Design, Synthesis and Biological Evaluation

of Peptidomimetic

Prenyl Transferase Inhibitors

Farid El Oualid

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Cover: Ternary substrate complex of protein:geranylgeranyl transferase-1 (PGGT-1) with the tetrapeptide substrate Cys-Val-Ile-Leu (sequence of the GTPase Rap2B) and 3'-azageranylgeranyl pyrophosphate, which is a non-reactive geranylgeranyl pyrophosphate analog that binds similarly in the active site.

In memoriam Prof. dr. Jacques H. van Boom
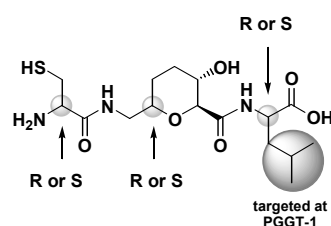
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List of Abbreviations

δ	chemical shift
Ac	acetyl
AcOH	acetic acid
amu	atomic mass unit
anh.	anhydrous
aq.	aqueous
ATR	attenuated total reflectance
$BF_3 \cdot OEt_2$	borontrifluoride diethyl etherate
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
Boc_2	*tert*-butyloxycarbonyl anhydride
BOP	benzotriazolyl-*N*-oxy-tris(dimethyl-amino)phosphonium hexafluoro phosphate
bs	broad singlet
bt	broad triplet
Bu	butyl
c	concentration
calc.	calculated
cat.	catalytic
COSY	correlation spectroscopy
CSA	camphorsulfonic acid
C_q	quaternary carbon atom
d	doublet
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCE	1,2-dichloroethane
DCM	dichloromethane
DIC	*N,N'*-diisopropylcarbodiimide
dd	doublet of doublets
ddd	double doublet of doublets
DEAD	diethyl azodicarboxylate
DHP	dihydropyran
DIAD	diisopropyl azodicarboxylate
DIBAL-H	diisobutylaluminium hydride
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-(*N,N*-dimethylamino)pyridine
DMAPP	dimethylallyl diphosphate
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulphoxide
dt	double triplet
DTT	dithiotreitol
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
e.g.	exempli gratia (for example)
ESI	electronspray ionisation
Et	ethyl
Et_2O	diethyl ether
Et_3N	triethylamine
et al.	et alii (and others)
equiv.	(molar) equivalent(s)
Fmoc	9-fluorenylmethoxycarbonyl
FPP	farnesyl pyrophosphate
g	gram(s)
GAP	GTPase activating protein
GDP	guanosine 5'-diphosphate
GEF	guanine-nucleotide exchange factor
GGPP	geranylgeranyl pyrophosphate
GTP	guanosine 5'-triphosphate
GP	general procedure
GPP	geranyl pyrophosphate
h	hour(s)
HATU	2-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCTU	2-(6-chloro-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMG-CoA	β-hydroxy-β-methylglutaryl-Coenzyme A
HOBt	1-hydroxybenzotriazole
HR-MS	high-resolution mass spectrometry
HRP	horseradish peroxidase
Hz	Hertz
IC_{50}	inhibitor concentration resulting in 50% inhibition
i.e.	id est (that is)
IPP	isopentenyl diphosphate
*i*Pr	isopropyl
Icmt	isoprenylcysteine carboxyl methyltransferase
IR	infrared
J	coupling constant
kDa	kiloDalton
LC-MS	liquid chromatography-mass spectrometry
m	multiplet
M	molar
Me	methyl

mg	milligram(s)
MHz	megahertz
min	minute(s)
MS	mass spectrometry
MS4Å	molecular sieves 4Å
m/z	mass to charge ratio
n	normal
NCBP(s)	nitrogen containing bisphosphonate(s)
NMP	N-methyl-2-pyrrolidinone
NMR	nuclear magnetic resonance
nOe	nuclear Overhauser effect
NOESY	nuclear Overhauser enhancement spectroscopy
NTP	nucleoside triphosphate
Ns	nitrobenzenesulfonyl
o	ortho
p	para
p.a.	pro analysi
PE	petroleum ether (40-60)
PFT	protein:farnesyltransferase
PGGT-1	protein:geranylgeranyl transferase-1
PGGT-2	protein:geranylgeranyl transferase-2
Ph	phenyl
PP	pyrophosphate
ppm	parts per million
PPTS	pyridinium p-toluenesulphonate
pyBOP	benzotriazolyl-N-oxy-tris (pyrrolidino)phosphonium hexafluorophosphate
q	quartet
REP	Rab escort protein
Ras	rat sarcoma
Rce	Ras and a-factor converting enzyme
ref.	reference(s)
R_f	retardation factor
R_t	retention time
RP-HPLC	reversed phase-high performance liquid chromatography
rt	room temperature
s	singlet
SAA(s)	sugar amino acid(s)
sat.	saturated

SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPPS	solid phase peptide synthesis
Su	succinimide
t	triplet
TBAI	tetra-n-butylammonium iodide
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy (free radical)
TFA	trifluoroacetic acid
Tf	trifluoromethanesulfonyl
THF	tetrahydrofuran
THP	tetrahydropyran-2-yl
TLC	thin layer chromatography
TMS	trimethylsilyl
Tr	triphenylmethyl (trityl)
Tris	Tris(hydroxymethyl)aminomethane
TsOH	toluenesulfonic acid
UV	ultraviolet
vs	versus
wt	weight

Three and one-letter codes amino acids*

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

* Unless stated otherwise, amino acid building blocks have the L-configuration.

Chapter 1

General Introduction

1.0 The Isoprene Metabolism
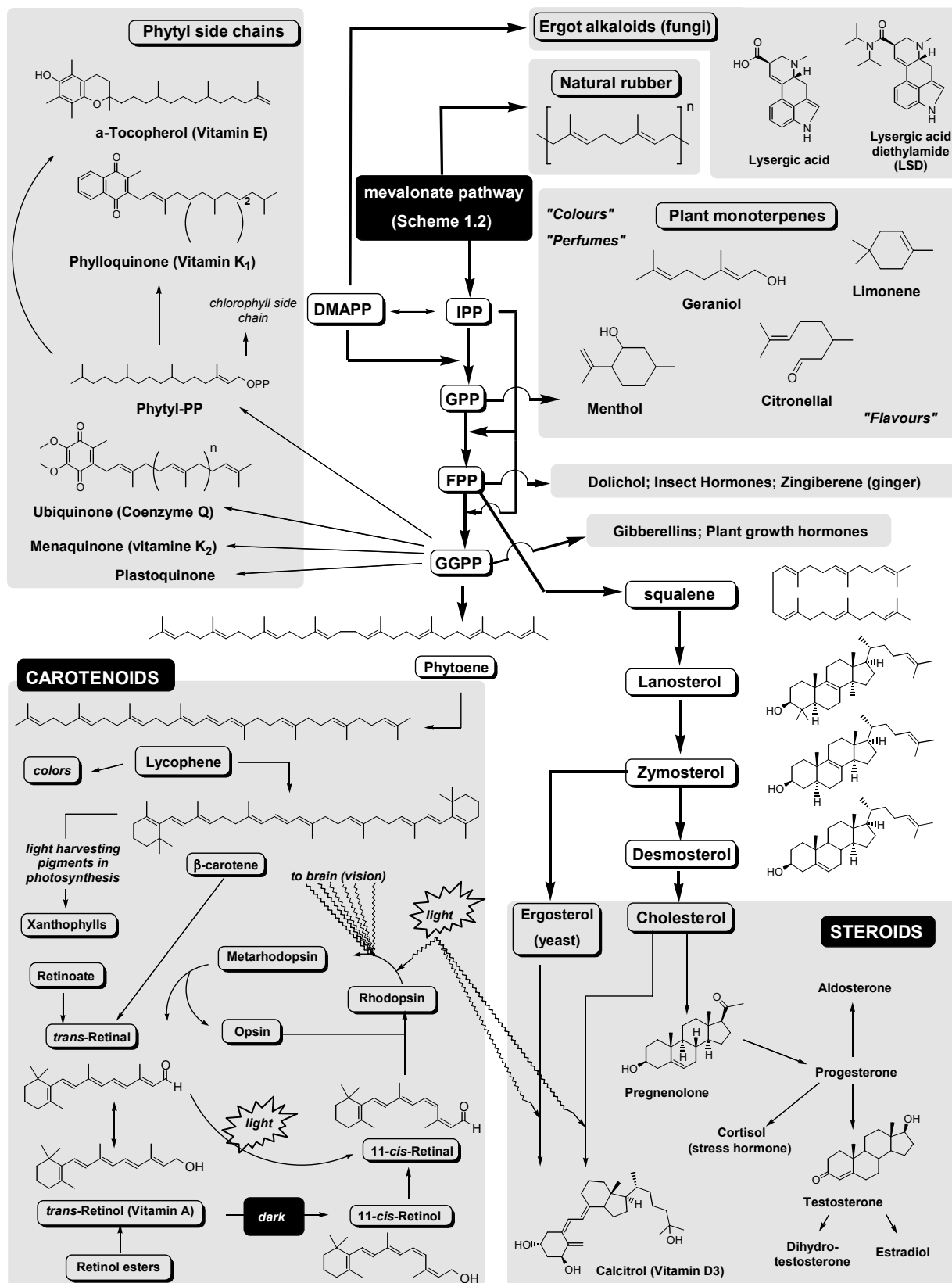
Scheme 1.1 Biochemical pathway of isoprene metabolism.

1.0.1 Introduction

The isoprenoids form one of the largest families of naturally occurring compounds.[1] In Scheme 1.1 a metabolic map of the isoprene metabolism is depicted.[2] The biosynthesis of these isoprenoids starts from two common precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Scheme 1.2). After the synthesis of IPP and DMAPP, which in eukaryotes are synthesised *via* the mevalonate pathway (Scheme 1.2),[3] the enzyme geranyl pyrophosphate synthase (GPP synthase) catalyses the head-to-tail addition of IPP to DMAPP yielding geranyl pyrophosphate (GPP). Condensation of GPP with IPP by farnesyl pyrophosphate synthase affords farnesyl pyrophosphate (FPP, Figure 1.1) and subsequent condensation of FPP with IPP by GPP synthase affords geranylgeranyl pyrophosphate (GGPP).[4] The isoprenoids FPP and GGPP are the key intermediates from which most isoprene metabolism products are derived.
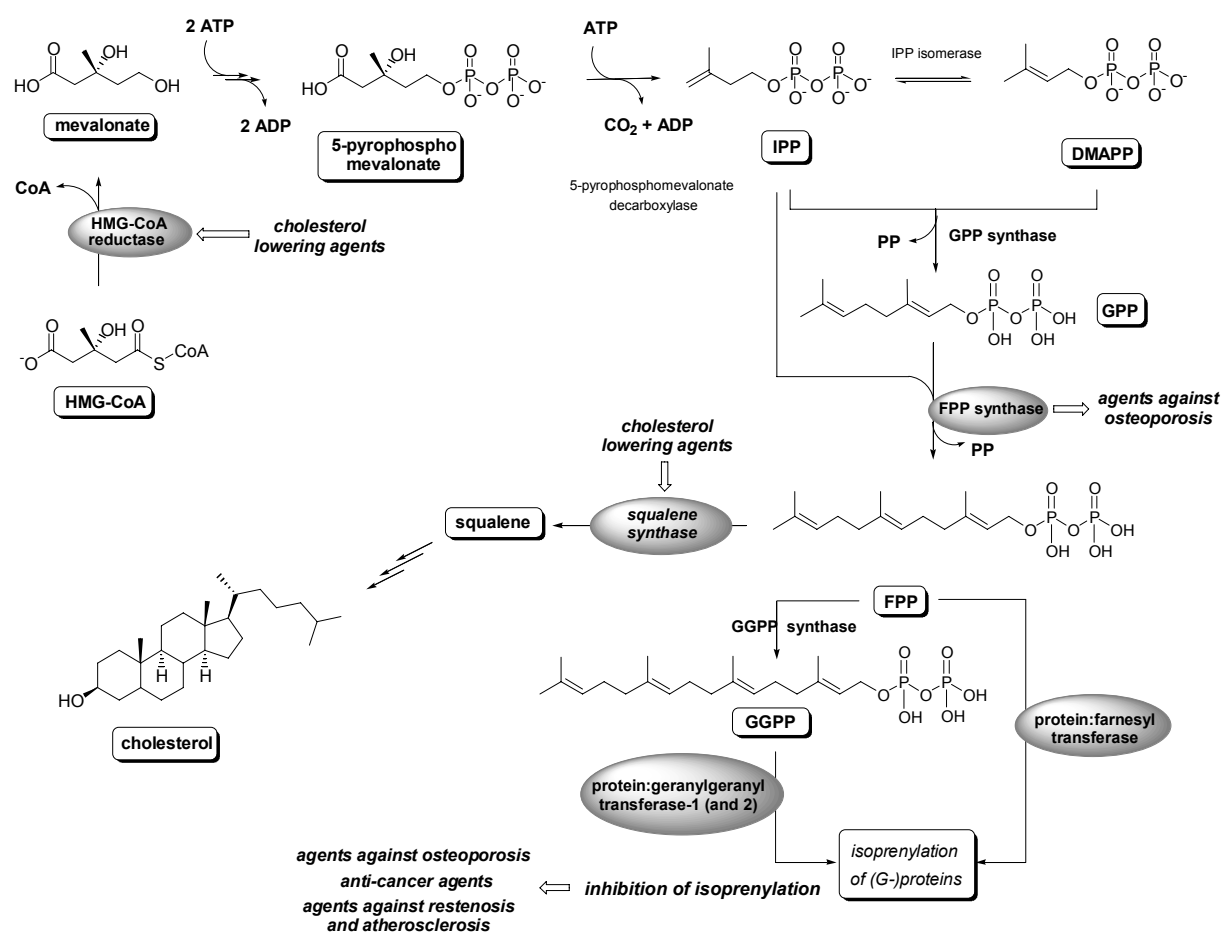
Scheme 1.2 A simplified representation of the IPP and DMAPP biosynthesis *via* the mevalonate pathway and their further processing (ATP= adenosine triphosphate, ADP= adenosine diphosphate).

Figure 1.1 Structures of FPP and GGPP.
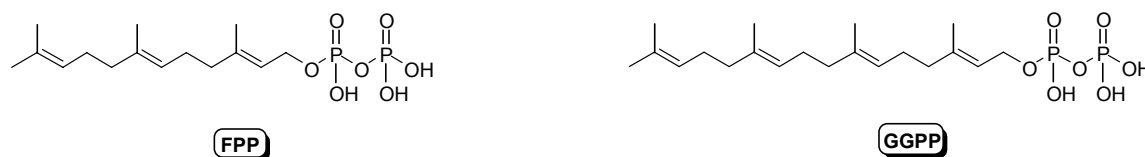
1.0.2 Drug Development and the Mevalonate Pathway

Interference in the mevalonate pathway is an attractive and rewarding approach for the development of drugs toward several pathological disorders that are related to isoprenoid functioning.

1.0.2.1 Cholesterol Lowering Agents

An important approach toward the treatment of elevated cholesterol levels in the blood plasma involves inhibition of cholesterol biosynthesis. Well known examples are the statins (Figure 1.2),[5ab] compounds that act by inhibiting HMG-CoA reductase (Scheme 1.2). As HMG-CoA reductase is situated early in the biochemical pathway, obstruction of this enzyme also influences the biosynthesis of other important products of the isoprene pathway. Next, inhibitors of squalene synthase were envisioned as more specific alternatives for the development of cholesterol lowering agents.[6ab] Squalene synthase catalyses the reductive dimerisation of two FPP molecules to squalene which is further processed to cholesterol (Scheme 1.2). To date, however, no squalene synthase inhibitor has reached the market due to serious toxicity.[6cd]
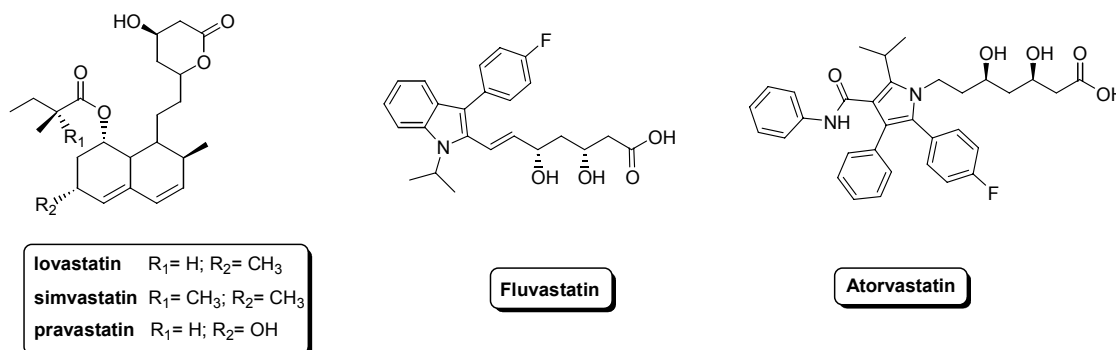

Figure 1.2 Examples of statins as HMG-CoA reductase inhibitors: lovastatin (Mevacor[®]), simvastatin (Zocor[®]), pravastatin (Lipostat[®]), fluvastatin (Lescol[®]) and atorvastatin[5cd] (Lipitor[®]).

1.0.2.2 Agents against Osteoporosis

Bones are continually remodeled by two types of cells: osteoblasts, which synthesise the collagen fibrils that form the scaffolding upon which the bone is formed and osteoclasts, which are responsible for bone resorption. During osteoporosis[7] there is an imbalance between these two cell types, for instance enhanced activity of osteoclasts, which can lead to an overall breakdown of bone tissue. A recently developed approach toward anti-osteoporosis agents is based on nitrogen containing bisphosphonates (NCBPs, Figure 1.3).[8] These compounds exert their activity against osteoclasts by inhibiting FPP synthase (Scheme 1.2) with apoptosis as effect.[9] As the earlier mentioned statins inhibit the formation of FPP precursors, these compounds are also envisioned as effective anti-osteoporosis agents.[10] However, until now there is insufficient evidence for the clinical use of statins as anti-osteoporosis agents which may be attributed to the fact that statins are mainly targeted to the liver.[5b]
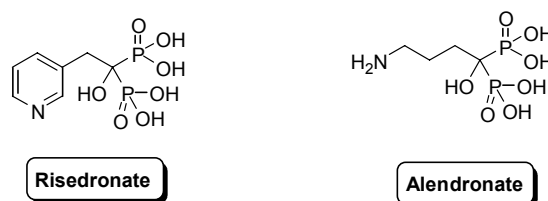
Figure 1.3 The nitrogen containing bisphosphonates Risedronate (Actonel®) and Alendronate (Fosamax®) as agents for the treatment of osteoporosis.

