$ = $J_{6u,NH}$ = 4.6 Hz, $J_{6u,6d}$ = 14.4 Hz), 3.08 (dd, 1H, H β _d ^DPhe₅, $J_{\beta d,\alpha}$ = 5.3 Hz, $J_{\beta d,\beta u}$ = 13.0 Hz), 2.99 (m, 1H, H δ _d Orn₃), 2.92 (m, 4H, H δ Orn₈, H δ _u Orn₃, H β _u ^DPhe₅), 2.47 (m, 1H, H δ _u Pro₆), 2.28 (m, 1H, H β Val₇), 2.07 (m, 1H, H β Val₂), 1.97 (m, 2H, H β _d Pro₆, H β _d Orn₃), 1.82 (m, 1H, H β _d Orn₈), 1.76 (m, 3H, H β _u, γ Orn₃), 1.68 (m, 2H, H β _u, γ _d Pro₆), 1.67 (m, 2H, H γ Orn₈), 1.65 (m, 3H, H β , γ Leu₉), 1.63 (m, 1H, H β _u Orn₈), 1.57 (m, 1H, H γ _u Pro₆), 1.54 (m, 2H, H β _d, γ Leu₄), 1.42 (m, 1H, H β _u Leu₄), 0.94 (m, 9H, H γ _d Val₇, H γ Val₂), 0.90 (m, 6H, H δ Leu₄), 0.86 (m, 9H, H δ Leu₉, H γ _u Val₇).

Biological activity: The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Bacteria were stored at -70°C and grown at 35°C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) overnight and diluted in 0.9% NaCl. Microtitre plates (96 wells of 100 μ L) as well as large plates (25 wells of 3 mL) were filled with Mueller Hinton II Agar (Becton Dickinson, Cockeysville, USA) containing serial two-fold dilutions of peptides **2a-c**. To the wells were added 3 μ L of bacteria, to give a final inoculum of 10^4 colony forming units (CFU) per well. The plates were incubated overnight at 35°C and the MIC was determined as the lowest concentration inhibiting bacterial growth.

Hemolytic Activity: Human blood was collected into EDTA-tubes and centrifuged to remove the buffy coat. The residual erythrocytes were washed three times in 0.85% saline. Serial two-fold dilutions of the peptides **2a-c** in saline were prepared in sterilized round-bottom 96-well plates (polystyrene, U-bottom, Costar) using 100 μ L volumes (500–0.5 μ M). Red blood cells were diluted with saline to 1/25 packed volume of cells and 50 μ L of the resulting cell suspension was added to each well. Plates were incubated while gently shaking at 37°C for 4 h. Next, the microtiter plate was quickly centrifuged (1000 g, 5 min) and 50 μ L supernatant of each well was transported into a flat-bottom 96-well plate (Costar). The absorbance was measured at 405 nm using a mQuant micro plate spectrophotometer (Bio-Tek Instruments). The A_{blank} was measured in the absence of additives and 100% hemolysis (A_{tot}) in the presence of 1% Triton X-100 in saline. The percentage hemolysis is determined as $(A_{\text{pep}} - A_{\text{blank}}) / (A_{\text{tot}} - A_{\text{blank}}) \times 100$.

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Chapter 8

General Discussion and Future Prospects

General Discussion

Bacterial resistance towards antibiotics demands the continual development of novel antimicrobial agents. As the existing resistance mechanisms are prone to adapt to accommodate new derivatives of earlier antibiotic classes, research should not only focus on the development of therapeutics based on novel structures. New molecular targets need to be addressed that may have reduced potential to generate drug-resistant variants as well. Cationic antimicrobial peptides (CAPs) are recognized as important components of innate immune defense mechanisms in the protection against pathogenic organisms. Considerable evidence exists that the principal target of these peptides is the lipid bilayer and that bactericidal activity is exhibited *via* membrane permeabilization. Due to the potent pharmacological activities of these compounds, there has been an overwhelming interest in exploring their mechanism of biosynthesis, interaction with their molecular target, and their structure-activity relationships.

Gramicidin S (GS, where the “S” stands for “Soviet”) is a cationic antimicrobial peptide that was first isolated from the aerobic sporulating bacteria *Bacillus brevis* discovered in Soviet garden soil.¹ Upon accretion on lipid bilayers GS inflicts loss of barrier functioning of cellular membranes.² GS has the primary sequence *cyclo*-(^DPhe-Pro-Val-Orn-Leu)₂ and adopts a C₂-symmetric β -sheet structure that is stabilized by four interstrand hydrogen bonds between the Leu and Val residues. The ^DPhe-Pro dipeptide sequences hold the *i*+1 and *i*+2 position in two type II' β -turns that further contribute to the stabilization of the pleated sheet structure. In this configuration, the hydrophobic (*i.e.* Val, Leu) and hydrophilic (*i.e.* Orn) residues of the two antiparallel β -strands are positioned on opposite sides of the molecule.³ Despite the capacity to lyse microbial cells, the therapeutic value of GS is restricted to topological applications,

since GS also exhibits strong toxicity against human erythrocytes. Due to its potent cidal action towards pathogenic bacteria and fungi, GS and analogues thereof have been extensively studied in order to elucidate the structure-function relationships.

The work described in this Thesis entails the design, synthesis, structural and biological evaluation of modulations made in the turn regions of GS. **Chapter 2** describes the synthesis of GS analogues containing additional functionalities in the β -turn moieties of GS through the incorporation of 2*S*,4*R*-azidoproline (*R*-Azp) or 2*S*,4*S*-azidoproline (*S*-Azp) residues. The design of these C_2 -symmetric GS analogues allowed the use of a biomimetic synthesis approach. Through a dimerization-cyclization reaction of the appropriate unprotected linear pentapeptide precursors, the desired cyclodecapeptides were obtained, albeit in low yields. In the next step, the azide moieties of the Azp-residues were transformed into cationic, anionic and hydrophobic functionalities. A loss of β -sheet character, deemed essential to biological activity, was not observed based on ^1H NMR experiments. However, the antibacterial activity of the GS analogues against various Gram-positive and -negative bacterial strains revealed that only those GS-analogues having Azp instead of Pro residues or $^{\text{D}}\text{Tyr}(\text{Bn})$ instead of $^{\text{D}}\text{Phe}$ residues were as active as native GS.

Chapter 3 describes the development of a Fmoc-based solid phase peptide synthesis strategy towards asymmetrically substituted GS-like peptides. Monomeric GS analogues, having additional functionalities in their turn regions, were prepared and subsequently converted into various covalently linked dimers. The motivation behind this dimerization strategy was that even though the *modus operandi* of GS has not yet been resolved, its accumulation onto lipid bilayers is widely accepted to be the first step in a series of events leading to bacterial lysis. By covalently linking monomeric GS peptides, a shift in dissociation/association equilibrium on the lipid bilayer might be induced. Another reason for producing dimeric species of GS analogue-containing building blocks was that membrane-spanning unimolecular channels could be accessed, as was amply demonstrated in the case of gramicidin A. However, the GS dimers exhibited adverse antimicrobial and hemolytic properties and upon conductivity measurements, no distinct ion channel formation was detected.

Chapter 4 describes the synthesis of a GS analogue containing a novel reverse turn motif. This turn structure is induced by a furanoid sugar amino acid (SAA) that replaced one of the native type II' β -turns. Particularly, the C_3 -hydroxyl function that stemmed from the parent sugar of the SAA plays a key role by participating in an intramolecular hydrogen bond. Consequently, the original H-bonding pattern found in native GS is disrupted, as was established by ^1H NMR and X-ray crystallographic analysis. Furthermore, upon close inspection of the molecular packing in the crystal structure, a hexameric β -barrel-like structure was observed. In the cyclic arrangement, that was stabilized through intermolecular H-bonds, the hydrophilic side chains extended into the core and the hydrophobic side chains created the periphery.

In **Chapter 5**, the versatility of the SPPS protocol towards linear decameric GS analogues and ensuing solution phase cyclization as presented in Chapter 3 and 4 was demonstrated with the synthesis of eight GS analogues having either a single or both ^DPhe-Pro reverse turn dipeptide sequences replaced by four distinct sugar amino acids. The fully protected peptides were obtained in moderate to good yields. The ¹H NMR characterization of the unprotected GS analogues having a single SAA substitution established that these peptides prevalently adopt a β -sheet secondary structure. Two SAA supplantations resulted in GS analogues that still have some propensity to form pleated sheet secondary structures. However, assaying their biological profile revealed a deleterious effect on the antimicrobial activity with a similar decrease in toxicity towards human erythrocytes for all analogues presented in this chapter.