1.0.2.3 Anti-cancer Agents

Protein isoprenylation[11] is a post-translational modification entailing the covalent attachment of an isoprenoid lipid to a protein-substrate (Scheme 1.2). To date more than 100 proteins that are involved in regulating various biological processes such as signal transduction,[12] cell growth, differentiation, cytoskeletal function and vesicular trafficking,[13] are known to be isoprenylated. In general, post-translational modifications transform a protein from a pre-mature state to a mature state by either regulating a proper translocation of the protein to a cellular membrane or inducing protein-protein interactions.[14] G-proteins are GTPases (guanine triphosphatases) which are located at the inner surface of the cell membrane and act as molecular switches in a large network of

signalling pathways.[15] Normally, they cycle between an active GTP bound state and an inactive GDP bound state (Figure 1.4). This process of GTP and GDP binding is regulated by a GTPase activating protein (GAP) and a guanine nucleotide exchange factor (GEF). GAP catalyses the hydrolysis of GTP and can be seen as a negative regulator of G-protein signalling (termination of signal),[16] while GEF is involved in the exchange of GDP for GTP.
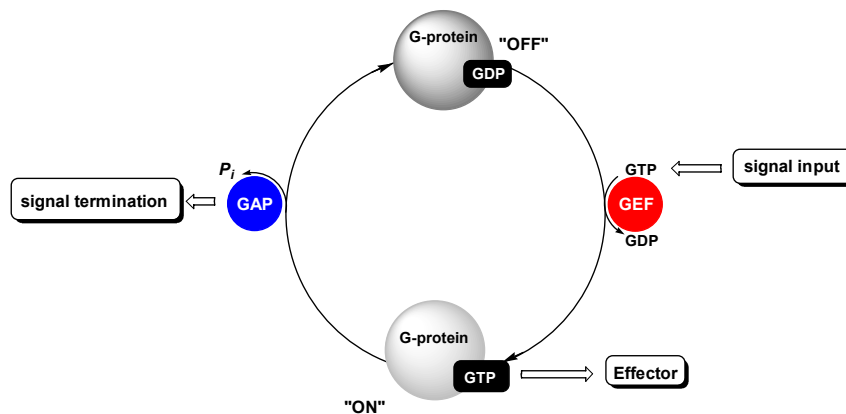

Figure 1.4 The GTPase cycle. GEF= guanine nucleotide exchange factor; GAP= guanine triphosphatase activating protein; GDP= guanosine diphosphate; GTP= guanosine triphosphate.

The wide interest in the interference of isoprenylation is primarily based on the finding that isoprenylated G-proteins[17] are involved in the malignant transformation of cells and that blocking these oncogenic G-proteins is a promising method for the development of anti-cancer agents. The involvement of G-proteins in tumorogenesis can be caused by the following: 1) mutations in the G-proteins themselves which invariably confer resistance to the binding and action of GAPs,[18] leading to a situation in which the signal induced by the G-protein is continually in the "ON" state; 2) alterations in upstream tyrosine receptor kinases leading to undesired activation of the G-protein; 3) alterations in downstream components such as GAP proteins which ultimately leads to a loss of negative regulation.[19]

The most important and abundant small G-proteins involved in human tumorogenesis[20] are members of the Ras family:* H-Ras (Harvey-Ras), N-Ras

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* The word Ras is an abbrevation of *rat sarcoma* as oncogenic Ras was first identified in rat.

(neuroblastoma-Ras), K-Ras (Kirsten-Ras), with K-Ras existing as two isoforms, K-RasA and K-RasB.[21] These small monomeric G-proteins (21 kDa) show a high sequence conservation and were first detected in human cancers in 1982. It is estimated that they are present in - and at least partially responsible for the proliferation of - about $30 - 40\%$ of all human tumors.[22] Since the finding of these oncogenic proteins in humans, there has been a considerable interest in the development of Ras inhibitors as anti-cancer agents.[23]

A widely used approach toward the inhibition of oncogenic Ras activity comes from understanding of the requirements for its activity. All Ras proteins are initially synthesised as inactive cytosolic proteins that have to undergo post-translational modifications to gain full biological activity (Scheme 1.3).[24] The first and most essential modification involves the isoprenylation of a *C*-terminal cysteine residue in a characteristic tetrapeptide motif, the "Ca_1a_2X-box", of the peptide substrate. Here C stands for cysteine, in general the a_1a_2 is an aliphatic hydrophobic dipeptide and the nature of residue X determines substrate specificity between the two responsible isoprenylating enzymes, protein:farnesyl transferase (PFT) and protein:geranylgeranyl transferase-1 (PGGT-1). PFT preferably modifies substrates where X is Met, Ser or Gln whereas PGGT-1 has a high propensity for modifying substrates having Leu or Phe as X residue.

Although the isoprenylation step is the major determinant for proper functioning of Ras proteins, subsequent post-translational modifications are also important for full transforming activity.[25] These post-translational modifications comprise a number of steps (Scheme 1.3): first, there is the proteolytic cleavage of the a_1a_2X tripeptide by the protease Rce1[26] which is followed by the methylation of the formed *C*-terminal carboxylic group by isoprenylcysteine carboxyl methyltransferase (Icmt).[27] In the case of H-Ras,[28] N-Ras and K-RasA, upstream cysteine residues (for H-Ras two cysteines) are then palmitoylated through the action of a palmitoyl transferase[29] and this additional hydrophobicity promotes further association to the cell membrane.[30] K-RasB on the other hand, contains a stretch of upstream located positively charged lysine residues which allow K-RasB to interact with the negatively charged heads of the lipid bilayer.[31] It is assumed that this electrostatic interaction substitutes for the palmitoyl interaction present in H-Ras, N-Ras and K-RasA.[32]
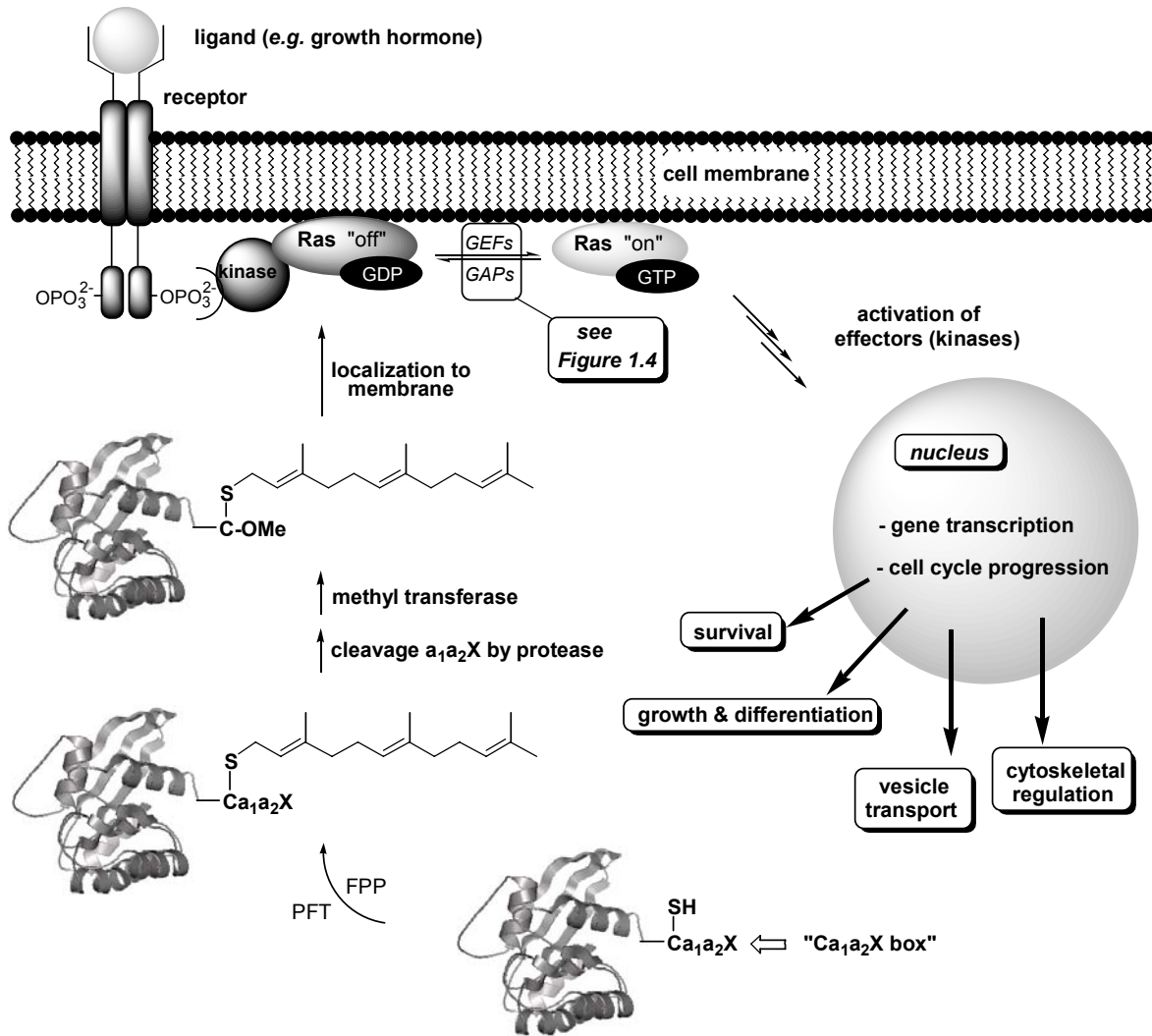
Scheme 1.3 Simplified representation of Ras processing and its role as molecular switch in signal transduction.

1.1 The Protein Prenyl Transferases [33]

1.1.1 Introduction

PFT and PGGT-1 catalyse the vast majority of protein isoprenylation events encountered in nature. Their role in post-translational modifications was identified for the first time in 1990 by Brown and co-workers who noticed that [^{3}H] labeled mevalonate was incorporated into polypeptides.[34] In 1991 PFT was identified in the cytosolic fraction of bovine brain.[35] Since then PFT has been isolated from rat brain,[36,37,38] pig[39], yeast,[40] plants[41] and human.[42] PGGT-1 was identified in 1991 and has since then been purified from bovine brain,[43] yeast (*Saccharomyces cerevisiae*),[44] rat,[45] human[46] and plants.[47]

The crystal structures of PFT[48] and PGGT-1[49] (Figure 1.5) show a heterodimeric metalloprotein consisting of an α-subunit (48 kDa) and a β-subunit (PFT: 46 kDa, PGGT-1: 43 kDa). PFT and PGGT-1 share the same α-subunit while the β-subunit of PFT has 25% homology with the β-subunit of PGGT-1. The α-subunit is build up by a set of helices which are arranged in α-helical hairpin pairs. This results in a crescent like shape which wraps extensively around the β-subunit. The β-subunit forms a globular and compact α-α barrel domain with a central cavity in which the active site resides.

Figure 1.5 Ribbon representations of human PFT and human PGGT-1 subunits.[50]

| α-subunit of PFT and PGGT-1 | β-subunit of PFT | β-subunit of PGGT-1 |
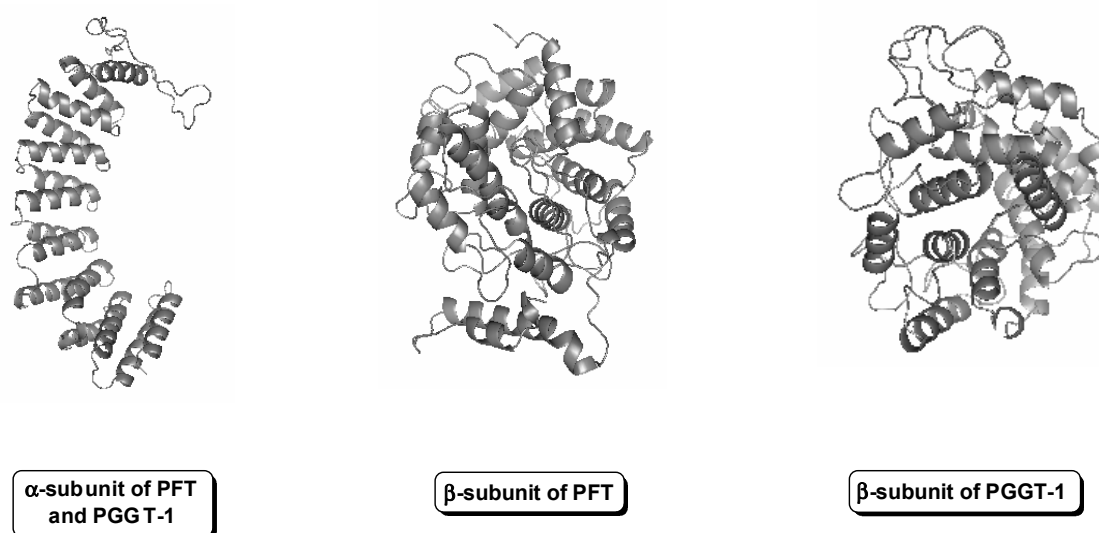
1.1.2 Reaction cycle and Mechanism of PFT and PGGT-1

From steady-state kinetic studies and X-ray crystallographic studies, a general reaction cycle for PFT[51] and PGGT-1[49] has been formulated. The catalytic cycle commences with the binding of the isoprenoid pyrophosphate substrate[52] followed by binding of the peptide substrate (**I→II→III**, Scheme 1.4). During the following turn-over process, metals play an important role: a Zn^{2+} ion is essential for catalytic activity and directs the binding of the peptide substrate and enhances the nucleophilicity of the cysteine-thiol functionality by lowering its pK_a (**A→B**, Scheme 1.4);[53] a Mg^{2+} ion is required in PFT for maximal activity by coordinating the isoprenoid pyrophosphate group and facilitating its nucleophilic displacement. PGGT-1 does not require any Mg^{2+} for activity, instead the active site of PGGT-1 contains a lysine residue for coordination with

the pyrophosphate group.[54] Initially, the pyrophosphate group and the zinc coordinated thiolate are separated by ~8Å. In the next step the isoprenoid substrate repositions the pyrophosphate group to close proximity of the thiolate allowing formation of the thioether by a S_n2 like displacement of the pyrophosphate group (**A→B**, Figure 1.6). The transition state of the product formation (**A**, Scheme 1.4) can be visualised as a metal bound cysteine bearing a partial negative charge on the thiol, the isoprenoid having a partial positive charge on C_1 and the bridging oxygen between the α-phosphate and C_1 having a partial negative charge.§ Upon binding of a new isoprenoid substrate (**IV→II**, Scheme 1.4) the isoprene part of the isoprenylated protein is moved to an exit groove. In the exit groove the conformation of the Ca_1a_2X part of the prenylated protein changes from an extended to a type 1 β-turn and this alteration is believed to be important for release of the product.
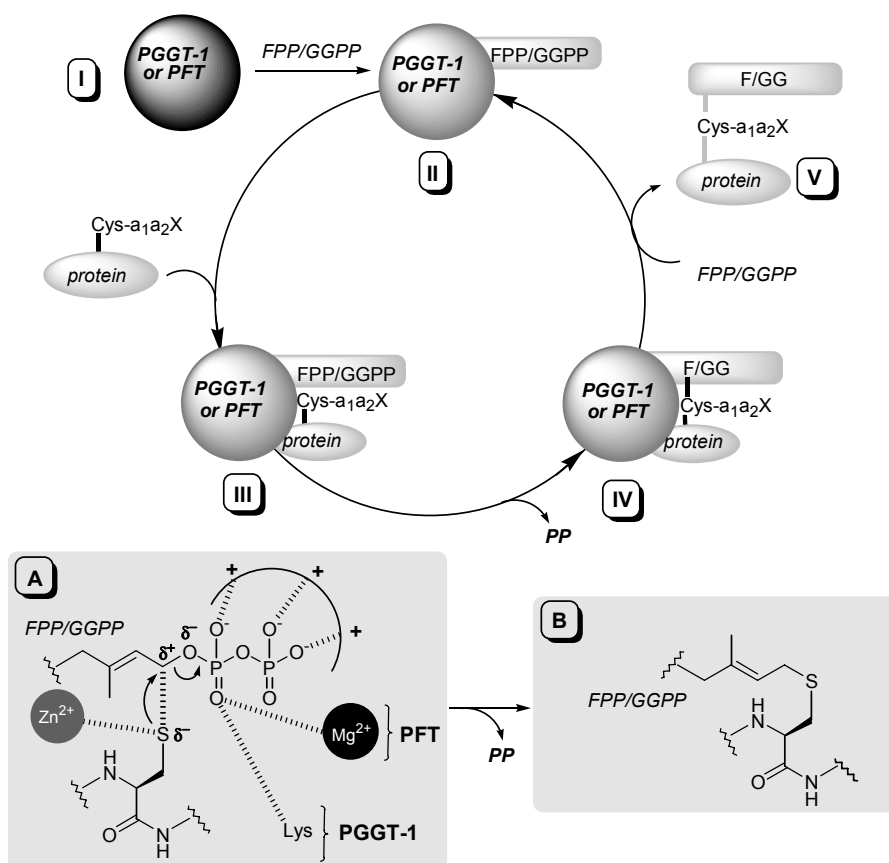
Scheme 1.4 Reaction sequence and mechanism of isoprenylation by PFT and PGGT-1.

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§ This negative charge on the bridging oxygen is stabilised by a tyrosine residue in the active site.
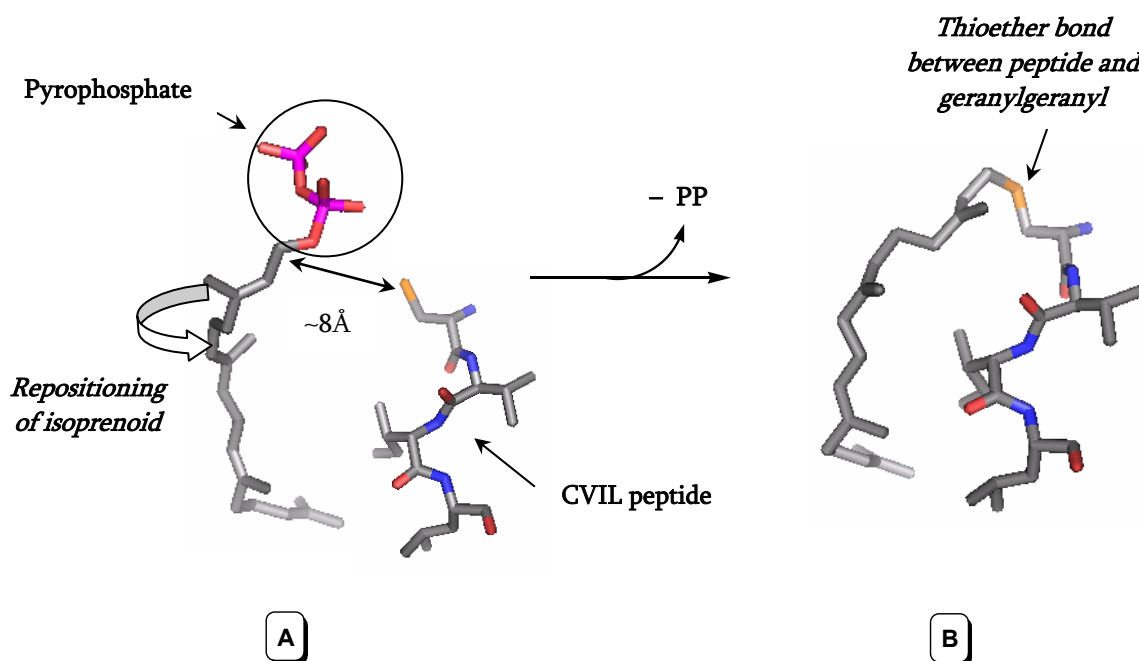
Figure 1.6 Rotation of first two isoprene units brings the isoprenoid in the productive conformation (exemplified for GGPP and CVIL).[50]

1.1.3 Factors that Determine Substrate Specificity of PFT and PGGT-1

In order to develop any substrate based prenyl transferase inhibitors it is important to understand the factors by which PFT and PGGT-1 discriminate between their peptide and isoprenoid substrate. In this section the factors governing this specificity will be outlined in some detail.