Next to the contribution of remnant SAA hydroxyls to secondary structure formation, the hydroxyl functionalities appended from these peptidomimetic compounds can be functionalized in order to make them resemble the native peptide sequence more closely.

Chapter 6 describes a strategy towards such decoration by using a two-step oxidative glycol cleavage / reductive amination approach, in which an ϵ -SAA having a *cis*-diol system was transformed into ϵ -morpholine amino acids (MAAs) having various substituents on the endocyclic nitrogen. The use of a *cis*-diol containing δ -SAA gave a mixture of diastereoisomeric δ -MAA. Epimerisation could be circumvented by installation of the carboxylic acid of the MAA after the glycol cleavage and ensuing reductive amination steps. The application of these second-generation carbohydrate-derived peptidomimetics was demonstrated both through the direct incorporation of an ϵ -MAA in the reverse turn of GS and through applying the two-step glycol cleavage / reductive amination strategy on a completely assembled GS analogue bearing a ϵ -SAA in its reverse turn, resulting in spectroscopically and spectrometrically identical peptides.

In **Chapter 7**, the crystal structure of the SAA-containing GS analogue described in Chapter 4 formed the basis for the design of SAA-containing GS mimics that more closely resemble the ^DPhe-Pro dipeptide sequence present in native GS. Adaptation of the synthetic scheme towards the previously described 2,5-anhydroglucitol-based furanoid SAA provided selective access to the C₄-hydroxyl for decoration with aromatic moieties. The GS analogues containing such aromatic SAAs prevalently adopted a β -sheet secondary structure with the SAA again involved in its characteristic reverse turn conformation, as was judged from ¹H NMR spectroscopic comparison. The deleterious effect of SAA-incorporation into the GS peptidic scaffold on their antimicrobial activity as observed in Chapter 5 was completely averted, making the GS analogues with aromatic appendages from the SAAs equally biologically active as GS itself.

Future prospects

In the unique turn structure induced by a furanoid SAA in GS analogue B (Figure 1, **B**), described in **Chapter 4** of this Thesis, a H-bond is observed between the C₃-hydroxyl of the SAA, functioning as H-bond acceptor, and the NH moiety from the SAA, acting as H-bond donor. This deviation from the original type II' β -turn in native GS (Figure 1, **A**) was also observed in the GS analogues described in **Chapter 7**.

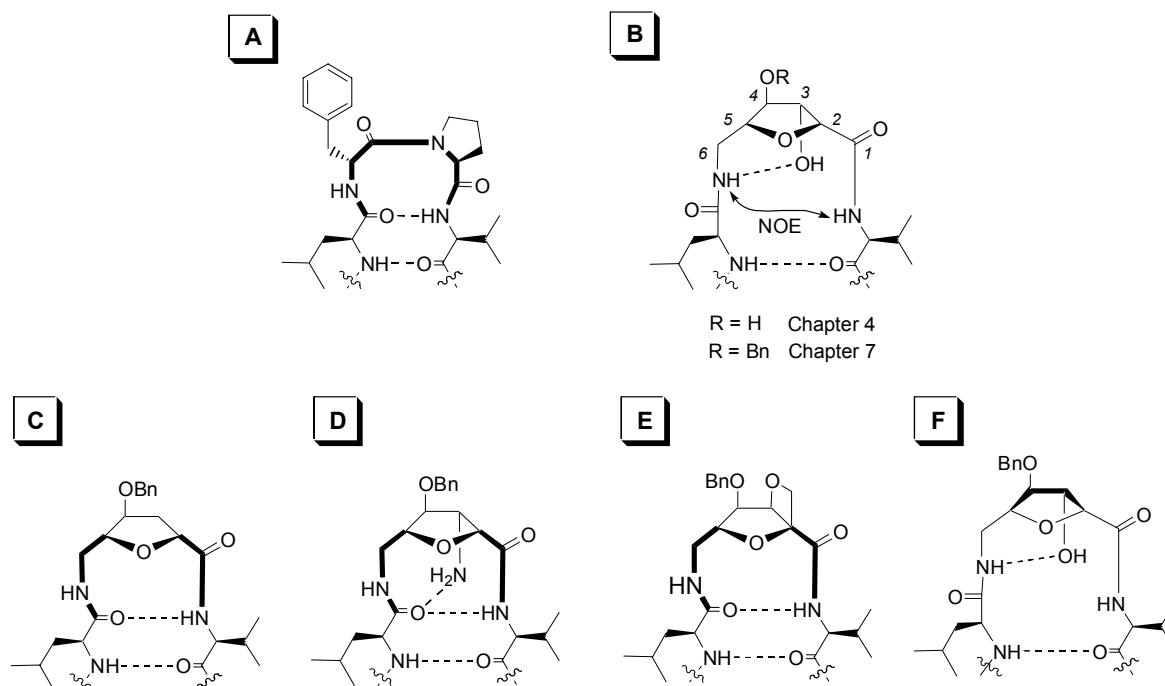
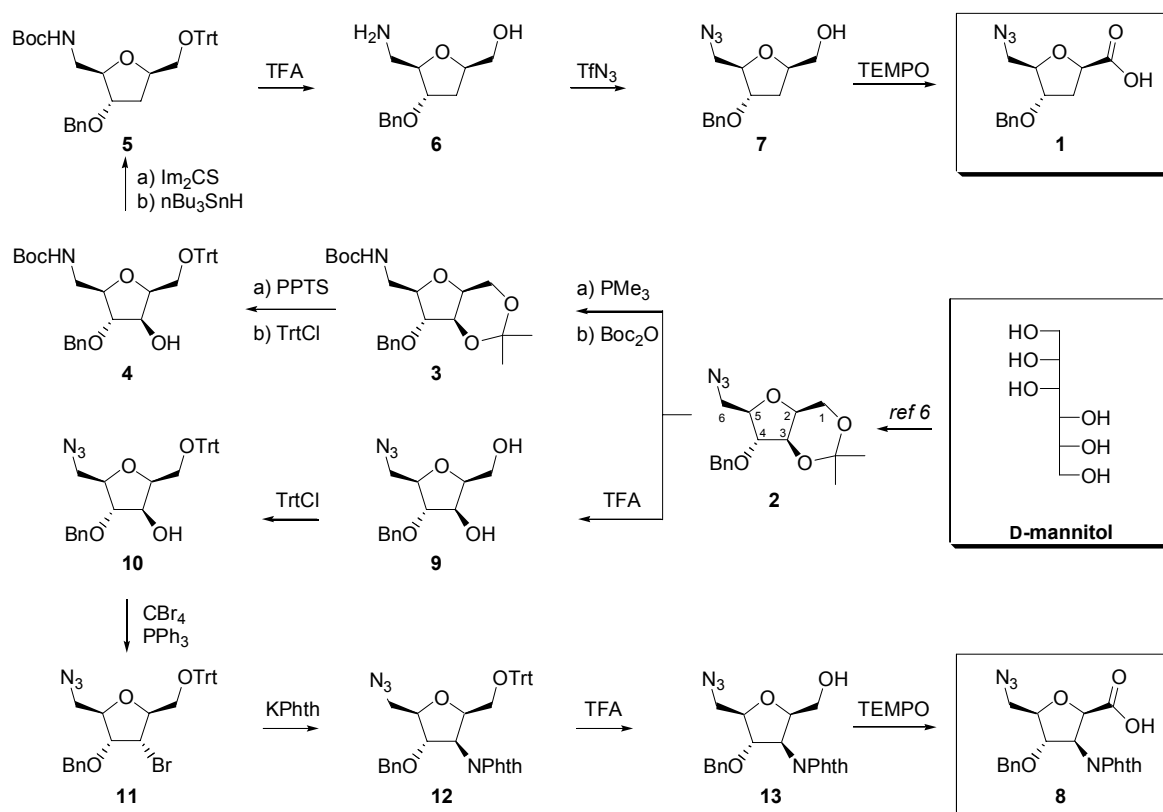


Figure 1: Reverse turn structure of GS (**A**), GS-analogue with a furanoid SAA (**B**), GS-analogue with a deoxygenated SAA (**C**), GS-analogue with an amino-functionalized SAA (**D**), GS-analogue with a locked SAA (**E**), GS-analogue with an enantiomeric furanoid SAA (**F**).