1.1.3.1 Peptide substrate specificity

The first difference between PFT and PGGT-1 concerns the selective binding of the peptide substrate. Beese and co-workers[13] defined a set of rules directing substrate selectivity by crystallographic analysis of a set of eight substrate peptides. For PFT and PGGT-1 the a_1 position is oriented toward the solvent and in theory should be able to accommodate any amino acid (Figures 1.7 and 1.8). In both enzymes the a_2 residue binds in a hydrophobic pocket, excluding polar or charged residues in this position. In addition, too large or too small residues are also not compatible with the a_2 pocket. In PFT (Figure 1.7) the a_2 pocket has a highly aromatic character and aromatic substituents at the a_2 position of the Ca_1a_2X-box can interact strongly with this pocket.
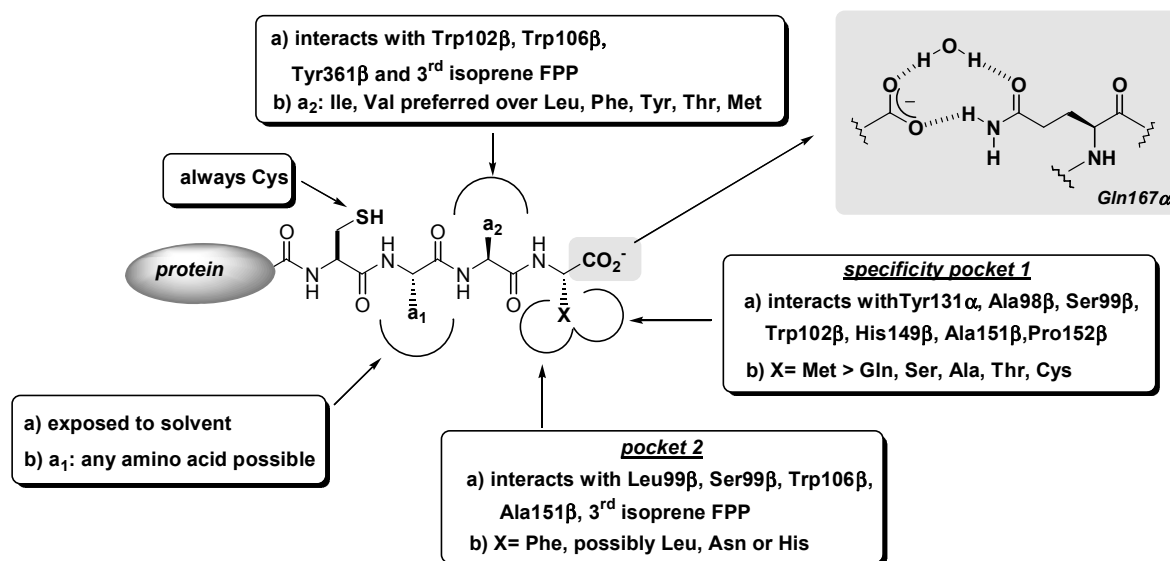
Figure 1.7 Surrounding of Ca_1a_2X tetrapeptide motif in active site of PFT.

The most important moiety governing peptide specificity for PFT and PGGT-1 is the side-chain of the X-residue. In the specificity pocket (pocket 1, Figure 1.7) of PFT van der Waals and electrostatic interactions allow the binding of Met, Gln or Ser, the X- residues found in the majority of mammalian PFT substrates.[55] Although natural PFT substrates containing a Phe as X-residue are known, pocket 1 is not able to accommodate this residue.[56] An explanation is that the Phe side-chain binds in an alternative pocket (pocket 2, Figure 1.7) and the empty space of specificity pocket 1 is filled with two water molecules. This alternative pocket can also be used by PFT to accommodate a Leu, Asn or His residue at the X-position without steric clashes or distortion of the Ca_1a_2X-box backbone.

Figure 1.8 Surrounding of Ca_1a_2X tetrapeptide motif in active site of PGGT-1.

In PGGT-1 (Figure 1.8) the hydrophobic pocket for residue a_2 is smaller and has less aromatic character than the corresponding pocket in PFT. Stabilis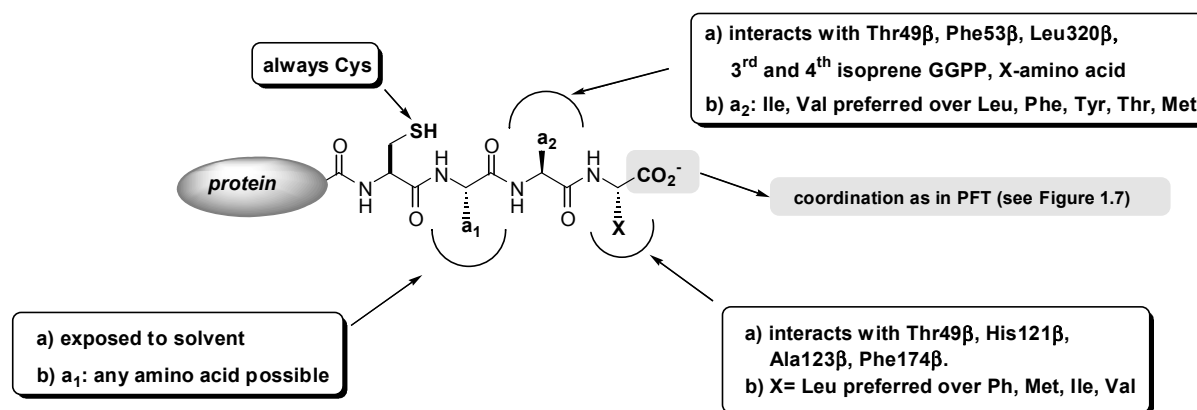ation of the *C*-terminal X-residue is governed by one pocket in which hydrophobic and van der Waals interactions allow binding with a Leu, Phe or Met moiety in the peptide substrates. The majority of PGGT-1 substrates have a Leu as X-residue, but Ile[57] and Val[58] are also tolerated by PGGT-1 as X-residue.

1.1.3.2 Isoprenoid substrate specificity[13,49]
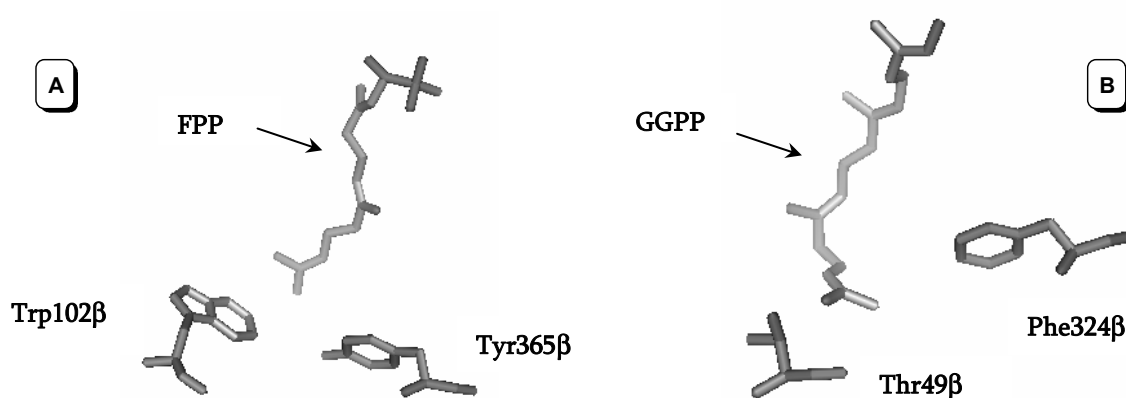
Figure 1.9 Left: surrounding FPP in PFT (**A**); right: surrounding GGPP in PGGT-1 (**B**).

The second difference between PFT and PGGT-1 concerns the selective binding of the isoprenoid substrate. In PFT, the terminal isoprene in FPP is surrounded by a Tyr(365β) and Trp(102β) residue (**A**, Figure 1.9). In PGGT-1 the Trp residue is a smaller Thr residue and the Tyr is Phe (**B**, Figure 1.9). This enables accommodation of the 4th isoprene of GGPP and thus productive binding of GGPP. In all PGGTs-1 isolated from different species, the 49β residue is found to be a small amino acid (Thr, Val, Ser, Ala) whereas in PFT it is always Trp. To underline the importance of Trp102β in PFT, it is reported that when this residue is mutated in PFT to a Thr residue the corresponding PFT shows the same isoprenoid substrate specificity as PGGT-1.[38a,46] Thus, as PFT discriminates between FPP and GGPP by steric factors,[59] how does PGGT-1 exclude the binding of the smaller FPP? The mechanism by which PGGT-1 releases its product determines the preference for GGPP binding: the lipid binding pocket is always occupied

with either the isoprenoid substrate or the product (see Scheme 1.4) and GGPP is much more able to displace the product from the active site in PGGT-1 than PFT; thus PGGT-1 uses its product release to direct isoprenoid specificity.[13]

1.1.3.3 Substrate cross-specificity

PFT and PGGT-1 exhibit cross-specificity that can be explained with the aid of structural information given in paragraph *1.1.3.1* and *1.1.3.2*. First, PFT can bind *C*-terminal Leu residues in pocket 2 (Figure 1.7), an alternative pocket to the one used for the Met residue. For example the small G-protein RhoB with CKVL as Ca_1a_2X sequence is a substrate of both PGGT-1 and PFT.[60] A model composed by Beese and co-workers[13] shows that Leu can nicely be accommodated in the second binding pocket of PFT. It is important to mention that this type of cross-specificity is strongly regulated by the a_1a_2 dipeptide sequence: some dipeptide sequences do not allow a correct positioning of the Leu side-chain in the alternative pocket of PFT. This is exemplified by the finding that Rap2b, having CVIL as Ca_1a_2X box, is not a substrate for PFT. Second, PGGT-1 is able to accommodate Met in its X-binding pocket, explaining the observed cross-specificity for K-RasB with Ca_1a_2X= CVIM and N-Ras with Ca_1a_2X= CVVM. Finally, in contrast to the peptide substrate, cross-specificity of the isoprenoid substrates appears to be less pronounced.[61]

1.1.3.4 Protein:geranylgeranyl transferase 2

Protein:geranylgeranyl transferase-2 (PGGT-2) is the third member of the protein prenyl transferases and exclusively isoprenylates Rab proteins, which are small G-proteins involved in subcellular localisation and vesicular transport.[62] In contrast to PFT and PGGT-1, the isoprenylation step catalysed by PGGT-2 (also called RabGGT) involves the transfer of two geranylgeranyl isoprenoids to two closely spaced cysteine moieties located at the *C*-terminus of the Rab proteins. These cysteines are arranged in motifs such as C-C, C-a-C, C-C-a, or a-a-X-X (with C= cysteine and a is any amino acid).[63] An additional difference with PFT and PGGT-1, is the required complexation of PGGT-2 with a Rab escort protein (REP) prior to substrate binding.[64]

1.2.1 Inhibition of PFT and PGGT-1 in Drug Development

As Ras proteins (*i.e.* H-Ras, N-Ras, K-RasA and K-RasB) are normally farnesylated, it is not surprising that the majority of research activities aimed at disabling protein isoprenylation is directed at the design of PFT inhibitors. In addition, the majority of proteins is geranylgeranylated,[11b,65] indicating that blocking of geranylgeranylation will affect a broader range of biological processes. However, PGGT-1 has emerged as an important alternative target for several reasons.[66] First, there is the observation that upon blocking PFT, N-Ras and the most abundant human oncogenic Ras protein K-RasB are geranylgeranylated through the action of PGGT-1.[67] This indicates that effective therapies based on preventing K-RasB (and N-Ras)[68] functioning may require the inhibition of both PFT and PGGT-1. Besides K-RasB, several natural PGGT-1 substrates (*e.g.* RhoA) may also be involved in mediating oncogenesis and/or metastasis.[69] Second, PGGT-1 inhibitors hold promise as anti-osteoporosis agents. This is based on the observation that geranylgeraniol (GGOH), but not FPP, was able to prevent the action of nitrogen containing bisphosphonates (NCBPs) on oscteoclasts.[8b,70] The GGOH is transformed *in vivo* to GGPP,[71] which then restores the geranylgeranylation of certain proteins important for osteoclast growth (such as Rab, Rho and Rac).[72]

A third therapeutic field promoting the targeting of PGGT-1 entails atherosclerosis and restenosis. Atherosclerosis is a general term for the thickening and hardening of arteries while restenosis involves the rethickening of a coronary artery after percutaneous transluminal coronary angioplasty.[73] During these processes the proliferation of vascular smooth muscle cells plays an important role. Because isoprenylated G-proteins are involved in the regulation of vascular smooth muscle cell proliferation, the inhibition of these proteins by blocking PFT and/or PGGT-1 is regarded to be a viable approach toward the development of therapeutic agents for atherosclerosis and restenosis.[74]

1.2.2 Design and Development of PFT and PGGT-1 inhibitors

1.2.2.1 The Ca_1a_2X box as lead

Since the early 90's it is known that PFT and PGGT-1 recognise and are inhibited by Ca_1a_2X box tetrapeptides and thus can serve as lead for the design of potential inhibitors

(Figure 1.10). The intrinsic potency of the Ca_1a_2X tetrapeptide as recognition motif for the corresponding prenyl transferase (PFT and/or PGGT-1) is further underscored by the early observation that a Ca_1a_2X sequence such as CVLS (from PFT substrate H-Ras) is farnesylated by PFT.[34,36,75] The presence of an *N*-terminal amine, for example in the case of CVLS, is generally tolerated by the prenyl transferase. Longer Ca_1a_2X containing peptides, *e.g.* SSGCVLS,[35] are also farnesylated. As small peptides exhibit a low cellular permeability and a high sensitivity toward proteolytic degradation, this renders them unuseful for therapeutic applications. To overcome these obstacles the Ca_1a_2X motif has been used extensively as lead for the development of numerous Ca_1a_2X peptidomimetics aimed at inhibiting PFT (Figure 1.11) and/or PGGT-1 (Figure 1.12).[76] Ca_1a_2M analogs **4**[77] and **8**[78] illustrate an interesting feature of the Ca_1a_2X template: compound **4** has an extended conformation while **8** adopts a β-turn like conformation. As mentioned in paragraph **1.1.2**, the Ca_1a_2X tetrapeptide adopts both type of conformations in both PFT and PGGT-1. Therefore Ca_1a_2X based inhibitors may adopt an extended or turn-like motif. Besides modification of the a_1a_2 part the cysteine and X-residue have also been replaced by non-peptidic mimics. Compounds **9–12** (Figure 1.11) and **16–20** (Figure 1.12) are examples in which the cysteine residue is replaced by an alternative Zn^{2+} chelating moiety, the imidazole functionality. In general, the X-residue is replaced by a hydrophobic residue which binds either in the pocket involved in the interaction with the X moiety or to other hydrophobic residues present in the active site. In compound **13** a tetrazole[79] is incorporated as a carboxylic acid isostere. Although the Ca_1a_2X tetrapeptide motif is a valuable and rewarding template for a peptidomimetic approach toward isoprenyl transferase inhibitors, the structural determinants of enzyme recognition and selectivity are complex[80] (Figure 1.7 and 1.8).

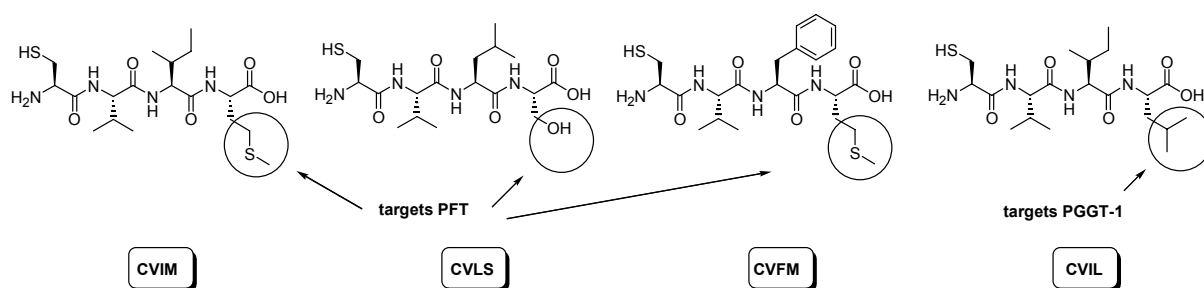**Figure 1.10** Ca_1a_2X box tetrapeptide sequences used for the development of PFT/PGGT-1 inhibitors.
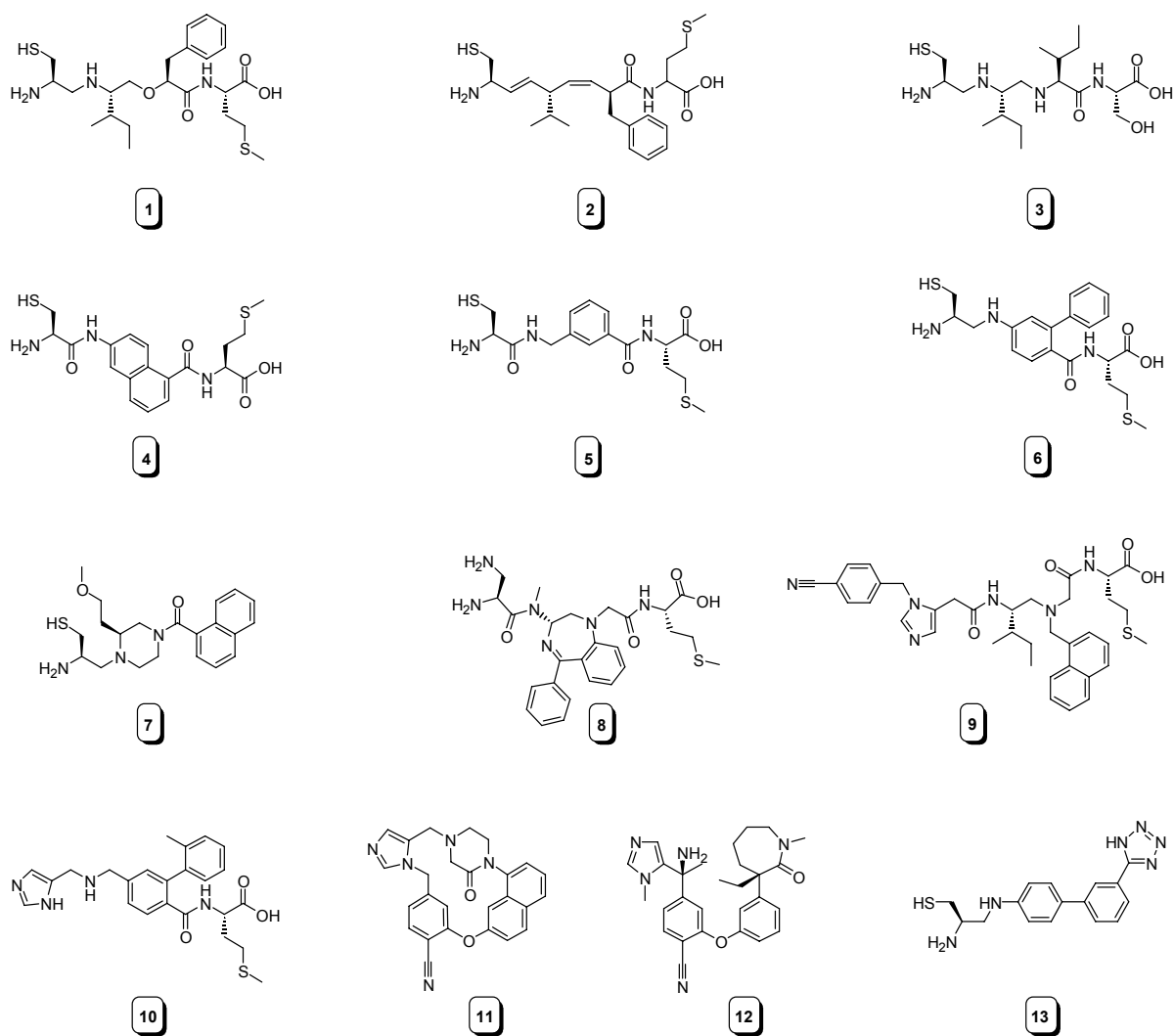
Figure 1.11 Inhibitors of PFT which bind in the Ca_1a_2X pocket.[76]
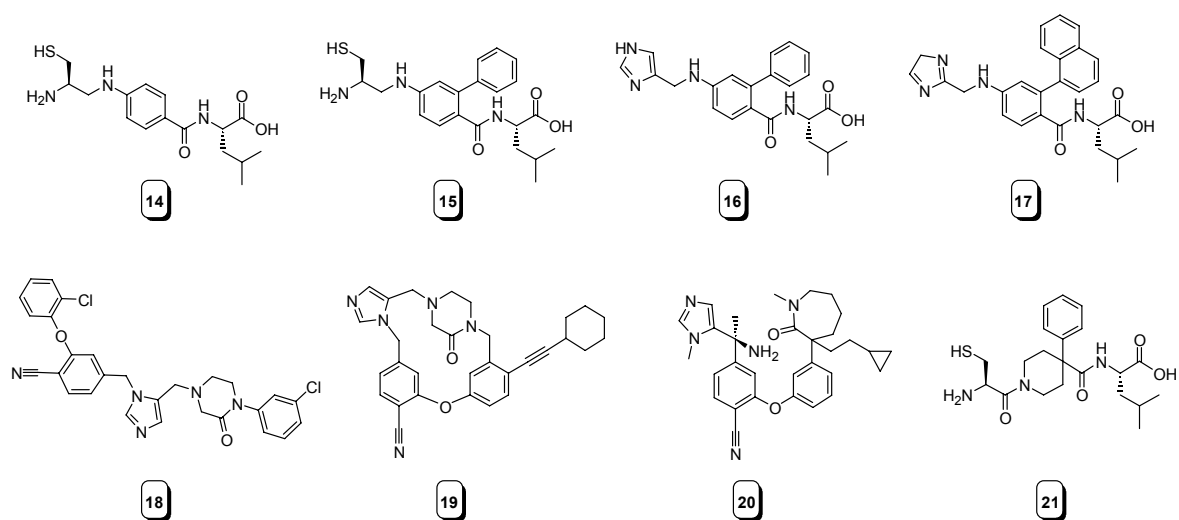
Figure 1.12 PGGT-1 inhibitors which bind in the Ca_1a_2X pocket: **14**,[67c] **15**,[81] **16/17**,[82] **18**,[83] **19**,[84] **20**,[85] **21**[86].