Several factors may contribute to the formation of this unusual turn structure. For example, both steric demands of the furanoid ring structure and electronic factors governing the H-bonding pattern, or a combination thereof may favour such a conformation. In order to independently appraise the factors in question, the synthesis, structural and biological evaluation of novel SAA-containing GS analogues is required. Hence, four novel SAAs are proposed to be incorporated in GS (Figure 1, **C-F**). To investigate the importance of the hydrogen bonding contributions, the first SAA (**C**) maintains its 2,5-anhydro-D-glucitol scaffold. However, the C₃-hydroxyl is removed, thus enabling the carbonyl functionality of the Leu residue to partake in its original H-bonding pattern with of the Val residue. In a different approach to remove the ability to act as H-bond acceptor, the second SAA (**D**) is designed to bear an amine functionality at the C₃-position. This H-bond donor might further entice the Leu residue's carbonyl into H-bonding.⁴ In the third SAA (**E**), an additional oxetane-ring will be responsible for structurally restricting the SAA from adopting the C₃-

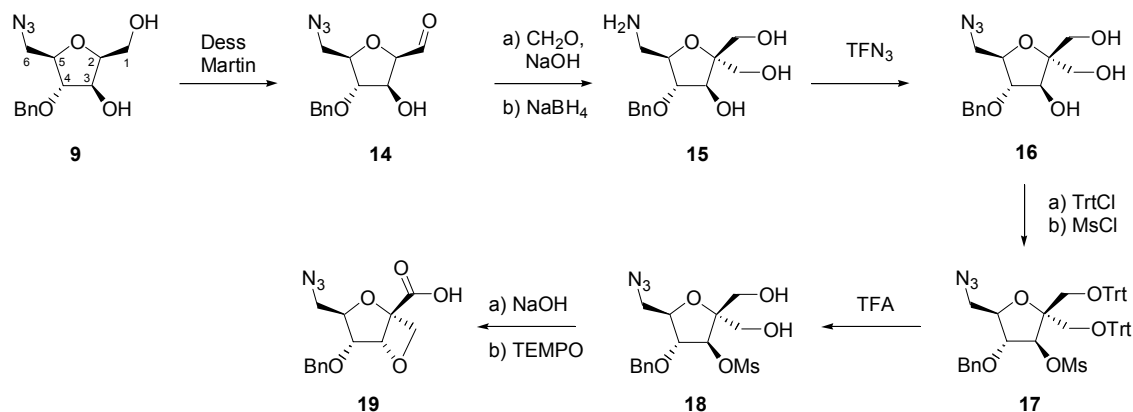
endo conformation originally found in **B**. Rather, the C₃-*exo* conformation will be enforced with the C₃-oxygen simultaneously being inverted, thereby possibly restoring the interstrand H-bonding pattern found in native GS.⁵ Finally, the synthesis and incorporation of the enantiomeric SAA (**F**) is proposed. In this design, the C₃-hydroxyl may still be able to interact with the amide bond between the SAA- and Leu-residue thereby inducing the exclusive turn structure. However, the furan ring of the SAA will no longer be positioned on the hydrophilic face of the GS analogue. Rather, it will be located on the hydrophobic face, being similarly positioned as the 5-membered ring of the Pro-residue in opposite type II' β -turn. This might consequently induce a smaller twist in the overall β -sheet structure of the GS analogue.



Scheme 1: Synthesis of a deoxygenated and an amine-functionalized SAAs.

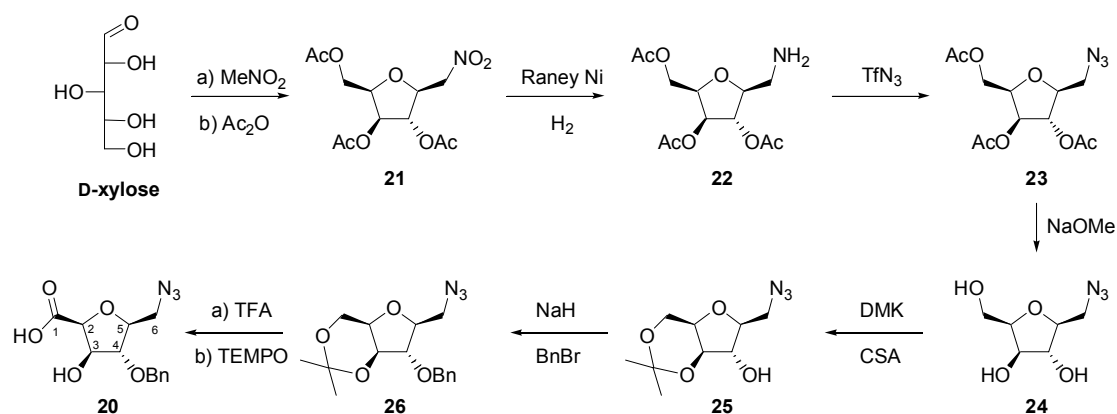
As is depicted in Scheme 1, the envisaged deoxygenated SAA **1** (Figure 1, **C**) can be prepared from fully protected **2**.⁶ The aromatic moiety on this SAA is retained as it is considered to be a requisite feature for biological activity (see **Chapter 7**). The 2,5-anhydro-D-glucitol scaffold **2**, that is derived from D-mannitol should first be transformed into Boc-protected **3**, as the azide is not expected to be compatible with the reductive conditions of deoxygenation. Scaffold **4** can then be subjected to Barton deoxygenation in which the thiocarbonyl derivative can undergo free radical scission upon treatment with tri-*n*-butyltin hydride. Reinstallation of the primary azide on **6** and final TEMPO-oxidation will furnish SAA **1**. The amine-functionalized 2,5-anhydro-D-gluconic acid **8** (Figure 1, **D**) can equally be accessible from **2** by selective protection of the primary hydroxyl in **9** followed by an Appel reaction,⁷

which proceeds with inversion of configuration at the C₃-position. A second inversion by nucleophilic displacement of the bromide in **11** using a Gabriel procedure provides the phthalimide-protected amine **12**, that upon acidic cleavage and TEMPO-oxidation will give SAA **8**.



Scheme 2: Synthesis of a locked SAA based on a D-gluconic acid scaffold.

To introduce additional conformational strain on the reverse turn of SAA-containing GS analogues (Figure 1, E), a SAA based on a furano-oxetane core structure is envisaged that may be obtainable following synthetic procedure previously reported by Van Well *et al.*⁸ As revealed in Scheme 2, the primary hydroxyl of diol **9** can be selectively oxidized to aldehyde **14**, using Dess-Martin periodinane (previously described in **Chapter 4**) followed by an aldol-type condensation with formaldehyde for the introduction of the hydroxymethylene function. Reduction of the initially formed β -hydroxy aldehyde gives triol **15** that will require restoration of the azide functionality. Selective protection of the primary alcohols in **16** and subsequent mesylation then affords **17**. Having set the stage for the formation of the oxetane ring, the primary hydroxyls can be unveiled and ring closure may take place under alkaline conditions. Final installation of the carboxylate through TEMPO-oxidation will provide locked SAA **19**.



Scheme 3: Synthesis of a SAA based on a L-gluconic acid scaffold.

For the synthesis of the enantiomeric SAA **20** (Figure 1, **F**), a procedure developed by Brandenburg and coworkers may be applied for the construction of the initial 2,5-anhydro-L-glucitol scaffold.⁹ As is shown in Scheme 3, a base-promoted aldol-type condensation of nitromethane with the aldehyde functionality of D-xylose followed by dehydration and Michael addition gives, after crystallization and acetylation, nitrohexitol **21**. Hydrogenation towards amine **22** and ensuing Cu-catalyzed diazo-transfer may provide azide **23**. In a five-step procedure involving standard functional group manipulations: deacetylation ($\rightarrow$ **24**), selective acetonation ($\rightarrow$ **25**), benzylation of the C₄-hydroxyl ($\rightarrow$ **26**), acidic removal of the isopropylidene and TEMPO-oxidation of the primary hydroxyl, may furnish SAA **20**.