1.2.2.2 The isoprenoids FPP and GGPP as lead

Next to the Ca_1a_2X tetrapeptides, FPP (Figure 1.13) and GGPP (Figure 1.14) can be used as templates for the development of prenyl transferase inhibitors. In general, the pyrophosphate is replaced by a more stable and cell permeable isosteric group (as in **22 – 24** and **28 – 30**). Compounds **26** and **27** are examples of PFT inhibitors which were developed from potential squalene synthase inhibitors.
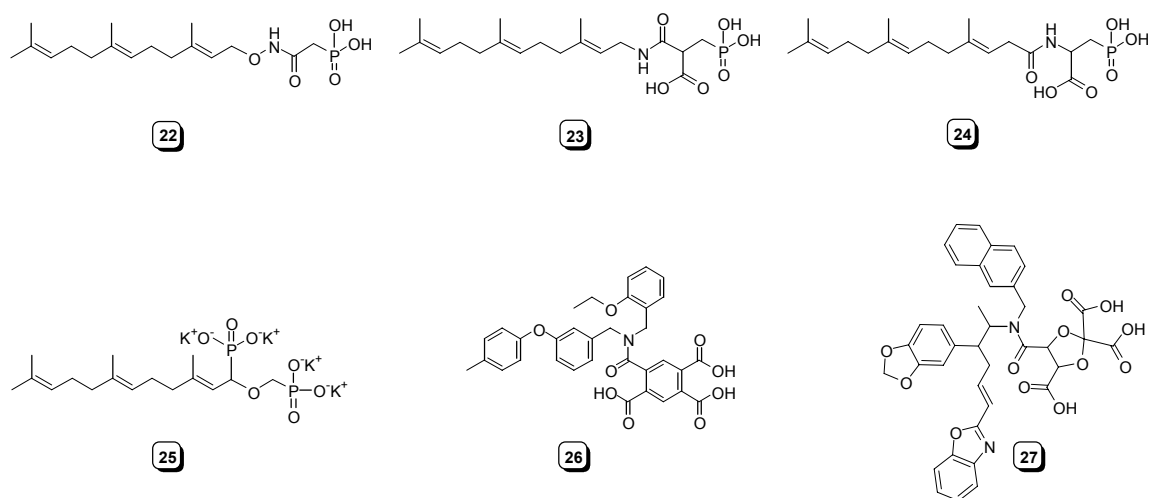
Figure 1.13 Examples of PFT inhibitors based on FPP: **22**[87], **23** and **24**[88], **25**[89], **26**[90], **27**[91].

Figure 1.14 Examples of PGGT-1 inhibitors based on GGPP: **28 - 30**[92], **31**[93], **32**[89], **33**[94], **34**[95].

1.2.2.3 Bisubstrate Inhibitors

Potential inhibitors of PFT and PGGT-1 can also be based on the characteristics of both the Ca_1a_2X and isoprenoid substrates.[96] Such bisubstrate inhibitors (**35–38**, Figure 1.15) offer opportunities for achieving high specificity, as combining the features of both substrates makes it more likely that neither component 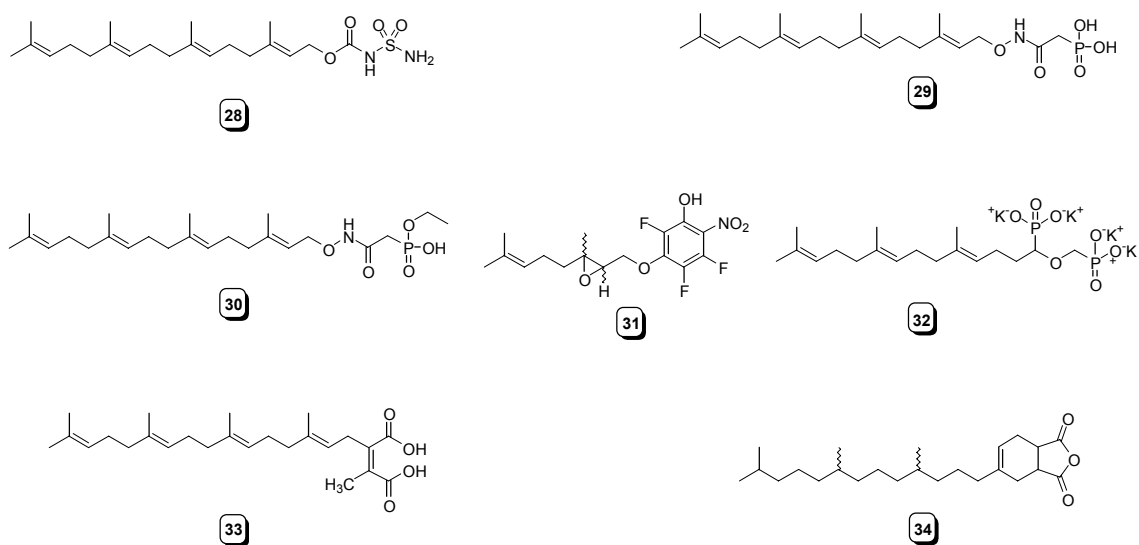will be recognised by untargeted enzymes which also use the same substrate (*e.g.* squalene synthase).[97] Note that to date only PFT has been the focus of potential bisubstrate inhibitors.[96]
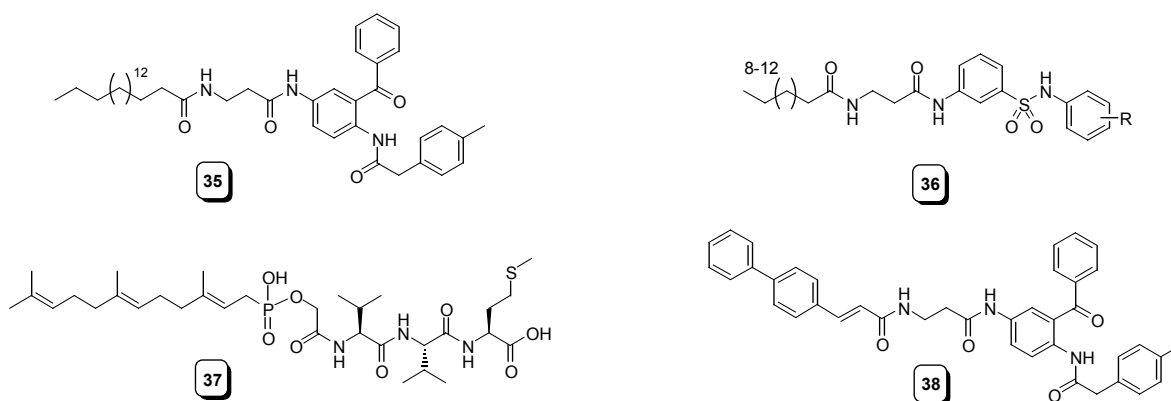
Figure 1.15 Examples of bisubstrate inhibitors targeted at PFT: **35**[98], **36**[99], **37**[100], **38**[101].

1.2.2.4 Inhibitors from library screening

Lead compounds for the development of PFT and/or PGGT-1 inhibitors have also been obtained by the screening of libraries. In general, these compounds exhibit a large variety in structural identity.[102] Compound **39** (Figure 1.16) is an example of a potent PFT inhibitor obtained from library screening.[103] Despite a high peptidic character, kinetic analysis showed **39** to be competitive for FPP. The very low cell permeability of **39** led to the development of the more cell permeable compound **40**.[104]
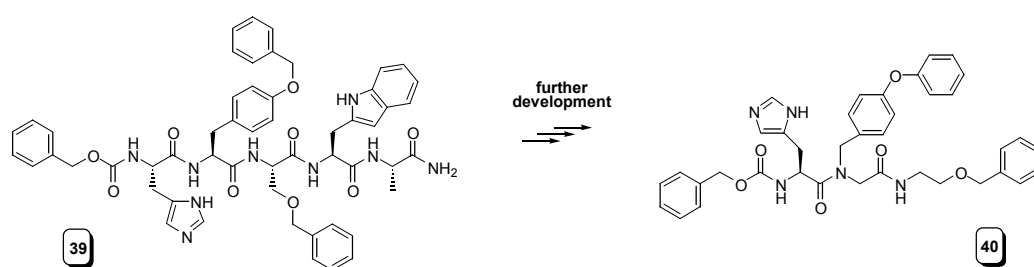
Figure 1.16 Compound **39**: PFT inhibitor from library screening; compound **40** is an analog of **39** with improved cellular permeability.

1.2.2.5 Inhibitors from natural sources

Several natural inhibitors of PFT[105] (Figure 1.17) and PGGT-1[106] (Figure 1.18) have been isolated from microorganisms, plants and soils. Natural source inhibitors are seldom used as lead compounds for the development of more potent analogs which may be attributed to their complex structure and low inhibitory potency.
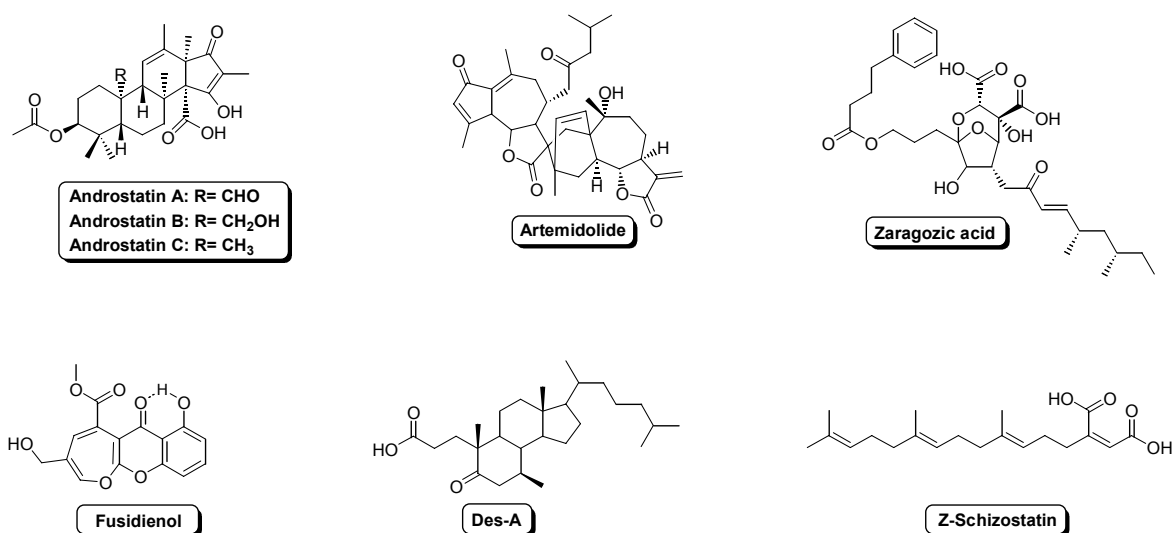
Figure 1.17 Natural inhibitors of PFT.
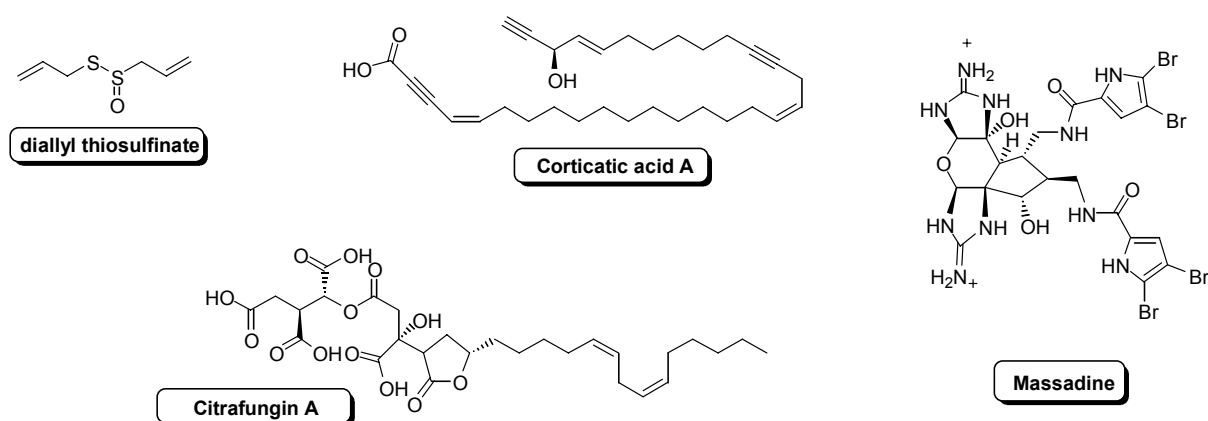
Figure 1.18 Natural inhibitors of PGGT-1.

1.2.3 Inhibitors of isoprenylation in clinical trials.

To date, four inhibitors of PFT have been subjected to evaluation in clinical trials (phase I-III).[106e,107] Compound **L-778,123** (Figure 1.19)[84] was initially designated a selective

inhibitor of PFT (PFT: K_i= 0.9 nM, PGGT-1: K_i= 10 µM) which competes with the Ca$_1$a$_2$X peptide substrate. Later it was found that when anions (such as adenosine triphosphate, phosphate, sulfate) are present, **L-778,123** behaves like a dual inhibitor of both PFT and PGGT-1 (PGGT-1: K_i= 4 nM) indicating that anions have a synergistic effect on the activity of **L-778,123** against PGGT-1.[84,108]
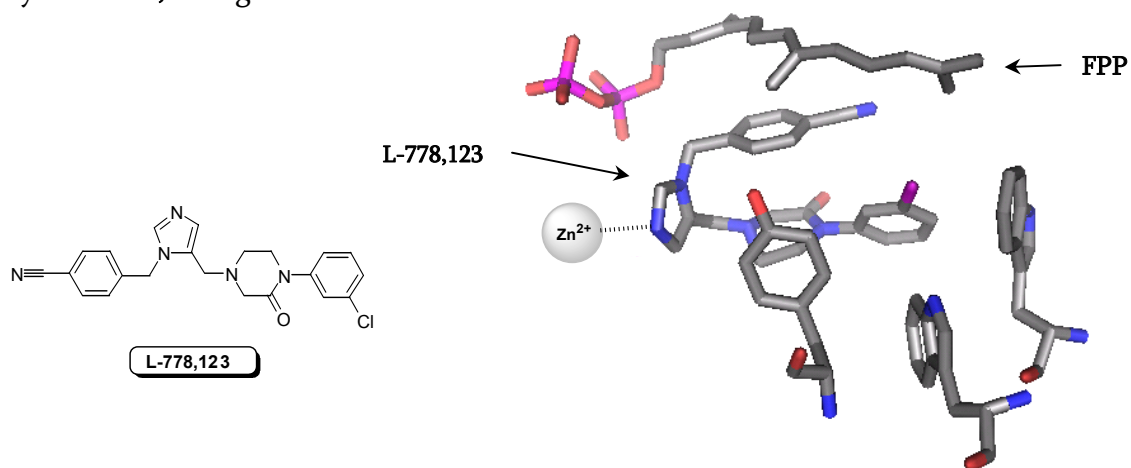
Figure 1.19 Structure of **L-778,123** and its binding mode in PFT.

The trihalobenzocycloheptapyridine **SCH66336** (Lonafarnib/Sarasar®,Figure 1.20) is a selective inhibitor of PFT (IC$_{50}$= 1.9 nM). The design of **SCH66336** started from **SCH44342**, a compound obtained by random library screening.[109] The binding mode of **SCH66336** has been elucidated in detail by crystallographic studies[110] showing that the upper part is involved in interactions with bound FPP while the lower part has contacts with amino acid residues of PFT (right part Figure 1.20).
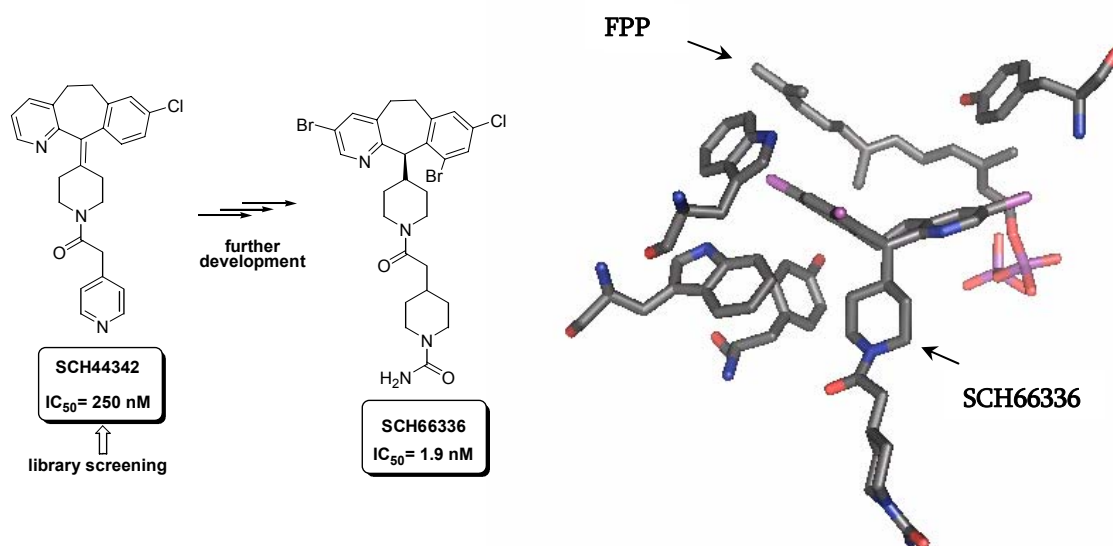
Figure 1.20 Left: structure of **SCH44342** and **SCH66336**. Right: binding mode of **SCH66336** in PFT.

BMS-214662 (Figure 1.21)[111] is a selective inhibitor of PFT (IC$_{50}$= 1.4 nM). Crystallographic studies showed that **BMS-214662** binds in the Ca$_1$a$_2$X box binding site, in line with its peptide-competitive behaviour, as previously determined by kinetic analysis.[112] The general design of this compound is based on an imidazole group for interaction with the Zn^{2+} in the active site and a core which is functionalised with aromatic residues for stacking interactions with aromatic residues of the a$_2$ binding pocket. As for **SCH66336**, the inactivity of **BMS-214662** against PGGT-1 is believed to be caused by its inability to form aromatic stacking interactions with the aromatic residues of **BMS-214662**.
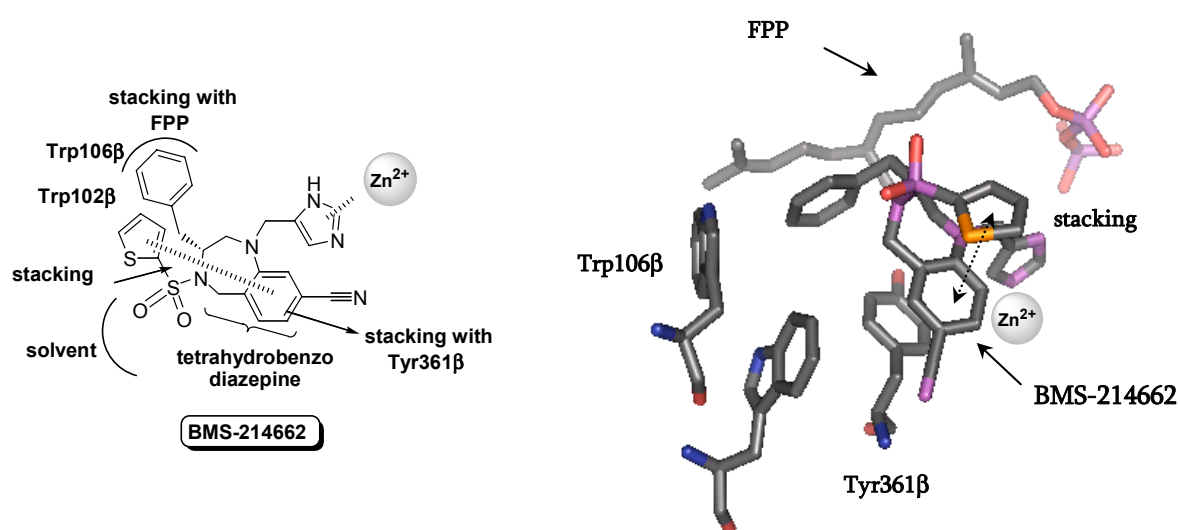
Figure 1.21 Left: key interactions of **BMS-214662** in PFT. Right: binding mode from X-ray studies.

Finally, **R115777** (Tipifarnib®, Figure 1.22)[113,*] is a selective inhibitor of PFT (IC$_{50}$= 1.4 nM) that binds to the peptide binding pocket as clarified by kinetic analysis and crystallographic studies.[111,113] **R115777** is U-shaped when bound to the active site of PFT and besides polar interactions, aromatic stacking of the aromatic residues are involved in binding. The selectivity of **R115777** against PFT is also governed by the aromatic residues of the a$_2$ binding pocket.

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* As from january 24th 2005, Tipifarnib® has been submitted to the US Food and Drug Administration (FDA) for approval for the treatment of acute myeloid leukemia (see www.drugs.com/nda/tipifarnib 050124.html).
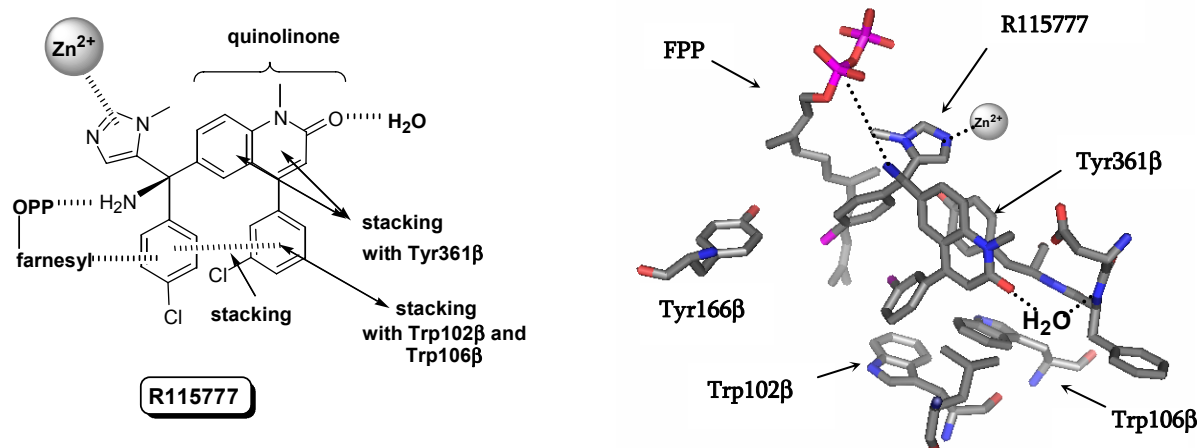
Figure 1.22 Left: key interactions of **R115777** in PFT. Right: binding mode from X-ray studies.

1.2.4 Why is there selectivity for tumor cells versus normal cells?

The phenomenon that tumor cells show an enhanced sensitivity for inhibition is not uncommon. At the moment the exact reasons for the observed selectivity of PFT and PGGT-1 inhibitors for tumor cells is not fully clear. Some observations made with PFT inhibitors, however, are worth mentioning.[114] First, not all farnesylated proteins exhibit the same sensitivity to PFT inhibition in cells.[78,115] Second, redundant pathways in normal cells may be responsible for the compensation of the functional loss of proteins.[116] Thirdly, the functions of farnesylated proteins involved with cellular transformation may be more susceptible to the action of PFT/PGGT-1 inhibitors than are the functions of those same proteins in normal cells. For example, a dominant negative form of Ras has been found to exhibit a much greater inhibitory effect on cellular transformation induced by oncogenic H-Ras function than on normal cellular Ras function.[117]

1.3 Outline of the Thesis

Van Boom and co-workers demonstrated for the first time the viability of (partially deoxygenated) sugar amino acids (SAAs) as peptidomimetic building blocks in the construction of novel PFT inhibitors based on the Ca_1a_2X-box.[118] In line with this approach, **Chapter 2** describes a novel route towards two dideoxy SAAs (**41** and **42**, Figure 1.23) which were used to synthesise analogs of the Ca_1a_2L motif thereby aiming to target PGGT-1 (**43**). Next to the (stereochemical) nature of the SAA building block (*i.e.* R or S at

C_6), the configuration (L or D) of the Cys and Leu pharmacophores was varied, leading to a set of eight Ca_1a_2L analogs. It is demonstrated that the nature of the SAA building block, in conjunction with the stereochemistry (L or D) of the Cys and Leu pharmacophores, has a distinct influence on the ability of the compounds with general structure **43** to inhibit PGGT-1.
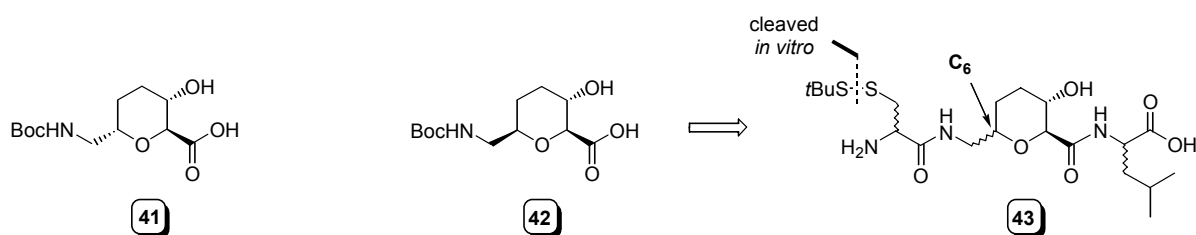
Figure 1.23 Sugar amino acids **41** and **42** as dipeptide isosters in the Ca_1a_2L motif.

Chapter 3 describes the use of SAAs in the development of both PFT and PGGT-1 inhibitors. It was envisioned that enhancing the hydrophobicity of the a_1a_2 dipeptide mimics might lead to hydrophobic interactions between the Ca_1a_2X analogs and the hydrophobic residues surrounding the a_2 pocket. As aromatic groups reside in this pocket it was decided to attach a benzyl group to the C_3 hydroxyl group (**44**, Figure 1.24). In addition, the importance of the amide linkage between the SAA and X-residue was probed by the synthesis of a set of Ca_1a_2X analogs (**49**) in which the corresponding amide was replaced by an amine.[119] The methyl ester analog of one of the developed compounds was evaluated *in vivo* and showed inhibitory activity against protein farnesylation in cultured cells.
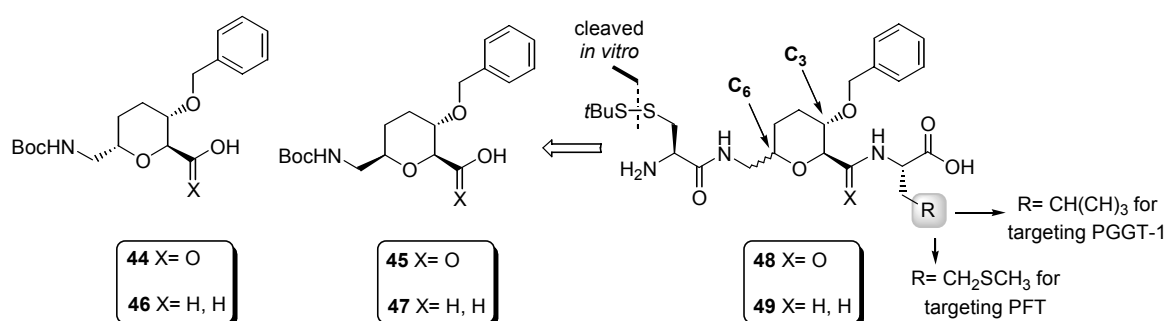
Figure 1.24 Benzylated SAAs (**44** and **45**) and corresponding amine analogs (**46** and **47**) as dipeptide isosters in the Ca_1a_2X motif.

Chapter 4 presents the synthesis of lipophilic Ca_1a_2L analogs as potential bisubstrate inhibitors of PGGT-1. The general structure of the presented compounds is depicted in Figure 1.25 (50): Ca_1a_2L analogs presented in Chapter 2 are connected, directly or *via* a linker (Gly or γ-Abu), to a simple fatty chain (lauric acid or palmitic acid) which may function as GGPP mimic.
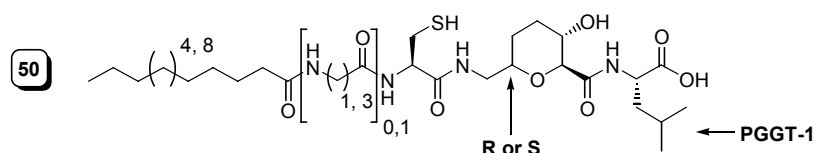
Figure 1.25 General structure of lipophilic Ca_1a_2L analogs presented in Chapter 4.

In Chapter 5 the effect of introducing a tetrazole[79] as carboxyl bioisostere in the Ca_1a_2X box is investigated (Figure 1.26). Compound 51 is an analog of the Ca_1a_2X box sequence CVIM (K-RasB) and compound 52 is derived form a potent and selective inhibitor of PFT presented in chapter 3. As is shown, in both compounds the *C*-terminal carboxylic functionality was replaced by the pharmacological advantageous tetrazole.
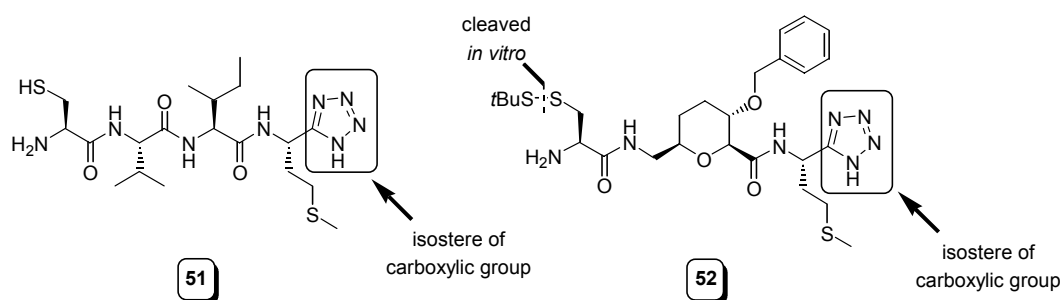
Figure 1.26 Tetrazole analogs of CVIM (51) and a potent PFT inhibitor presented in Chapter 3 (52).

Chapter 6 presents a labeling strategy for detecting the *in vivo* isoprenylation of proteins by a Bertozzi-Staudinger reaction[120] between an azide substituted FPP moiety (53) and phosphine reagent 54 (Scheme 1.5). After incubation of a mouse macrophage cell line with FPP analog 53, PFT recognises 53 as alternative substrate and consequently processes it. The cell lysate was then treated with reagent 54 thereby covalently binding any azidofarnesylated proteins with 54. Next, avidin conjugated to horseradish peroxidase

allowed detection of any azidofarnesylated proteins by chemiluminescence. Analysis by SDS-PAGE showed the azidofarnesylated proteins as separate bands and by addition of PFT inhibitors, the labeling efficiency was decreased indicating that the developed strategy also shows potency for the evaluation of PFT inhibitors.
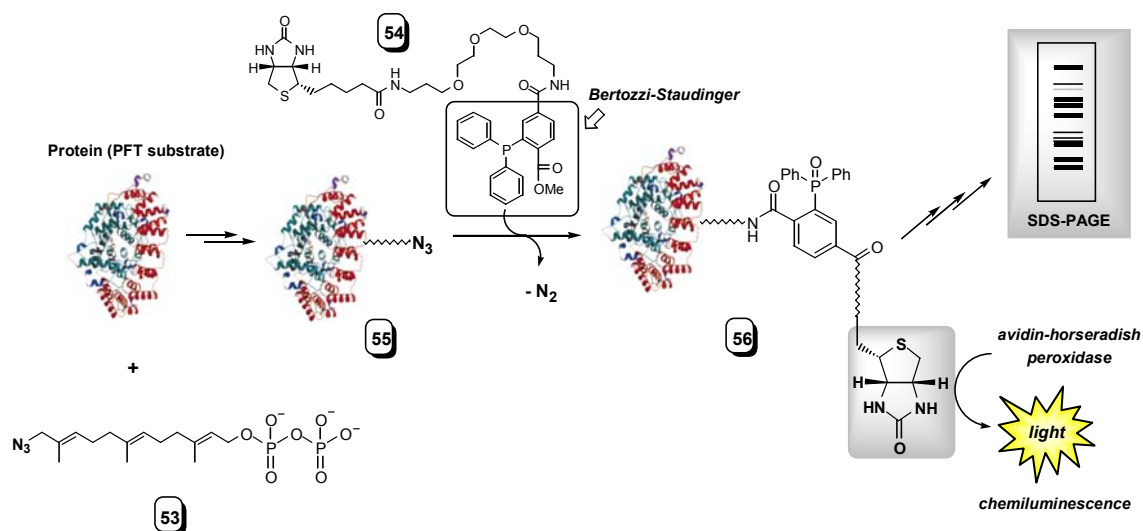
Scheme 1.5 Two-step Bertozzi-Staudinger ligation for the identification of isoprenylated proteins.

Chapter 7 describes a combinatorial approach toward a library of ambiphilic peptide-based compounds of general structure in which an *in silico* iterative optimisation procedure was used for the rapid construction of potential inhibitors of PGGT-1 (Scheme 1.6). The iterative optimisation procedure involves the arbitrary replacement of randomly chosen building blocks. By repetitive cycles of the process a progressive improvement of the average inhibitory potency of the compounds against PGGT-1 was observed.
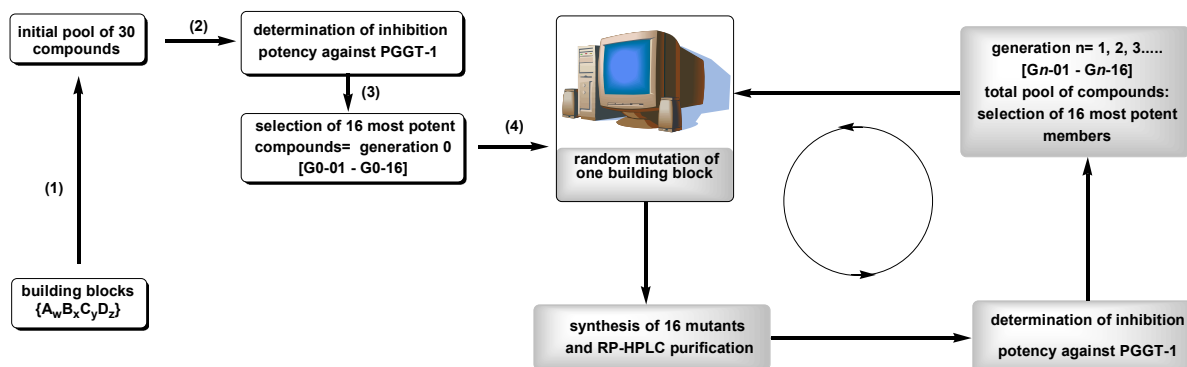
Scheme 1.6 Schematic presentation of optimisation procedure presented in Chapter 7.