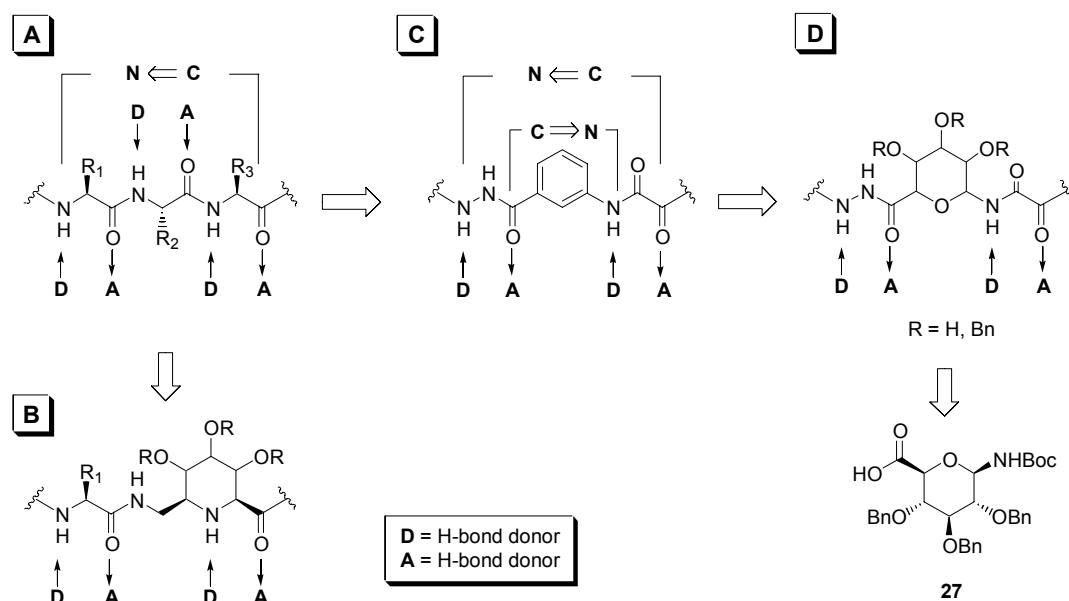


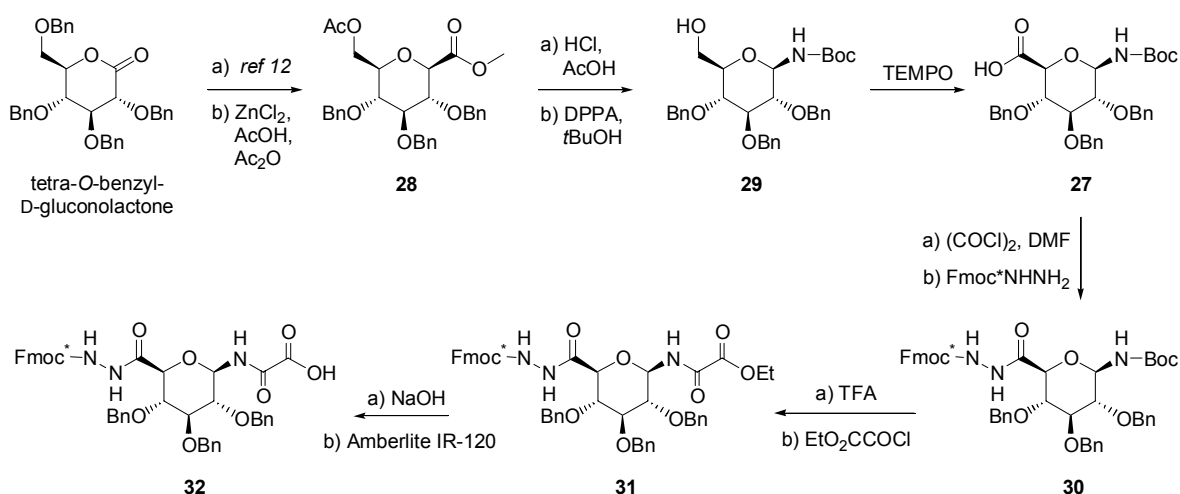
Figure 2: General β -strand tripeptide structure (**A**), iminosugar-based β -strand mimic (**B**), Nowick's design for tripeptide β -strand mimic (**C**), SAA-containing tripeptide β -strand mimic (**D**).

An important goal in peptidomimetic research is the design of compounds that nucleate or propagate peptide folding thereby mimicking structural elements such as α -helices, β -sheets or reverse turns that are commonly found in the native folding structures of proteins. Thus far, homooligomers and mixed oligomers of carbohydrate-based peptidomimetics have been shown to be excellent reverse turn mimetics and could also be successfully incorporated into helical structures.¹⁰ With the aim to generate intramolecular hydrogen-bonded β -sheet-like structures with a SAA incorporated in the β -strand, the design of a molecular template based on a SAA can be envisaged as presented in Figure 2.

A peptide strand involved in a β -sheet hydrogen-bonding pattern typically consists of an H-bond donor and acceptor of the first amino acid being directed towards one side of the β -strand and the following donor-acceptor pair being directed towards the opposite side (Figure 2, **A**). The amino acid side chains (R_1 , R_2 , R_3 etc) are positioned on alternating faces of the resulting sheet. To mimic the H-bonding pattern of a single edge of a peptide β -strand, an iminosugar-containing peptide can be envisaged (Figure 2, **B**) in which the endocyclic amine

and its α -positioned carboxylic acid moiety form a H-bond donor-acceptor pair. The H-bonding pattern can be continued by coupling the next amino acid on the N-terminus of this dipeptide isostere.

In several publications from the group of Nowick, peptidomimetic β -sheet mimicry is described that duplicates the hydrogen-bonding pattern of a tripeptide β -strand by applying an aminobenzoic acid derivative functionalized with a hydrazide and oxalamide group (Figure 2, C).¹¹ This design allows the use of a γ -amino acid, as the peptide chain direction inside the tripeptide sequence is temporarily reversed. However, the tripeptide mimics such as those designed by Nowick *et al.* are flat due to their aromatic template. A β -strand structure comprised of a γ -sugar amino acid (Figure 2, D) integrates the pleated nature of β -sheets and should therefore complement these pioneering results of templates that stabilize β -sheet-like structures. The equatorial positioning of the amine and carboxylic acid functionalities in pyranoid SAA **27** ensures the extended conformation as found in β -strands.



Scheme 4: Synthesis of a β -strand mimicking SAA-containing tripeptide analogue.

As can be gauged from Scheme 4, SAA **27** can be obtained by transformation of 2,3,4,6-tetra-*O*-benzyl-D-gluconolactone into methyl ester **28** via a route developed by Dondoni and co-workers and subsequent selective debenzoylation of the primary hydroxyl moiety.^{12,13} Ensuing Curtius rearrangement of the carboxylate derivative might be effected by treatment with diphenylphosphoryl azide (DPPA) as described earlier by van Well *et al.*¹⁴ In this reaction, which is known to proceed with retention of configuration, the intermediate acyl azide rearranges into an isocyanate, which can subsequently be trapped with *t*BuOH to provide the Boc-protected **29**. Installation of carboxylic acid in **27** paves the way for functionalization with the hydrazide and oxalamide groups. Thus, employing Vilsmeier-Haack reagent to create an intermediate acid chloride that can consequently be reacted with 2,7-di-*t*-butylfluorenylmethyloxycarbonyl (Fmoc*)-protected hydrazine to give **30**. Acidic release of

the anomeric amine and condensation with ethyl oxalyl chloride ($\rightarrow$ **31**), followed by saponification and acidification by ion exchange resin finally furnishes SAA-containing tripeptide analogue **32**.

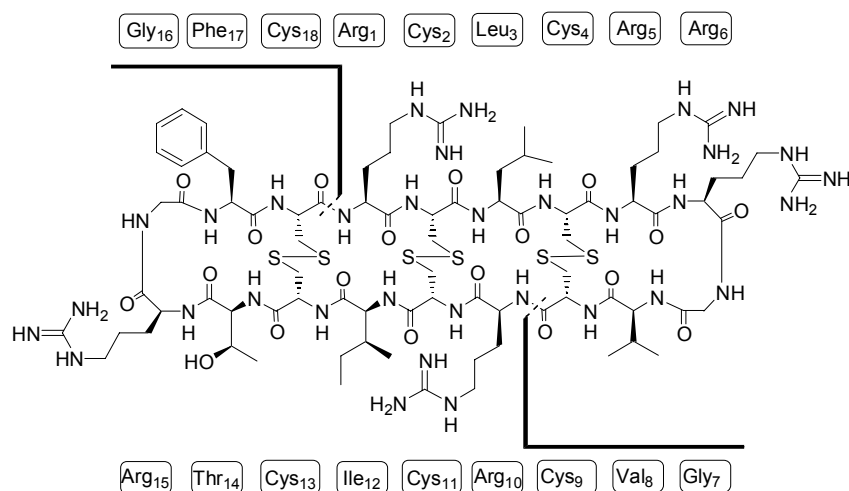


Figure 3: Rhesus theta defensin-1 as novel synthetic β -sheet based target.

Recently, there have been numerous reports on β -sheet containing antibiotics originating from mammalian innate immune systems.¹⁵ In this respect, the octadecapeptide rhesus theta defensin-1 (RTD-1, Figure 3), that has been identified in rhesus macaque leukocytes,¹⁶ was shown to be a cyclic CAP that contains five cationic arginine residues and three disulfide bridges that aid in the stabilization of the β -sheet structure. By resolving its three-dimensional solution structure (see Figure 4) through simulated-annealing calculations, the β -sheet structure was shown to possess a substantial degree of conformational freedom as is demonstrated by two lowest-energy structures A and C.¹⁷

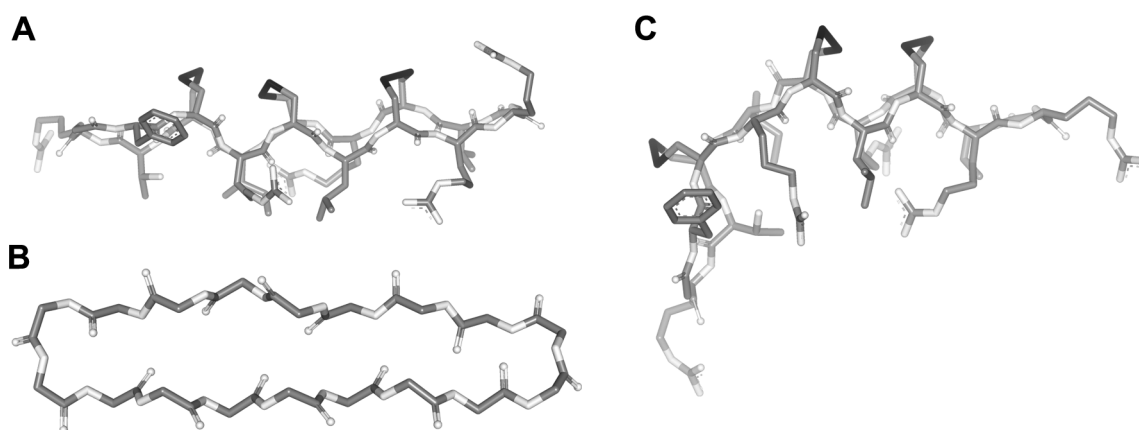


Figure 4: NMR-derived structures of RTD-1 (A) pleated-sheet viewed from the side, (B) pleated-sheet viewed from the top with the amino acid side chains omitted, (C) structure of a curved β -sheet viewed from the side, as reported by Trabi *et al.*¹⁷

In an effort to elucidate the biosynthetic pathway towards RTD-1, it was found that no sequence in a rhesus macaque genomic library coded for the complete 18 amino acid sequence. Rather, RTD-1 appears to be a posttranslationally processed gene product that is constructed through the ligation of two distinct nonapeptide precursor protein fragments (see Figure 3).¹⁶ This is clearly reminiscent of the biosynthetic pathway of GS, although GS is nonribosomally synthesized by the NRPS. Furthermore, human bone marrow has been shown to contain mRNA that has a high homology to the rhesus θ -defensin gene (DEFT) transcripts.¹⁸ However, a stop codon mutation within its signal sequence blocks the subsequent translation, thereby silencing the translation of this gene. The genetic information could nevertheless be used for the synthesis of putative ancestral hominid θ -defensins that have been dubbed retrocyclins. These retrocyclins are of particular pharmacological interest as it has been demonstrated that they display activity against G^+ and G^- bacteria. Moreover, they interfere with (retro)viral uptake of HIV-1, thereby protecting human cells *in vitro* from infection. Retrocyclins have also been shown to act as carbohydrate-binding peptides (lectins) with affinity for gp120 (a glycoprotein found in the outer envelope of HIV particles), CD4 (a glycoprotein present on T-cells) and galactosylceramide which is also implicated in HIV-1 uptake.¹⁹

As the posttranslational processing of RTD-1 requires the ligation of two distinct nonapeptide fragments it can be speculated that a predisposition towards β -sheet formation, analogous to that found in the biosynthesis GS, is present in these nonapeptide precursors. To probe the factors that influence the dimerisation and ensuing cyclisation, the application of a biomimetic synthesis strategy (as discussed in the General Introduction and Chapter 2) can be envisaged, which results in the generation of RTD-1 and retrocyclin analogues. By reacting two equal active ester nonapeptides **33** (depicted in Figure 5) with varying amino acid composition, several C_2 -symmetric octadeca-peptides may be accessible.

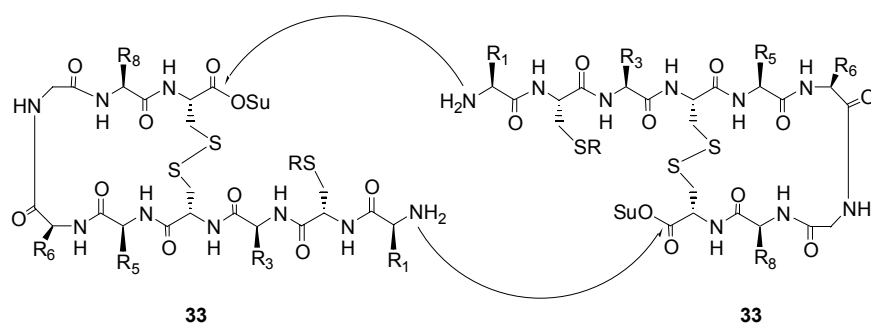


Figure 5: Dimerization-cyclization strategy towards C_2 -symmetric retrocyclin analogues.

In order to increase metabolic stability for RTD-1 or retrocyclin analogues, the disulfide bridges that stabilize the separate β -strands of the octadecapeptides in the general structure **34** (Figure 6) can be replaced by alkane (**35**) and alkene (**36**) isosters. This may be accomplished by employing a ring-closing metathesis (RCM) strategy for the cyclization of oligopeptides

containing allylglycine residues.²⁰ As the cyclic octadecapeptides are predisposed towards forming a β -sheet structure, according to the postulation of β -sheet periodicity in cyclic peptides,²¹ it is expected that six allylglycine residues in **37** exclusively form the three cystine isosters in **36** during RCM.

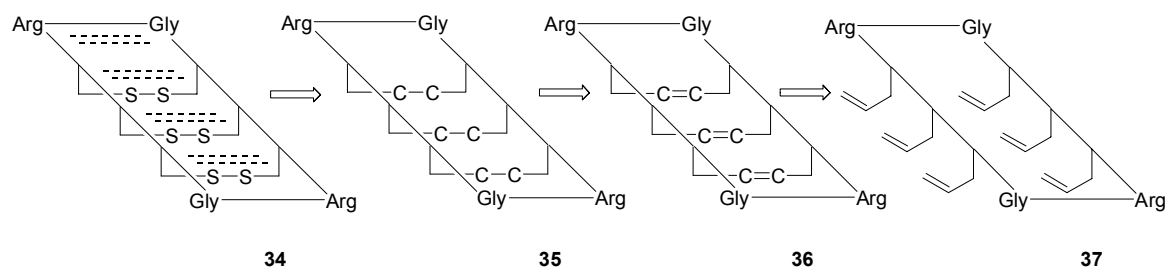


Figure 6: Schematic representation of the ring-closing metathesis strategy towards RDT-1 analogues with alkene and alkane isosters replacements of the disulfide bridges.

To determine the role of the disulfide bridges in inducing an appropriate conformation in the biomimetic strategy, the cystine bridge closest to the reverse turn region can be installed before the dimerization-cyclization reaction takes place or replaced by an alkene isoster (*vide supra*) precursor **38** (see Figure 7). Finally, when these reactions are preformed under oxidative conditions, the intermolecular formation of the central disulfide bridges could act as a driving force for this biomimetic synthesis.