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Chapter 2

Synthesis and Biological Evaluation of Protein:geranylgeranyl Transferase-1 Inhibitors –
Incorporation of Sugar Amino Acids as Dipeptide Isosters

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Abstract. A novel route towards two dideoxy sugar amino acids (SAAs) is presented. The suitably protected SAA building blocks were used to synthesise eight analogs of the Ca_1a_2L motif present in natural substrates of protein:geranylgeranyl transferase 1 (PGGT-1). Two Ca_1a_2L analogs (*i.e.* 2,6-trans **9** and 2,6-cis **14**) which structurally differ at the C_6 position of the central SAA residue and at C_α of the cysteine residue, show comparable inhibition potency against PGGT-1 ($IC_{50}= 68 \pm 16$ μM and 69 ± 20 μM, respectively). The results indicate that both 2,6-cis and 2,6-trans SAA building blocks can be used for the development of potent PGGT-1 inhibitors.

2.1 Introduction

An attractive strategy for the generation of PGGT-1 inhibitors is based on the design of structural analogs of the Ca_1a_2L motif present in natural PGGT-1 substrates (see Chapter 1). A generic structure of these Ca_1a_2L based inhibitors consists of the important pharmacophoric groups, *i.e.* the cysteine and leucine side chains, interconnected *via* a predesigned peptidomimetic linker (**I**, Figure 2.1). Desirable properties in terms of biostability (resistance towards proteolytic degradation) and bioactivity (*e.g.* inducement of a bioactive conformation)[1] can be introduced by the choice of an appropriate peptidomimetic linker.[2]
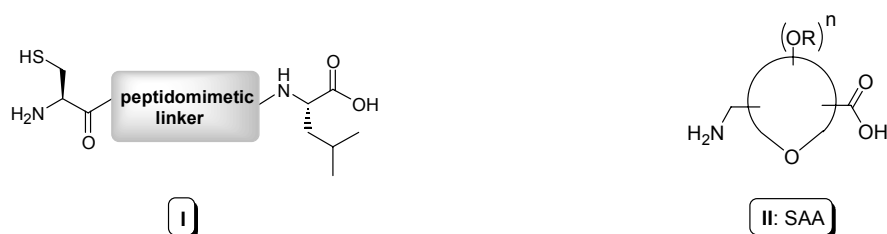

Figure 2.1 Peptidomimetic replacement of a_1a_2 dipeptide (**I**); schematic representation of SAA (**II**).

Sugar amino acids (SAAs, **II**, Figure 2.1), modified carbohydrates featuring an amine and a carboxylate, have emerged as useful building blocks for the construction of a wide variety of potentially biologically active molecules,[3] such as oligosaccharide mimetics,[4] enkephalin analogs,[5] integrin inhibitors[6] and scaffolds.[7] Moreover, van Boom and co-workers demonstrated the viability of partially deoxygenated gluconic amino acids (having either a 2,6-cis or 2,6-trans configuration) as building blocks in the construction of novel PFT inhibitors.[8]

In this chapter the use of SAAs **III** (2,6-cis) and **IV** (2,6-trans) as an isosteric replacement of the a_1a_2 dipeptide present in the Ca_1a_2L motif (**V**, Figure 2.2) of natural PGGT-1 substrates is presented. Both the 2,6-cis and 2,6-trans configuration in the SAA building blocks were selected.[9,10] Next to the (stereochemical) nature of the SAA building block, the configuration (L or D) of the Cys and Leu pharmacophores was varied, leading to eight novel tetrapeptide analogs of the general design **VI** (Figure 2.2). It is demonstrated that the nature of the SAA building block, in conjunction with the stereochemistry (L or D) of the Cys and Leu pharmacophores, has a distinct influence on the ability of compounds based on design **VI** (Figure 2.2) to inhibit PGGT-1.
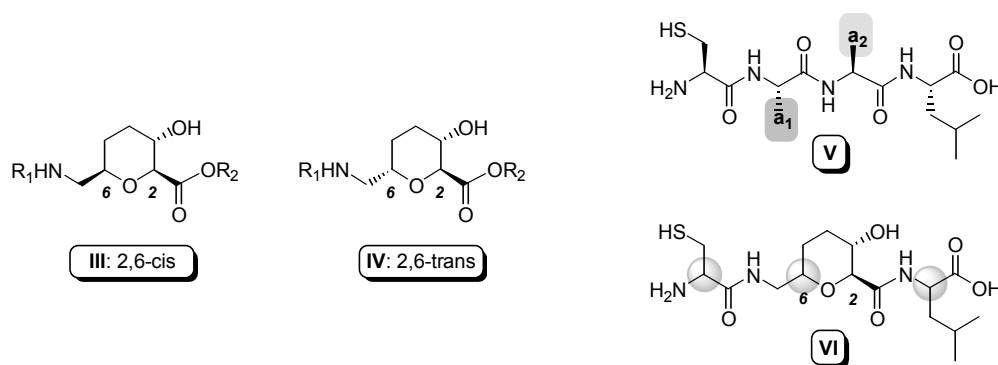
Figure 2.2 Incorporation of SAAs **III** and **IV** as dipeptide isosters in the Ca$_1$a$_2$L motif (**V**) furnishing potential PGGT-1 inhibitors of type **VI**.

2.2 Results and Discussion

2.2.1 Synthesis – In the first approach toward the synthesis of SAAs **3** (Scheme 2.1), glucal **1**, readily prepared from D-(+)-glucuronic acid-γ-lactone,[11] was treated with TMSCN and BF$_3$·OEt$_2$ furnishing 4,5-dideoxy glucopyranosyl cyanides 2,6-cis/trans **2** (86%, cis/trans ~3/5) as an inseparable mixture.[12] Reduction of the olefin and cyanide functionalities[13] in **2** followed by protection of the resulting primary amine as the *tert*-butoxy carboxylate (Boc), and final saponification of the acetate gave SAA **3** as an inseparable mixture of diastereoisomers in 30% yield over the four steps.

Although this route of synthesis proved to be an efficient process, the anomeric mixture obtained after the Ferrier rearrangement in the first step (2,6-cis/trans **2**) could not be separated after the three subsequent transformations. Therefore the removal of the 3-*O* acetate in an earlier stage of the synthetic route was investigated. Base mediated removal of the acetate in 2,6-cis/trans **2** is excluded because of the instability of **2** under these conditions.[14] Gratifyingly, acid-induced hydrolysis[15] of the acetate furnished a separable mixture of the desired 2,6-cis/trans cyanides **5**.

In an alternative approach, the individual C-glycosides 2,6-cis and 2,6-trans **5** proved to be accessible from the known, partially deprotected methyl glucuronate-D-glucal **4**.[16] Treatment of **4** with Pd(OAc)$_2$ and TMSCN in CH$_3$CN at 80°C gave cyanides 2,6-cis **5** and 2,6-trans **5** in a ratio of ~1/1 in 76% yield.[17] After separation by silica gel chromatography, the individual isomers were transformed (10% Pd/C, H$_2$; Boc$_2$O, Et$_3$N, DCM) into the corresponding orthogonally protected SAA building blocks (2,6-cis **3** and 2,6-trans **3**) in good overall yield.

**Scheme 2.1** Synthesis of SAA building blocks 2,6-cis **3** and 2,6-trans **3**.[a]
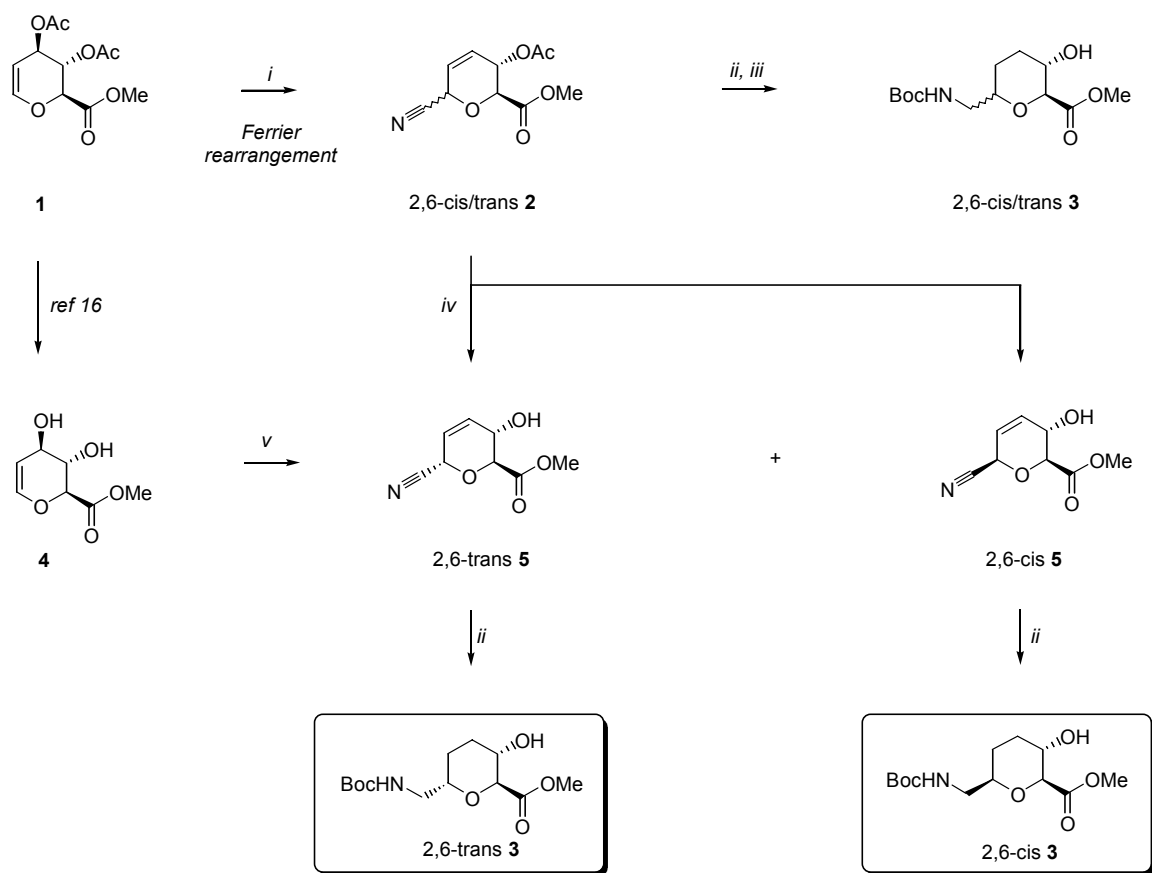
[a]Reagents and conditions. (*i*) 1.1 equiv. TMSCN, 0.2 equiv. BF$_3$·OEt$_2$, DCM (2,6-cis/trans **2**: 86%); (*ii*) (a) 10% Pd/C, MeOH/CHCl$_3$ (10/1 v/v), H$_2$ atm. (45 *psi*), 24 h, (b) Boc$_2$O, Et$_3$N, DCM (2,6-trans **3**: 55%, 2,6-cis **3**: 51%); (*iii*) KO*t*Bu, MeOH (2,6-cis/trans **3**: 87%); (*iv*) 0.05M HCl in MeOH, 60°C, 5 h (combined yield 2,6-cis **5** and 2,6-trans **5**: 89%); (*v*) 5 mol% Pd(OAc)$_2$, 5 equiv. TMSCN, CH$_3$CN, 80°C, 48 h (combined yield 2,6-cis **5** and 2,6-trans **5**: 76%).

The general route of synthesis to the fully protected Ca$_1$a$_2$L mimetics is exemplified by the preparation of 2,6-trans **8** (Scheme 2.2). Saponification of the methyl ester in 2,6-trans **3** and condensation of the resulting acid (2,6-trans **6**)[8] with Leu-OMe·HCl under the influence of PyBOP and DIPEA in DMF afforded dimer 2,6-trans **7** in 87% yield over the two steps. Removal of the Boc protective group in 2,6-trans **7** and condensation of the resulting ammonium salt with Fmoc-L-Cys(S*t*Bu)-OH gave protected 2,6-trans **8**. By the same synthetic procedure and with comparable efficiency, protected trimers 2,6-trans **13** (from 2,6-trans **7**), 2,6-cis **8** and 2,6-cis **13** (starting from 2,6-cis **3**) were readily prepared (Scheme 2.4).

Scheme 2.2 Synthesis 2,6-trans **8**.[a]
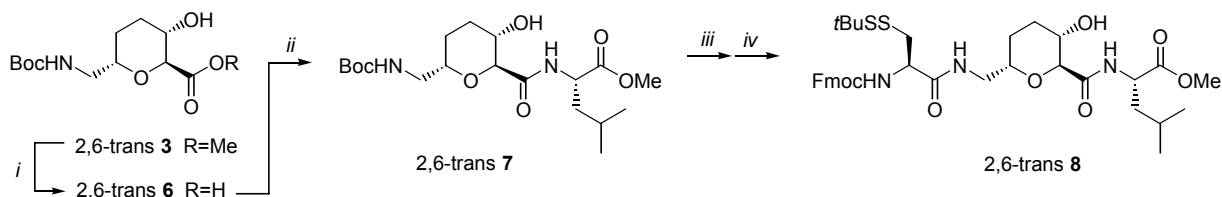
[a]Reagents and conditions. (*i*) 1.0 equiv. LiOH (1.0M aq.), H_2O/1,4-dioxane, 0°C (>99%); (*ii*) L-Leu-OMe·HCl, PyBOP, DIPEA, DMF (87%); (*iii*) 50% TFA in DCM, *i*Pr$_3$SiH; (*iv*) Fmoc-L-Cys(S*t*Bu)-OH, PyBOP, DIPEA, DMF (87%).

Scheme 2.3 Synthesis 2,6-cis **11** and 2,6-trans **11**.[a]
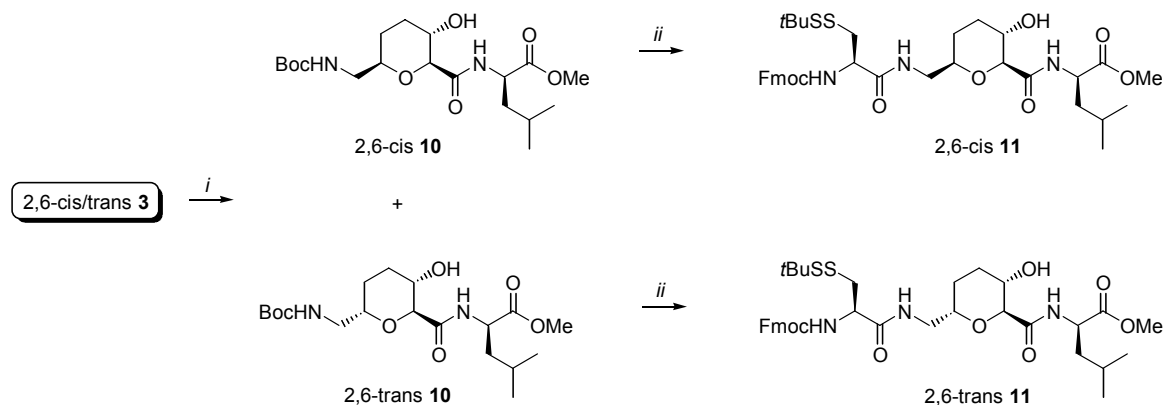
[a]Reagents and conditions. (*i*) (a) LiOH, H_2O/1,4-dioxane, 0°C (>99%), (b) D-Leu-OMe·HCl, PyBOP, DIPEA, DMF (combined yield 2,6-cis **10** and 2,6-trans **10**: 74%); (*ii*) (a) TFA, DCM, *i*Pr$_3$SiH, (b) Fmoc-L-Cys(S*t*Bu)-OH, PyBOP, DIPEA, DMF (2,6-cis **11**: 79%, 2,6-trans **11**: 77%).

In an alternative route to the Ca_1a_2L analogs (**VI**, Figure 2.2), the diastereoisomeric mixture of SAAs **3** was used in the initial peptide formation followed by separation at a later stage (Scheme 2.3). Saponification of SAAs 2,6-cis/trans **3** followed by condensation with D-Leu-OMe·HCl (PyBOP, DIPEA, DMF) gave a mixture of diastereoisomeric dimers 2,6-cis **10** and 2,6-trans **10** (cis/trans ~3/5), which could be readily separated by silica-gel chromatography. Elongation of the individual isomers with Fmoc-L-Cys(S*t*Bu)-OH using standard condensation gave the corresponding fully protected trimers 2,6-cis **11** and 2,6-trans **11** in good yield. According to this protocol 2,6-cis **15** and 2,6-trans **15** were prepared, completing the set of Ca_1a_2L mimetics (Scheme 2.4).

In a final deprotection step, concomitant removal of the Fmoc and hydrolysis of the methyl ester protecting groups in the eight Ca_1a_2L mimetics (**8**, **11**, **13**, **15**) proceeded smoothly with Tesser's base mixture (MeOH/1,4-dioxane/4M aq. NaOH 15/4/1 v/v/v). The corresponding deprotected trimers 2,6-cis **9**, **12**, **14**, **16** and 2,6-trans **9**, **12**, **14**, **16** (Scheme 2.4) were subsequently purified by RP-HPLC.