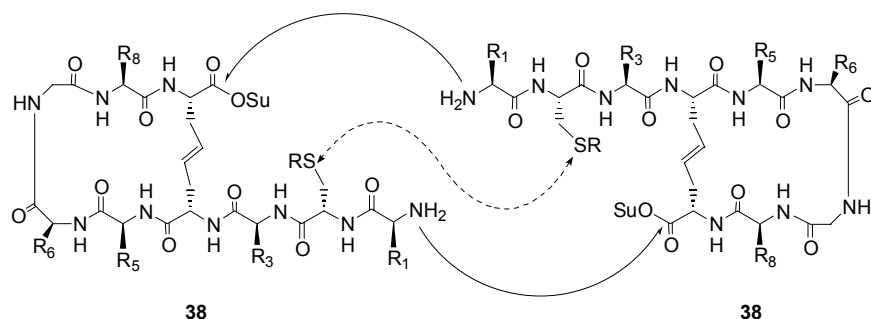


Figure 7: Dimerization-cyclization strategy towards C_2 -symmetric retrocyclin analogues.

References and Notes

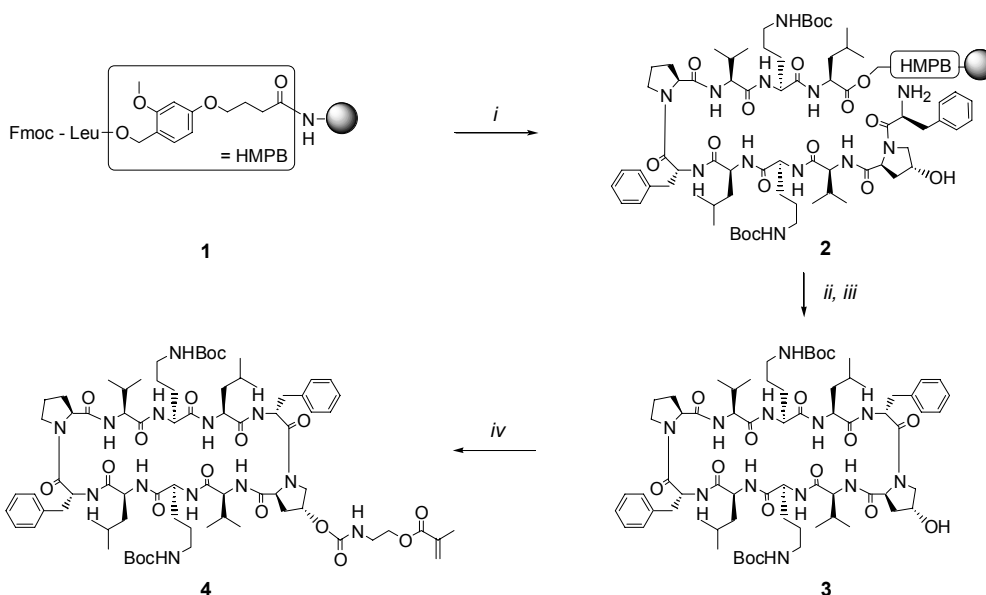
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Addendum

Gramicidin S and Bio-inspired Materials

The work described in this Thesis is part of a collaborative research effort, funded by the Netherlands Technology Foundation (STW) and in which the groups of Prof. Dr. Rutjes and Prof. Dr. van Hest (both Radboud University Nijmegen) and the Leiden bioorganic chemistry group participate. Aim of this project, entitled “*design and synthesis of modified, linear and cyclic oligo(glyco)peptides, a bio-inspired approach towards the development of novel silk-like materials*” is to emulate advantageous properties present in natural polymers in the development of their synthetic equivalents. Specifically, the aim is to mimic in synthetic polymers the architecture of spider silk, being small, defined β -sheet peptide regions to which amorphous polypeptides are appended. This architecture is thought to be at the basis of the unique properties of spider silk: its great strength combined with its remarkable flexibility. In one specific synthetic design aimed for in the research program, gramicidin S (GS) was selected as the mimetic of the spider silk β -sheet regions, with the amorphous stretches projected to be connected to these cyclic decapeptide core by means of atom transfer radical polymerisation (ATRP). My research objective in this particular project was to provide the Nijmegen researchers with sufficient quantities of GS monomer **4** equipped with a single methacrylate functionality as a suitable ATRP substrate. The generation of ‘poly-GS’ from derivative **4**, the nature of this material and its physical properties falls outside the scope of this Thesis and will be discussed in detail in the Thesis of Lee Ayres from the van Hest group. Full experimental details on the synthesis of **4**, performed on a gram scale, are provided in the experimental section. The route of synthesis that was found to be most effective for the preparation of **4** essentially follows the general scheme as presented in **Chapter 3**. Briefly, nonapeptide **2** (Scheme 1) was assembled on 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) functionalized 4-methylbenzhydrylamine (MBHA) resin **1** using standard Fmoc-based SPPS protocols. Acidolytic cleavage from the solid support and subsequently cyclization under highly dilute conditions gave cyclic GS analogue **3** in 92% yield. The protected peptide was subsequently treated with 2-isocyanatoethyl methacrylate (IEM) to quantitatively furnish urethane **4**.



Scheme 1: *Reagents and conditions:* (i) Repetitive deprotection; piperidine / NMP (1/4 v/v), condensation; Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-^DPhe-OH, Fmoc-Leu-OH or Fmoc-Hyp-OH (3 equiv), BOP (3 equiv), HOBt (3 equiv), DiPEA (3.6 equiv), NMP; (ii) TFA / DCM (1/99 v/v), 4× 10 min; (iii) PyBOP (5 equiv), HOBt (5 equiv), DiPEA (15 equiv), DMF, 16 h, 92%; (iv) IEM, pyridine, 55°C, 16 h, quant.

Experimental Section

Compound 2. Resin 1 (2.0 g, 0.5 mmol/g, 1.0 mmol) was subjected to nine cycles of solid-phase synthesis: (a) piperidine in NMP (1/4 v/v, 25 mL, 15 min); (b) NMP wash (25 mL, 3× 3 min); (c) Fmoc amino acid (2.5 mmol, 2.5 equiv), BOP (1.10 g, 2.5 mmol, 2.5 equiv), HOBt (338 mg, 2.5 mmol, 2.5 equiv) and DiPEA (467 μ L, 2.75 mmol, 2.75 equiv) were premixed for 2 min in NMP (25 mL) and shaken for 90 min; (d) NMP wash (25 mL, 3× 3 min), using the commercially available building blocks in the following order: Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-^DPhe-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Hyp-OH and Fmoc-^DPhe-OH. *N*-Terminal amine liberation with piperidine in NMP (1/4 v/v, 25 mL, 15 min) was followed by an NMP wash (25 mL, 3× 3 min) and DCM wash (25 mL, 3× 3 min). Val-residues were standardly immobilized using a double coupling procedure and all couplings were monitored by the Kaiser test.

Compound 3. Resin-anchored peptide 2 was suspended in TFA/DCM (1/99, v/v, 30 mL, 4× 10 min). The filtrates were collected and coevaporated with toluene (200 mL) thrice, to give the crude linear peptide. This was taken up in DMF (50 mL), slowly added to a solution of PyBOP (2.60 g, 5.0 mmol, 5 equiv), HOBt (675 mg, 5.0 mmol, 5 equiv) and DiPEA (2.61 mL, 15 mmol, 15 equiv) in DMF (750 mL) over the period of an hour and allowed to stir for 16 h. The solvents were removed by evaporation and the mixture was directly applied to a Sephadex[®] size exclusion column (50.0 mmD × 1500 mmL) that was eluted with MeOH, to produce title compound 3 as white amorphous solid (1.25 g, 0.92 mmol, 92%).

Compound 4. Cyclic peptide 3 (1.13 g, 0.83 mmol) was dissolved in freshly distilled pyridine (20 mL) and 2-isocyanatoethyl methacrylate (IEM, 0.24 mL, 1.66 mmol, 2 equiv) was added. The mixture was stirred at 55°C for 16 h and concentrated *in vacuo*. The product was purified by silica gel column chromatography (0%→10% MeOH in DCM) to quantitatively yield the title compound 4 (1.26 g, 0.83 mmol) as off-white foam.

Samenvatting

Antibiotica zijn veel gebruikte medicijnen waarmee antibacteriële infecties kunnen worden bestreden. Deze geneesmiddelen zijn, in het algemeen, selectief toxisch voor bacteriën omdat hun werkingsmechanisme gebruik maakt van verschillen tussen de prokaryotische en eukaryotische eiwitsynthese, het celmembraan en de celwand, ofwel in de cellulaire stofwisseling. Omdat bacteriestammen zich eenvoudig aanpassen aan nieuwe omstandigheden kunnen zij hun gevoeligheid voor specifieke antibiotica verliezen en treedt er resistentie op. Bij de ontwikkeling van therapeutica met antibacteriële eigenschappen is het daarom van belang niet alleen nieuwe derivaten van bestaande antibiotica te genereren, maar ook nieuwe klassen te identificeren en te onderzoeken om zodoende de kans op resistentie te verkleinen.

Kationische antimicrobiële peptiden (KAPs) worden geproduceerd door organismen van uiteenlopende taxonomische rijken (prokaryota, protocista, fungi, animalia en plantae) en hebben een belangrijke immunologische functie. Deze peptiden hebben een amfifiele structuur die gekoppeld is aan hun activiteit tegen vijandige micro-organismen. Namelijk, doordat één zijde van de KAPs hydrofiel (kationisch) en de andere zijde hydrofoob is, worden deze moleculen door elektrostatische attractie naar de negatief geladen fosfolipiden van het bacteriële membraan gedirigeerd, waarna verstoring van de integriteit van de lipide-bilaag plaatsvindt en het membraan zijn functie als barrière verliest. Omdat KAPs als een klasse van antibiotica worden beschouwd waarmee het probleem van resistentie van pathogene bacteriën kan worden omzeild, wordt veel wetenschappelijk onderzoek verricht naar de biosynthese, de interactie met biologische membranen en de structuur-activiteitsrelaties van KAPs.

Gramicidine S (GS) is een membraan-actief kationisch antimicrobieel peptide dat voor het eerst werd geïsoleerd uit een russische *Bacillus brevis* stam (waarbij de “S” staat voor “Sovjet”). Dit cyclische decapeptide, met als primaire sequentie *cyclo*-(^DPhe-Pro-Val-Orn-Leu)₂, heeft een karakteristieke β -sheet-conformatie als secundaire structuur. Hierbij hebben twee β -strengen, bestaande uit een lineaire peptideketen met de sequentie Val-Orn-Leu, een niet-covalente interactie door middel van vier waterstofbruggen tussen de valine en leucine aminozuur residuen. Deze β -strengen worden door twee “reverse turns” (een bocht in de peptideketen waardoor deze van richting verandert) bestaande uit ^DPhe-Pro dipeptide-sequenties met elkaar verbonden. De zijketens van de aminozuurresiduen in de β -streng steken afwisselend naar boven en naar onder ten opzichte van de β -sheet, waardoor er een polaire (Val- en Leu-zijketens) en een apolaire (Orn-zijketens) zijde ontstaat. De β -sheet-configuratie met deze specifieke aminozuurdistributie is verantwoordelijk voor de amfipaticiteit en daaraan gekoppelde membraanactiviteit van GS.

Het onderzoek dat in dit proefschrift is beschreven, was gericht op het ontwerpen en synthetiseren van modificaties in het reverse turn-gedeelte van GS, alsmede op de structurele en biologische evaluatie van de daaruit voortkomende GS-analoga. In de algemene inleiding (**Hoofdstuk 1**) wordt een overzicht gegeven van enkele ontwikkelingen op het terrein van antibiotica onderzoek, met een bijzondere aandacht voor KAPs. Ook worden de syntheseroutes met elkaar vergeleken die over de jaren verschenen zijn voor de bereiding van GS en analoga daarvan. Hierbij wordt een onderscheid gemaakt tussen veranderingen die zijn aangebracht in de reverse turn van GS en die in het β -sheet gedeelte daarvan. Tenslotte wordt besproken hoe de starre structuur van GS is gebruikt om specifieke chemische functionaliteiten op een voorspelbare manier ten opzicht van elkaar te positioneren.

In **Hoofdstuk 2** wordt de introductie van nieuwe groepen in de Pro-^DPhe dipeptide-turn-sequenties van GS beschreven. Dit kon tot stand worden gebracht door de proline-residuen te vervangen door niet-natuurlijke 2S,4R-azidoproline (R-Azp) of de 2S,4S-azidoproline (S-Azp) residuen. Het ontwerp van deze C_2 -symmetrische peptiden stond het gebruik toe van een biomimetische synthese route, waarbij volledig onbeschermde lineaire pentapeptiden, via een dimerisatie-cyclisatiereactie, de gewenste producten gaven. De nieuwe functionaliteiten in de turn werden na cyclisatie voorzien van kationische, anionische en hydrofobe groepen. ¹H-NMR-analyse toonde aan dat het β -sheetkarakter van deze nieuwe GS-analoga door deze modificaties niet werd verstoord.

Hoofdstuk 3 beschrijft de succesvolle synthese van gefunctionaliseerde GS-analoga die de oorspronkelijk aanwezige C_2 -symmetrie niet bezitten. Deze GS-analoga bevatten een gemaskeerde carbonzuur- en aminegroep. Met behulp van deze functies konden de GS-analoga gecondenseerd worden tot covalent gebonden dimeren. Aangezien de ophoping van GS op membranen een vereiste is voor biologische activiteit, zou dimerisatie een verandering van het dissociatie/associatie equilibrium kunnen bewerkstelligen. Daarnaast zouden dimeren mogelijkwerwijs membraan-overspannende unimoleculaire kanalen kunnen vormen. Echter, de dimersatie van GS-analoga bleek geen voordelige invloed op de antimicrobiële en hemolytische eigenschappen te hebben en kanaalvorming werd niet waargenomen.

Hoofdstuk 4 behandelt de synthese van een GS-analagon waarbij een Pro-^DPhe dipeptide werd vervangen door een suikeraminozuur ("Sugar Amino Acid"; SAA). Deze klasse van gemodificeerde bouwstenen, waarbij een saccharide is voorzien van tenminste één amine en één carbonzuurgroep, kan via standaard-peptidesynthese protocollen ingebouwd worden in peptide-achtige constructen. Het furanoïde SAA dat de natieve type II' β -turn verving bleek een ongewone reverse turn te induceren waarbij de C_3 -hydroxyl van het SAA participeerde in een intramoleculaire waterstofbrug. Dit veroorzaakte een verstoring in het natuurlijke H-brug patroon van GS, zoals vastgesteld met behulp van ¹H NMR en Röntgenkristallografische analyse. Daarnaast werd in de kristalstructuur een macromoleculaire structuur, lijkend op een β -barrel, gevonden waarbij zes kristallografisch gelijke GS-analoga zich ordenden in een ringvormig kanaal met een hydrofiel centrum en een hydrofobe periferie.

In **Hoofdstuk 5** wordt het eerder ontwikkelde synthese protocol (zie hoofdstuk 3 en 4) aangewend om acht GS-analoga te construeren waarvan één enkele of beide reverse turn-sequenties door vier verschillende suikeraminozuren werden vervangen. De volledig beschermde cyclische peptiden konden in een redelijk tot goede opbrengst worden verkregen. Dit onderstreept dat dit milde syntheseprotocol goed samen gaat met verschillende beschermgroepmanipulaties en daarom uitermate geschikt is voor de synthese van GS analoga. Analyse, met behulp van ^1H NMR, laat zien dat de GS-analoga met SAA substituties in de turn voornamelijk een β -sheet secundaire structuur hebben. Bij de evaluatie van de biologische activiteit bleken de enkele SAA-substituties een nadelig effect op de antibiotische werking te hebben met een evenredige reductie in hemolytische toxiciteit. Dubbele vervanging van de turns met de SAAs bleek de activiteit nog ernstiger te doen afnemen.

Hoofdstuk 6 laat een nieuwe applicatie zien van de vrije hydroxylgroepen van een SAA residue die afkomstig zijn van het oorspronkelijke suiker. Met behulp van een twee-staps oxidatieve glycolsplitsing- / reductieve amineringsstrategie werd een ϵ -SAA met een *cis*-diol systeem omgezet in een ϵ -morpholino-aminozuur ("Morpholine Amino Acid"; MAA). De strategie bleek toepasbaar met verschillende aminereagentia, waardoor diverse ϵ -MAAs werden verkregen met uiteenlopende substituenten op de endocyclische stikstof. Wanneer echter een δ -SAA als substraat werd gebruikt leverde de tweestaps-strategie een mengsel van diastereoisomere δ -MAAs op. In een alternatieve procedure, waarbij eerst de morpholine ring werd gevormd uit een *C*-glycoside, gevolgd door installatie van de carbonzuurgroep, kon epimerisatie worden voorkomen en waren beide epimere δ -MAAs afzonderlijk toegankelijk. De toepasbaarheid van deze peptidomimetica werd gedemonstreerd door de directe incorporatie in de reverse turn van GS. Daarnaast werd een GS-analoga met een ingebouwd ϵ -SAA onderworpen aan het oxidatieve glycolsplitsing / reductieve amineringsprotocol waarbij het spectroscopisch en spectrometrisch identieke product werd verkregen.