Scheme 2.4 Structures of fully protected and deprotected trimers.[a]
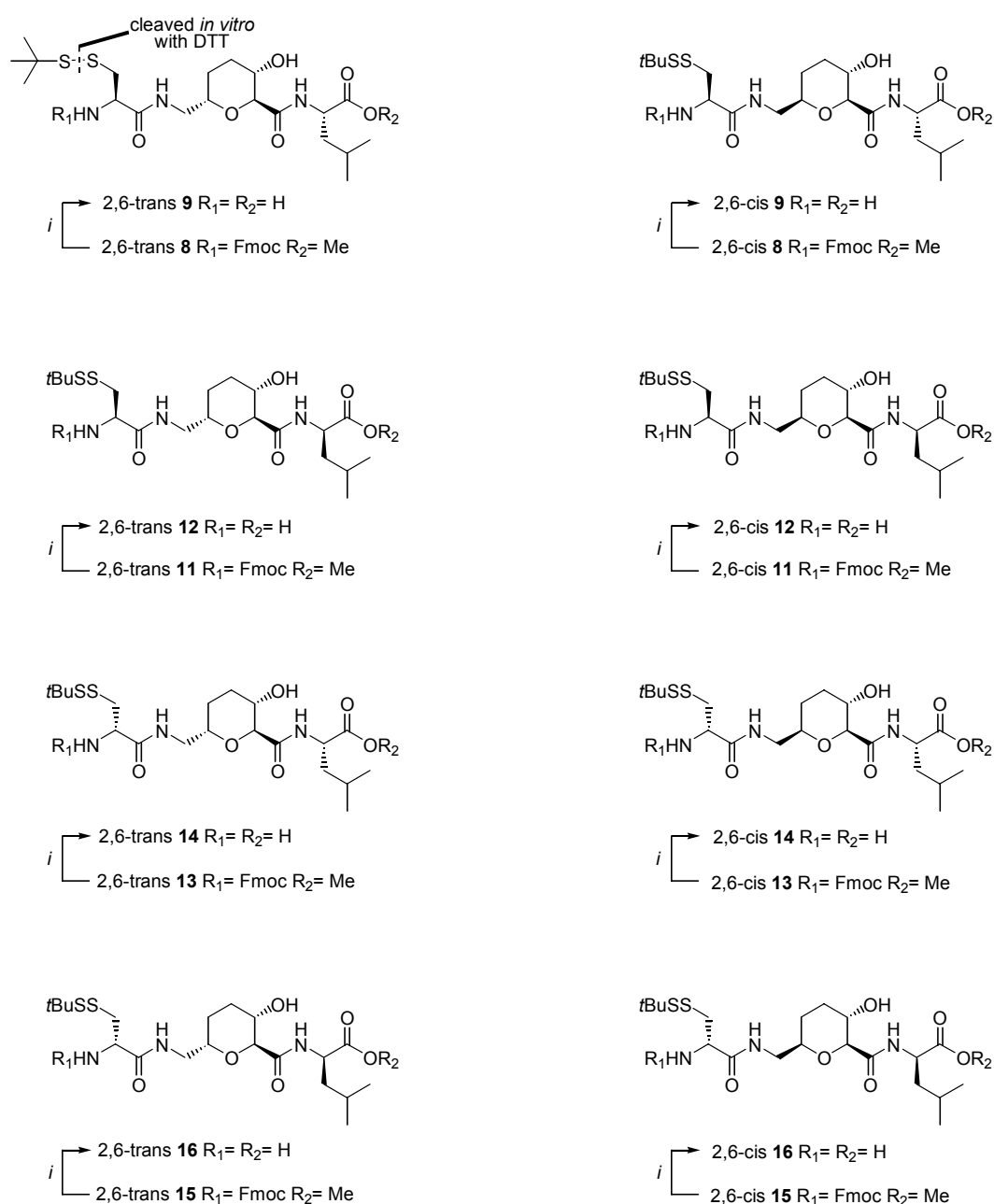
[a]Reagents and conditions. (*i*) (a) MeOH/1,4-dioxane/4M NaOH (15/4/1 v/v/v), (b) RP-HPLC purification.

2.2.2 **NMR analysis** – An important element in the design of the Ca_1a_2L mimetics was the absolute configuration at C_2 relative to C_6 of the incorporated SAA building block. As epimerisation may occur at various stages of the synthetic sequences employed to prepare the Ca_1a_2L analogs, various NMR techniques were applied in order to ensure that the individual stereochemical integrity of the SAA building blocks (2,6-cis and 2,6-trans **3**) is conserved in the final products. The initial cis/trans configuration was introduced into the SAA scaffold in the course of the Ferrier rearrangement. To establish the absolute stereochemical outcome of the reaction, nOe experiments were applied on the individual diastereoisomers of the SAA precursors 2,6-cis and 2,6-trans **5**. Irradiation of H_2 (δ= 4.03 ppm, Figure 2.3) in the more polar product (TLC analysis) of the 4,5-unsaturated glycopyranosyl cyanides showed an enhancement of the signal of H_6 (δ= 5.17 ppm), which was in agreement with the 2,6-cis configuration of **5**.
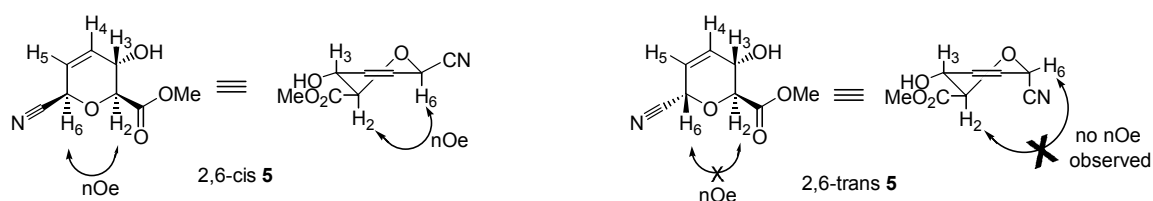
Figure 2.3 Structural assignment of 2,6-cis and 2,6-trans **5** by nOe experiments.

As expected, irradiation of H_2 (δ= 4.25) and H_6 (δ= 5.18) ppm) in the less polar product did not show a nOe, which is in agreement with the 2,6-trans configuration. Furthermore, the negative $[\alpha]_D$ value found for 2,6-trans **5** ($[\alpha]_D^{25}$= –134.4, c= 1.0, $CHCl_3$) and positive $[\alpha]_D$ value for 2,6-cis **5** ($[\alpha]_D^{25}$= +112.2, c= 1.0, $CHCl_3$), are consistent with reported data for optical rotations of 4,5-unsaturated C-glucopyranosides.[18]

To ensure that the initial configuration of the incorporated SAA building blocks is unchanged in the final products, we subjected trimer 2,6-trans **8** to a NOESY correlation experiment (Figure 2.4). The anticipated axial disposition of C_6 in 2,6-trans **8** was confirmed by the observed nOe signal between H_2 (δ= 3.96 ppm) and the H_7 protons (δ= 3.55 and 3.39 ppm, Figure 2.4).

Figure 2.4 Configuration of C_6 in 2,6-trans **8** by NOE experiment.

In addition to 2D-correlation ^{1}H-NMR, simple 1D ^{13}C-NMR provides rapid insight into the stereochemistry of *C*-glycosidic compounds. This is based on the general observation that the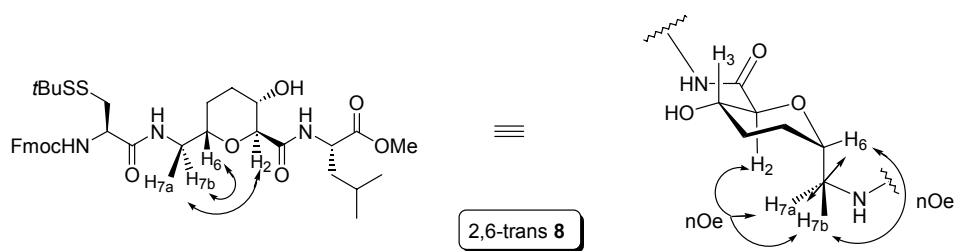 C_6 carbon resonances for α-*C*-glycosides (2,6-trans) appear at higher field than those for the corresponding β-*C*-glycosides (2,6-cis).[18] The chemical shifts of the pyranoid carbon atoms (*i.e.* C_{2-6}) of the eight fully protected trimers are listed in Table 2.1. The chemical shift of C_6 of the 2,6-trans compounds resonates at a higher field than that of the 2,6-cis compounds. Interestingly, this characteristic difference in chemical shift is also observed for the resonances of C_2, C_3, C_4 and C_5. Analysis of the chemical shifts of the pyranoid carbon atoms of the unprotected 2,6 cis **9** and 2,6-trans **9** revealed a similar trend.

Table 2.1 ^{13}C-NMR chemical shifts of C_2, C_3, C_4,C_5 and C_6 of compounds **8**, **9**, **11**, **13** and **15**.

compound	δ ^{13}C (ppm)					
	C_6	C_5	C_4	C_3	C_2	
2,6-trans **8**[a]	72.5	23.8	27.2	66.3	76.5	
2,6-trans **11**[a]	72.3	23.3	26.4	65.4	76.1	
2,6-trans **13**[a]	72.1	23.6	26.3	66.1	75.0	
2,6-trans **15**[a]	72.5	23.6	27.1	65.8	77.1	
2,6-trans **9**[b]	70.9	22.3	26.2	63.3	77.5	
2,6-cis **8**[a]	77.2	27.5	31.4	68.9	78.9	
2,6-cis **11**[a]	76.4	27.3	30.9	68.4	78.3	
2,6-cis **13**[a]	76.3	27.0	30.5	67.8	78.0	
2,6-cis **15**[a]	76.6	27.2	30.6	68.1	78.1	
2,6-cis **9**[b]	75.2	27.4	31.4	67.1	80.6	

[a]Measured in CDCl$_3$. [b]measured in DMSO-*d6*.

2.2.3 Biological Evaluation – The eight Ca_1a_2L peptidomimetics were tested for their inhibitory activity against bovine PGGT-1 using purified enzyme in an *in vitro* assay as published previously.[19] The S*t*Bu protective group in the cysteine residue is cleaved under the conditions of the assay (pH 7.4, DTT).[20] In Table 2.2 the IC_{50} values of the tested Ca_1a_2L analogs are listed. As can be seen from Table 2.2, four out of the eight Ca_1a_2L mimetics (*i.e.* 2,6-trans **9**, 2,6-trans **12**, 2,6-trans **14** and 2,6-cis **14**) proved to be capable of inhibiting PGGT-1 in the micromolar range, whereas the other four (*i.e.* 2,6-cis **9**, 2,6-cis **12**, 2,6-cis **16** and 2,6-trans **16**) showed little or no inhibition at millimolar concentrations. The two most potent inhibitors of the series, 2,6-trans **9** and 2,6-cis **14**, contain a 2,6-trans and a 2,6 cis SAA residue, respectively. They further differ in the stereochemistry of the cysteine residue (L *vs* D, respectively). Incorporation of both D-amino acids in both examples led to inactive compounds (*i.e.* 2,6-cis **16** and trans **16**).[21]

Table 2.2 IC_{50} values of tested Ca_1a_2L analogs.[a]

compound	IC_{50} (μM)	compound	IC_{50} (μM)
2,6-trans **9**	68 ± 16	2,6-cis **9**	~1000
2,6-trans **12**	241 ± 75	2,6-cis **12**	>1000
2,6-trans **14**	109 ± 30	2,6-cis **14**	69 ± 20
2,6-trans **16**	>1000	2,6-cis **16**	~1000

[a]IC_{50}: concentration of compound required to inhibit for 50% the PGGT-1 catalysed incorporation of [3H]-GGPP. All IC_{50} values are means of three determinations: one determination involves performing the assay at 5 concentrations of compound. By using a mathematical function fitting to the concentration/inhibition curve, the IC_{50} value was determined.

2.3 Conclusions

In summary, a useful strategy for the preparation of novel PGGT-1 inhibitors is presented. The approach is based on the incorporation of partially deoxygenated (2,6-cis/trans) SAA building blocks, which in turn were prepared from the common starting compound glucal **1** (Scheme 2.1). Variation of the SAA, in combination with the introduction of the cysteine and leucine pharmacophores as the D- and L amino acid derivatives, furnished 8 novel peptidomimetics resembling the Ca_1a_2X motif present in PGGT-1 substrate proteins. Of these, 4 were found to be inhibitors of PGGT-1 in the

micromolar range, whereas the other 4 showed no inhibition at millimolar concentrations. The two most potent inhibitors of the series, 2,6-trans **9** and 2,6-cis **14**, differ both in the nature of the SAA building block and the stereochemistry of the cysteine residue. This observation indicates that both 2,6-trans **3** and 2,6 cis **3** can be used for the development of novel PGGT-1 inhibitors.

2.4 Experimental Section

2.4.1 General – CH_3CN, $CHCl_3$, DCE, DCM, DMF, 1,4-dioxane, toluene were all of p.a. quality (Baker) and were stored on 4Å molecular sieves. Methanol (p.a. Baker) was stored on 3Å molecular sieves. PE (40-60 fraction) and EtOAc were of technical grade and distilled before use. Et_3N (99%, Acros) was distilled over CaH_2 when necessary or used as received. DIPEA (peptide grade) and TFA were purchased from Biosolve and used without purification. Leu-OMe·HCl, Fmoc-Cys-(S*t*Bu)-OH and pyBOP were from Novabiochem. D-Leu-OMe·HCl and D-Fmoc-Cys-(S*t*Bu)-OH were from Bachem. $BF_3 \cdot OEt_2$ (Aldrich), Boc_2O (Fluka), D-(+)-glucuronic acid-γ-lactone (Fluka), *i*Pr_3SiH (Aldrich), TMSCN (Fluka), $Pd(OAc)_2$ (Fluka) and 10% Pd/C (Aldrich) were used as received. RP-HPLC analysis and purification were performed on a Jasco HPLC system equiped with a Merck Lichrosphere C18 100Å column (4×250 mm). ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AC-200 (^{1}H-NMR 200 MHz, ^{13}C-NMR 50 MHz), Bruker DPX-300 (^{1}H-NMR 300 MHz; ^{13}C-NMR 75 MHz), Bruker AV-400 (^{1}H-NMR 400 MHz, ^{13}C-NMR 100 MHz), Bruker DMX-600 (^{1}H-NMR 600 MHz, ^{13}C-NMR 150 MHz) or Bruker DSX-750 MHz (^{1}H-NMR 750 MHz, ^{13}C-NMR 188 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. ^{1}H-NMR: resonance of $CDCl_3$ at 0.00 ppm as internal standard. ^{13}C-NMR: middle resonance of $CDCl_3$ set at 77.0 ppm as internal standard. MS (ESI): Perkin Elmer SCIEX API 165 quadrupole mass spectrometer, HR-MS: API QSTAR™ Pulsar (Applied Biosystems). Reactions were followed by TLC analysis on silica gel (Schleider & Schull, F 1500 LS 254) or HPTLC aluminium sheets (Merck, silica gel 60, F254), with detection by UV-absorption (254 nm) where applicable and charring at 150°C with 20% H_2SO_4 in ethanol (25 g L^{-1}), ninhydrin (3 g L^{-1}) in EtOH/AcOH (100/3 v/v), $NH_4(Mo)_7O_{24} \cdot 4H_2O$ (25 g L^{-1}) and $NH_4Ce(SO_4)_4 \cdot 2H_2O$ (10 g L^{-1}) in 10% aq. H_2SO_4 or 2% $KMnO_4$ in aq. K_2CO_3 (1%). Column chromatography was performed with silica gel (Baker; 0.063-0.200mm).

2.4.2 General procedures – *General procedure 1 (GP 1); hydrogenation and Boc protection:* to a 0.25M solution of the 4,5-unsaturated glucopyranosyl cyanide in MeOH/CHCl$_3$ (20 mL, 10/1 v/v) was added 10% palladium on activated carbon (25 mass%). The reaction mixture was shaken overnight under elevated hydrogen atmosphere (45 *psi*). After TLC analysis (EtOAc) showed complete conversion of the starting material into a ninhydrin positive product (base line spot), the mixture was filtrated over Celite® and the solution was concentrated under reduced pressure. The crude product was used without further purification for the next reaction. To a ~0.1M solution of the crude amine in DCM, 1.2 equiv. of Boc_2O and 2.2 equiv. of Et_3N were added. After TLC analysis showed consumption of the starting material (PE/EtOAc 1/1 v/v), water was added. The aqueous layer was extracted with EtOAc (2×) and the combined organic layers were washed with brine, dried ($MgSO_4$) and the solvent was removed *in vacuo*.

General procedure 2 (GP 2); saponification methyl ester SAA Building block: to a 0.1M solution of the methylester in 1,4-dioxane/H_2O (1/1 v/v) at 0°C, was added ≈1.1 equiv. aq. LiOH (1.0 M). After TLC

analysis (EtOAc) showed consumption of the starting material, the reaction mixture was neutralised (pH ≈7) by AcOH or Dowex-H⁺ and the solvents were removed *in vacuo*. The obtained residue was used without further purification for the amino acid coupling described in general procedure 3.

General procedure 3 (GP 3); coupling of SAA Building block to Leu-OMe·HCl: to a 0.1M solution of the SAA in DMF was added 1.2 equiv. Leu-OMe·HCl, 1.2 equiv. pyBOP and 4 equiv. DIPEA. After TLC analysis (PE/EtOAc 1/1 v/v) showed consumption of the starting material, the DMF was removed *in vacuo*. The residue was dissolved in EtOAc and washed with water (2×), sat. aq. $NaHCO_3$ (2×), 5% $KHSO_4$ (2×) and brine (sat. aq. NaCl). The organic phase was dried ($MgSO_4$) and the solvent was removed *in vacuo*.

General procedure 4 (GP 4); removal of the Boc protective group: to a 0.05M solution of the dimer in DCM were added 1.3 equiv. of iPr_3SiH and TFA (DCM/TFA 1/1 v/v). After TLC analysis (PE/EtOAc 1/1 v/v) showed consumption of the starting material, the reaction mixture was coevaporated 5× with PhMe.

General procedure 5 (GP 5); coupling of dimer to Fmoc-Cys-(StBu)-OH: to a 0.1M solution of the deprotected dimer in DMF were added 4 equiv. DIPEA, 1.2 equiv. Fmoc-Cys-(S*t*Bu)-OH and 1.2-1.5 equiv. pyBOP. After TLC analysis (PE/EtOAc 1/1 v/v) showed consumption of the starting material, the reaction was worked up as described in general procedure 2.

General procedure 6 (GP 6); Tesser's base mediated deprotection: a 0.01M solution of the trimer in MeOH/1,4-dioxane/4M aq. NaOH (15/4/1 v/v/v) is stirred at 0°C and the reaction mixture was allowed to reach room temperature. After TLC analysis (PE/EtOAc 1/1 v/v) showed consumption of the starting material, the reaction mixture was diluted with water and washed with DCM (3×). The aqueous layer was collected and neutralised with AcOH (pH ≈7). After lyophilisation, the crude product is purified by RP-HPLC (purity ≥95%, 10→40% linear gradient CH_3CN (= eluent B) in H_2O (= eluent A) and 1% aq. TFA (= eluent C, effective 0.1%).

Methyl (3,4-di-*O*-acetyl-D-glucuronate) glucal (1): white crystalline compound, 90% yield over 4 steps (250 mmol scale) from commercially available D-(+)-glucuronic acid-γ-lactone as described in reference 11.

(±)-(2S,3S,6SR) Methyl 3-*O*-acetyl-6-nitrile-4,5-dideoxyhex-4-eno-D-glucopyranuronate (2,6-cis/trans 2).
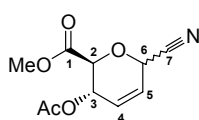
To a solution of glucal **1** (5.01 g, 19.4 mmol; 2× coevaporated with DCE) in DCM (50 mL) was added TMSCN (2.91 mL, 23.3 mmol, 1.2 equiv.) and $BF_3 \cdot OEt_2$ (0.49 mL, 0.2 equiv.). The reaction mixture was stirred for 1 h, after which TLC analysis (PE/aceton 4/1 v/v) showed complete conversion of **1**. The reaction mixture was quenched with sat. aq. $NaHCO_3$ (50 mL) and diluted with EtOAc (50 mL). The water layer was extracted with EtOAc (2×50 mL) and the combined organic phases were washed with sat. aq. $NaHCO_3$ (50 mL) and brine (50 mL) and dried on $MgSO_4$. The solvent was removed *in vacuo* and silica gel chromatography (PE/aceton 4/1 v/v) gave 2,6-cis/trans **2** as a colorless oil (3.76 g, 86%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.21-5.95 (m, 2H, H_4, H_5), 5.55 (m, 1H, H_3), 5.34, 5.22 (2×dd, 2H, $H_6^{\alpha/\beta}$, *J*= 2.6 and 7.1 Hz; *J*= 3.3 and 4.0 Hz), 4.47 (d, 1H, H_2^{α}, *J*= 6.3 Hz), 4.44 (d, 1H, H_2^{β}, *J*= 4.3 Hz), 3.81, 3.79 (2×s, 2×3H, $OCH_3^{\alpha/\beta}$), 2.12, 2.09 (2×s, 2×3H, $CH_3^{Ac, \alpha/\beta}$); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 169.4 (2×C=O), 126.7, 125.7 ($C_5^{\alpha/\beta}$), 124.8, 124.4 ($C_4^{\alpha/\beta}$), 115.1 (C≡N), 73.4, 72.4 ($C_2^{\alpha/\beta}$), 63.4, 62.8 ($C_3^{\alpha/\beta}$), 61.3, 60.9 ($C_6^{\alpha/\beta}$), 52.3, 52.2 ($OCH_3^{\alpha/\beta}$), 20.1 (CH_3^{Ac}); MS (ESI): *m/z* 248.1 (M+Na)⁺.