Hoofdstuk 7 beschrijft het ontwerp en de synthese van furanoïde SAAs die werden gefunctionaliseerd met een aromatische groep en vervolgens ingebouwd in de reverse turn van GS analoga. Het hydrofiele karakter van de SAA-residuen beschreven in Hoofdstuk 4 en 5 werd hiermee vervangen door SAA residuen met dezelfde aromaticiteit die ook aanwezig is in het $^{\text{D}}$ Phe-residu van natief GS. De GS-analoga voorzien van deze SAAs bleken na vergelijking van ^1H -NMR-spectra wederom de β -sheetstructuur te hebben, alsmede de ongewone turn-structuur beschreven in Hoofdstuk 4. Testen van de biologische activiteit lieten zien dat deze verbindingen niet alleen even actief als GS tegenover bacteriën zijn, maar tevens ook hemolytisch. Hiermee werd wederom aangetoond dat SAAs als geschikte peptidomimetica kunnen worden aangewend.

Het werk beschreven in dit proefschrift geeft nieuwe inzichten en richtlijnen aan het onderzoek naar antibiotica gebaseerd op gramicidine S. Waar de structuur van GS gerelateerd aan het biologische werking onopgehelderd blijft, waren de verbindingen beschreven in

hoofdstuk 4 en 7 goed te karakteriseren, met name wat betreft secundaire en tertiare structuur. Subtiële veranderingen in de turn konden direct gecorreleerd worden aan zowel de structuur als de biologische activiteit. Dergelijk onderzoek, waarvan nieuwe mogelijke richtingen in **Hoofdstuk 8** zijn aangegeven kan bijdragen aan opheldering van de vraag: hoe werkt GS? Onafhankelijk daarvan is er de goede hoop dat zulke studies de basis kunnen zijn van het genereren van nieuwe antibiotica.

List of Publications

Design and synthesis of gramicidin S analogues containing decorated sugar amino acids

Grotenbreg, G. M.; Buizert, A. E. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M.

Manuscript in preparation

Synthesis and application of pyranopyran sugar amino acids

Grotenbreg, G. M.; Witte, M. D.; Tuin, A. W.; Leeuwenburgh, M. A.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M.

Manuscript in preparation

Synthesis and controlled polymerisation of a novel gramicidin S analogue

Ayres, L.; Grotenbreg, G. M.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M.; van Hest, J. C. M.

Manuscript in preparation

Carbohydrates as versatile platforms in the construction of small compound libraries

Timmers, M. S. M.; Verhelst, S. H. L.; Grotenbreg, G. M.; Overhand, M.; Overkleeft, H. S.
Pure and Applied Chemistry, Manuscript in press

Synthesis and biological evaluation of gramicidin S dimers

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Curriculum Vitae

Gijsbert Marnix Grotenbreg was born in Alkmaar on the 1st of July 1975. After completing his secondary education in 1993 at the Petrus Canisius College in Alkmaar, he traveled in Zambia, Zimbabwe and South Africa. He started his academic studies in chemistry at Leiden University in September 1994. From August 1997 to August 1998, undergraduate research was conducted in the “Bio-organic Synthesis” group of Prof. Dr. J. H. van Boom under the supervision of Dr. Jeroen Verheijen. His undergraduate thesis describes the solution-phase synthesis of cyclic peptide nucleic acids.

From October 1998 to September 1999, he performed research at the RIKEN Institute in Japan, as part of the Frontier Research Program, in the group of Dr. Osamu Kanie and Prof. Dr. C.-H. Wong. Part of this work, which involved the debenzoylation of immobilized (oligo)saccharides through catalytic hydrogenolysis with stabilized palladium nanoparticles, was presented at the 15th International Symposium for Glycoconjugates (poster presentation) held at August 1999 in Tokyo.

After returning to Leiden University, he obtained his doctorandus (Master of Science) degree in August 2000. Subsequently, he was affiliated with Leiden University as a Ph.D. student during the period of September 2000 to December 2004, where the work described in this thesis was conducted under the supervision of Prof. Dr. H. S. Overkleeft, Prof. Dr. J. H. van Boom, Dr. G. A. van der Marel and Dr. M. Overhand. The research performed was part of a collaboration with the groups of Prof. Dr. J. C. M. van Hest and Prof. Dr. F. P. J. T. Rutjes of the Radboud University in Nijmegen with financial aid from Netherlands Technology Foundation (STW) and DSM Research. He partook in the “National Peptide Meeting” (April 2004, oral presentation) and “International Symposium on Advances in Synthetic, Combinatorial and Medicinal Chemistry” in Moscow (May 2004, poster presentation) where parts of the work described in the thesis were presented.

From March 2005, he will commence his post-doctoral studies as NWO-TALENT fellow at the Harvard Medical School in Boston in the group of Prof. Dr. H. L. Ploegh.

Nawoord

Ter afsluiting wil ik graag die personen noemen die elk op hun eigen manier een belangrijke bijdrage hebben geleverd aan de totstandkoming van dit proefschrift. Allereerst natuurlijk mijn ouders, die mijn opleiding mogelijk hebben gemaakt en mij altijd onvoorwaardelijk hebben gesteund.

Een bijzondere plaats in dit nawoord verdienen Martijn Kronemijer, Erwin Tuin, Martin Witte, Alphert Christina en Annelies Buizert, die in het kader van hun hoofdvakstage elk een wezenlijke bijdrage hebben geleverd aan het in dit proefschrift beschreven onderzoek. Het bonte gezelschap waaruit de werkgroep “Bio-organische Synthese” bestaat, heeft ervoor gezorgd dat ik gedurende de laatste vier jaar met bijzonder veel plezier op het lab heb rondgelopen. De verhelderende kijk op zowel wetenschappelijke als niet-wetenschappelijke zaken van Richard van der Berg, Kimberly Bonger, Leendert van den Bos, Silvia Cavalli, Jeroen Codée, Dima Filippov, Martijn de Koning, Bas Lastdrager, Michiel Leeuwenburgh, Remy Litjens, Farid El Oualid, Karen Sliedrecht-Bol, Mattie Timmer, Martijn Verdoes, Steven Verhelst, Peter de Visser en Tom Wennekes heb ik altijd enorm gewaardeerd. In dit verband wil ik ook graag de “oude garde” noemen: Begoña Aguilera, Nicole Kriek, Huib Ovaa, Marike van Roon, John Turner en Renate van Well.

De hulp van Hans van den Elst en Nico Meeuwenoord is onmisbaar geweest bij de synthese van peptides, alsmede de LC/MS analyse en de HPLC zuiveringen daarvan. Op Fons Lefebvre en Kees Erkelens kon ik altijd rekenen voor hulp bij het opnemen van, en verwerken tot, mooie NMR-spectra. Voor technische ondersteuning in het lab kon ik altijd terecht bij de ama's. Door de inzet van Mark van Raaij en Antonio Llamas-Saiz is niet alleen een kristalstructuur opgelost, maar kon ook de moleculaire pakking daarvan worden beschreven. Han de Neeling en Emile Spalburg van het RIVM in Bilthoven ben ik zeer erkentelijk voor de begeleiding met antibacteriële experimenten. Voor hemolytische experimenten kon ik altijd een beroep doen op Peter van Hooft en Daan Noort van TNO in Rijswijk.

Daarnaast wil ik ook mijn familie en vrienden buiten de wetenschap noemen, die voor de nodige ontspanning hebben gezorgd en allerlei scheikundige verhalen hebben willen aanhoren. In het bijzonder wil ik daarbij alle leden van zowel het Aikido Centrum Leiden als het Universitair Sport Centrum en met name Marc Jongsten noemen die voor de stimulerende omgeving hebben gezorgd waarin het trainen mogelijk werd gemaakt.

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