(+)-(2S,3S,6S) Methyl 3-hydroxy-6-nitrile-4,5-dideoxyhex-5-eno-D-glucopyranuronate (2,6-trans 5);
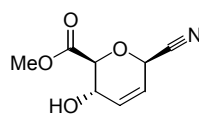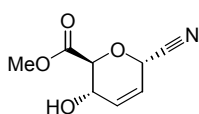
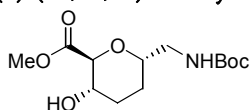**(-)-(2S,3S,6R) Methyl 3-hydroxy-6-nitrile-4,5-dideoxyhex-5-eno-D-glucopyranuronate (2,6-cis 5). Method 1:** To a solution of 2,6-cis/trans **2** (2.00 g, 8.88 mmol; 2× coevaporated with 10 mL PhMe) was added a 0.05M methanolic HCl solution (50 mL). The reaction mixture was stirred for 5 h at 60°C, after which TLC analysis (EtOAc) showed complete conversion of the starting material. The reaction

was quenched with sat NaHCO$_3$ (50 mL) and diluted with EtOAc (50 mL). The water layer was extracted with EtOAc (50 mL). The combined organic phases were washed with sat. NaHCO$_3$ (25 mL) and brine (25 mL), and dried on MgSO$_4$. The solvent was removed *in vacuo* and silica gel chromatography (PE/EtOAc 1/1 v/v) gave 2,6-trans **5** and 2,6-cis **5** both as a colorless oil (1.45 g, 89%).
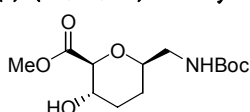
Method 2: To a solution of **4** (236 mg, 1.36 mmol (2× coevaporated with PhMe) in CH$_3$CN (20 mL) was added 5 mol% Pd(OAc)$_2$ (15 mg) and 5 equiv. TMSCN (0.85 mL, 6.80 mmol). The reaction mixture was stirred for 72 h at 80°C after which TLC analysis showed complete conversion of the starting material. The reaction was quenched with 0.1 N HCl (25 mL) and diluted with EtOAc (50 mL). The water layer was extracted with EtOAc (2×50 mL). The combined organic phases were washed with sat. aq. NaHCO$_3$ (50 mL) and brine (saturated aq. NaCl, 50 mL), and dried on MgSO$_4$. The solvent was removed *in vacuo* and silica gel chromatography (PE/EtOAc, 1/1 v/v) yielded 2,6-trans **5** and 2,6-cis **5** (189 mg, 76%). 2,6-trans **5**: $[\alpha]_D^{25}$= −134.4 (*c*= 1.0, CHCl$_3$), ^{1}H-NMR (300 MHz, CDCl$_3$) δ 6.12 (dt, 1H, H$_5$, *J*= 2.2 Hz), 5.83 (ddd, 1H, H$_4$, *J*= 2.0 and 3.2 Hz), 5.18 (dd, 1H, H$_6$, *J*= 2.3 and 5.4 Hz), 4.47-4.41 (m, 1H, H$_3$), 4.25 (d, 1H, H$_2$, *J*= 8.1 Hz), 3.87 (s, 3H, OCH$_3$); ^{13}C-NMR (75 MHz, CDCl$_3$) δ 169.7 (C=O), 131.8 (C$_5$), 121.9 (C$_4$), 115.4 (C≡N), 74.5 (C$_6$), 63.7 and 62.5 (C$_2$ and C$_3$), 53.0 (OCH$_3$); MS (ESI): *m/z* 184.2 (M+H)$^+$, 206.2 (M+Na)$^+$; HR-MS: calc. 184.0609 (C$_8$H$_9$NO$_4$+H)$^+$, found 184.0605; 2,6-cis **5**: $[\alpha]_D^{25}$= +112.2 (*c*= 1.0, CHCl$_3$), ^{1}H-NMR (300 MHz, CDCl$_3$) δ 6.13 (dt, 1H, H$_5$, *J*= 2.6 Hz), 5.93 (dt, 1H, H$_4$, *J*= 1.9 Hz), 5.17 (dd, 1H, H$_6$, *J*= 2.5 and 4.7 Hz), 4.54-4.49 (m, 1H, H$_3$), 4.03 (d, 1H, H$_2$, *J*= 7.6 Hz), 3.87 (s, 3H, OCH$_3$); ^{13}C-NMR (50 MHz, CDCl$_3$) δ 169.5 (C=O), 131.3 (C$_5$), 122.3 (C$_4$), 115.5 (C≡N), 76.6 (C$_6$), 63.2, 63.0 (C$_3$ and C$_2$), 52.9 (OCH$_3$); MS (ESI): *m/z* 184.2 (M+H)$^+$, 206.2 (M+Na)$^+$; HR-MS: calc. for 184.0609 (C$_8$H$_9$NO$_4$+H)$^+$, found 184.0647.

(+)-(2S,3S,6S)-Methyl 3-hydroxy-4,5-dideoxy-7-[*N*-(tert-butyloxycarbonyl)amino]-D-glucopyranuronate (2,6-trans 3). Treatmen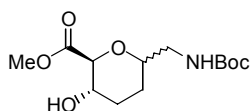t of 2,6-trans **5** (100 mg, 0.55 mmol) according to *GP 1* followed by silica gel chromatography (PE/EtOAc 1/1 v/v) yielded the title compound as a colorless oil (87 mg, 55%, 2 steps). ^{1}H-NMR (600 MHz, CDCl$_3$) δ 5.08 (bs, 1H, NH), 4.35 (bs, 1H, H$_6$), 4.14 (bs, 1H, H$_2$), 3.80 (m, 1H, H$_3$), 3.74 (s, 3H, OCH$_3$), 3.28 and 3.12 (2×m, 2H, H$_7$), 1.80, 1.70 and 1.50 (3×m, 4H, H$_5$, H$_4$), 1.41 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$) δ 170.7 (C=O^{ester}), 156.2 (C=O^{Boc}), 79.2 (C$_q$^{tBu}), 75.5 (C$_6$), 72.3 (C$_2$), 65.1 (C$_3$), 52.1 (OCH$_3$), 44.4 (C$_7$), 28.5 (*t*Bu), 26.6 (C$_4$), 22.1 (C$_5$); MS (ESI): *m/z* 312.1 (M+Na)$^+$. HR-MS: calc. for [C$_{13}$H$_{23}$NO$_6$+H]$^+$ 290.15981, found 290.15945. $[\alpha]_D^{20}$= +24.8 (CDCl$_3$, *c*= 1).

(-)-(2S,3S,6R)-Methyl 3-hydroxy-4,5-dideoxy-7-[*N*-(tert-butyloxycarbonyl)amino]-D-glucopyranuronate (2,6-cis 3). Treatment of 2,6-cis **5** (362 mg, 1.98 mmol) according to *GP 1* followed by silica gel chromatography (PE/EtOAc 1/1 v/v) gave 2,6-cis **3** as a colorless oil (271 mg, 51%). ^{1}H-NMR (400 MHz, CDCl$_3$) δ 5.07 (bs, 1H, NH), 3.82 (s, 3H, OCH$_3$), 3.80-3.60 (m, 3H, H$_6$, H$_3$, H$_2$), 3.46-3.32 (m, 2H, H$_7$), 2.20 (m, 1H, H$_5$), 1.70, 1.59-1.48 (m, 3H, H$_4$, H$_5$), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (100 MHz, CD$_3$OD) δ 172.6 (C=O^{ester}), 158.3 (C=O^{Boc}), 82.8, 77.8, 68.6 (C$_6$, C$_3$, C$_2$), 80.1 (C$_q$^{tBu}), 52.6 (OCH$_3$), 45.5 (C$_7$), 32.7 (C$_4$), 28.7 (*t*Bu), 28.6 (C$_5$); MS (ESI): *m/z* 312.1 (M+Na)$^+$. HR-MS: calc. for [C$_{13}$H$_{23}$NO$_6$+H]$^+$ 290.15981, found 290.15948. $[\alpha]_D^{20}$= −31.8 (CDCl$_3$, *c*= 1).

(±)-(2S,3S,6RS)-Methyl 3-hydroxy-4,5-dideoxy-7-[*N*-(tert-butyloxycarbonyl)amino]-D-glucopyranuronate (2,6-cis/trans 3). Treatment of 2,6-cis/trans **2** (1.50 g, 6.67 mmol) according to *GP 1* followed by silica gel chromatography (PE/EtOAc 1/1 v/v) gave the 3-OAc precursor of the title compound as a colorless oil (1.26 g, 57% 2 steps). ^{13}C-NMR (50 MHz, CDCl$_3$) δ 169-168.8 (2×C=O^{ester}), 155.7 and 155.5 (C=O^{Boc}), 78.6, 78.5 (C$_q$^{tBu}), 77.9, 76.6, 74.9, 71.8, 68.1, 68.9 (C$_6$, C$_3$, C$_2$$^{\alpha/\beta}$), 51.8 (OCH$_3$$^{\alpha\beta}$), 44.5, 44.9 (C$_7$$^{\alpha/\beta}$), 28.1, 26.5, 23.9, 22.0 (C$_5$, C$_4$$^{\alpha/\beta}$), 27.8 (*t*Bu), 20.6 and 20.3 (CH$_3$^{Ac}); MS (ESI): *m/z* 332.2 (M+H)$^+$, 354.1 (M+Na)$^+$. The isomeric mixture (458 mg, 1.38 mmol) was dried by coevaporation with 1,4-dioxane (3×10 mL) and dissolved in MeOH (20 mL) followed by addition of KO*t*Bu (31 mg, 0.28 mmol). After TLC analysis (PE/EtOAc 1/1 v/v)

showed completion, the reaction mixture was neutralised (Dowex-H⁺) and the solvent was removed by evaporation. The product was washed with water (25 mL) and brine (25 mL), extracted with EtOAc (2×25 mL) and the collected organic phases were dried on MgSO₄. Silica gel chromatography (PE/EtOAc 1/1 v/v) afforded the title compound as a colorless oil (346 mg, 87%).

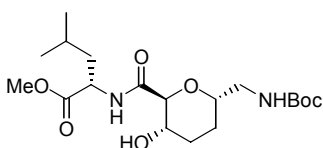### (+)-4,5-dideoxy-3-hydroxy-7-(tert-butyloxycarbonyl)aminomethyl-α-D-glucuronic acid (2,6-trans 6).
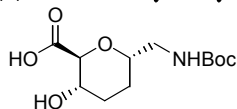
From 2,6-trans **3** (25 mg, 86 µmol) and aq. LiOH (1.0 M, 90 µL) according to *GP 2*, crude yield: >99%. 2,6-Trans **6** was used crude in *GP 3*. ¹H-NMR (300 MHz, CDCl₃/MeOD): δ 4.36-4.28, 4.22-4.13, 4.00-3.91 (3×m, 3H, H₆, H₃, H₂), 3.24-3.17 (m, 2H, H₇), 1.89-1.72 and 1,45 (m, 3H, H₅, H₄), 1.44 (bs, 9H, t-Bu); ¹³C-NMR (50 MHz, CDCl₃/MeOD): δ 173.0 (C=O, acid), 157.6 (C=O^{Boc}), 79.7 (C$_q$ t-Bu), 78.2 (C₆), 72.5 (C₂), 65.2 (C₃), 45.1 (C₇), 28.6 (*t*Bu^{Boc}), 27.0, (C₄), 22.4 (C₅); MS (ESI): *m/z* 298.2 (M+Na)⁺.

(-)-4,5-dideoxy-3-hydroxy-7-(tert-butyloxycarbonyl)aminomethyl-β-D-glucuronic acid (2,6-cis 6).
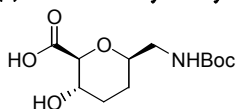
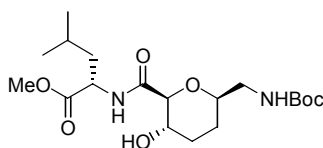From 2,6-cis **3** (25 mg, 86 µmol) and aq. LiOH (1.0 M, 90 µL) according to *GP 2*, crude yield: >99%. 2,6-cis **6** was used crude in *GP 3*. ¹³C-NMR (50 MHz, MeOD): δ 173.9 (C=O, acid), 158.0 (C=O^{Boc}), 81.5 (C₆), 77.7 (C₂), 68.3 (C₃), 45.1 (C₇), 32.4 (C₄), 28.1 (C₅), 28.7 (*t*Bu); MS (ESI): *m/z* 298.2 (M+Na)⁺.

N-(6-(*N*-tert-butyloxycarbonyl)-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-L-leucine methyl ester (2,6-trans 7).

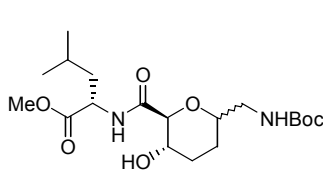From crude 2,6-trans **6** and L-Leu-OMe·HCl (19 mg, 0.1 mmol) according to *GP 3*, yield of the title compound after silica gel chromatography (EtOAc): 30 mg, 87%, 2 steps, colorless oil. ¹H-NMR (300 MHz, CDCl₃): δ 7.00 (d, 1H, NHC$_α^{Leu}$, *J*= 8.3 Hz), 4.80 (bs, 1H, NHBoc), 4.62 (m, 1H, H$_α^{Leu}$), 3.98 (m, 1H, H₆), 3.92 (d, 1H, H₂, *J*= 8.3 Hz), 3.74 (s, 3H, OCH₃), 3.71 (m, 1H, H₃), 3.47 and 3.26 (2×m, 2H, H₇), 1.95 and 1.90-1.56 (2×m, 6H, H₅, H₄, H$_{βγ}^{Leu}$), 1.45 (s, 9H, *t*Bu), 0.96 (dt, 6H, 2×CH₃^{Leu}, *J*= 3.2 Hz, 3.3 and 6.2 Hz); ¹³C-NMR (50 MHz, CDCl₃): δ 172.8, 171.8 (C=O$^{amide\&ester}$), 155.9 (C=O^{Boc}), 79.6 (C$_q^{tBu}$), 73.5 (C₆), 72.7 (C₂), 67.6 (C₃), 52.3 (C$_α^{Leu}$), 50.2 (OCH₃), 41.4 (C₇), 40.6 (C$_β^{Leu}$), 28.3 (*t*Bu), 26.4 (C₄), 24.9 (C$_γ^{Leu}$), 24.2 (C₅), 22.7 and 21.9 (2×CH₃^{Leu}); MS (ESI): *m/z* 403.5 (M+H)⁺; 425.3 (M+Na)⁺. HR-MS: calc. for [C₁₉H₃₄N₂O₇+H]⁺ 403.24388, found 403.24402. [α]$_D^{20}$= +8.2 (CDCl₃, *c*= 1).

N-(6-(*N*-tert-butyloxycarbonyl)-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-L-leucine methyl ester (2,6-cis 7).

From crude 2,6-cis **6** and L-Leu-OMe·HCl (19 mg, 0.1 mmol) according to *GP 3*, yield of the title compound after silica gel chromatography (EtOAc): 32 mg, 92%, 2 steps, colorless oil. ¹H-NMR (300 MHz, CDCl₃): δ 7.02 (d, 1H, NHC$_α^{Leu}$, *J*= 7.2 Hz), 4.85 (bs, 1H, NHBoc), 4.59 (m, 1H, H$_α^{Leu}$), 3.75 (s, 3H, OCH₃), 3.64 (d, 1H, H₂, *J*= 9.5 Hz), 3.54 (m, 2H, H₆ and H₃), 3.28 (m, 2H, H₇), 2.20 (m, 1H, H₄), 1.80-1.40 (m, 6H, H₅, H₄, H$_{βγ}^{Leu}$), 1.46 (s, 9H, *t*Bu), 0.96 (t, 6H, 2×CH₃^{Leu}, *J*= 5.8 Hz and *J*= 11.7 Hz); ¹³C-NMR (50 MHz, CDCl₃): δ 172.8, 172.2 (C=O$^{amide\&ester}$), 156.0 (C=O^{Boc}), 79.5 (C$_q^{tBu}$), 78.2 (C₆), 77.5 (C₂), 68.3 (C₃), 52.4 (OCH₃), 50.2 (C$_α^{Leu}$), 44.7 (C₇), 41.1 (C$_β^{Leu}$), 30.6 (C₄), 28.4 (*t*Bu), 27.1 (C₅), 24.9 (C$_γ^{Leu}$), 22.7, 21.9 (2×CH₃^{Leu}); MS (ESI): *m/z* 403.5 (M+H)⁺; 425.3 (M+Na)⁺. HR-MS: calc. for [C₁₉H₃₄N₂O₇+H]⁺ 403.24388, found 403.24338. [α]$_D^{20}$= −25.6 (CDCl₃, *c*= 1).

N-(6-(*N*-tert-butyloxycarbonyl)-aminomethyl-4,5-dideoxy-αβ-D-aminoglucuronopyranosyl)-L-leucine methyl ester (2,6-cis/trans 7).

From 2,6-cis/trans **3** (204 mg, 0.71 mmol) and aq. LiOH (1.0 M, 0.75 mL) according to *GP 2*. The crude SAA was used without purification for the coupling with L-Leu-OMe·HCl (154 mg, 0.85 mmol) according to *GP 3*. Purification by silica gel chromatography (PE/EtOAc 1/1 v/v) afforded 2,6-cis/trans **7** as a colorless oil (yield 241 mg, 84%, 2 steps).

N-(6-(*N*-tert-butyloxycarbonyl)-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-D-leucine methyl ester (2,6-trans 10); *N*-(6-(*N*-tert-butyloxycarbonyl)-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-D-leucine methyl ester (2,6-cis 10).
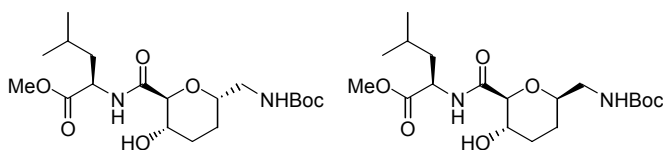
From 2,6-cis/trans **3** (75 mg, 0.26 mmol) and aq. LiOH (1.0 M, 0.3 mL) according to *GP 2*. The crude product was coupled to D-Leu-OMe·HCl (56 mg, 0.31 mmol) according to *GP 3*. Purification and separation by silica gel chromatography (PE/EtOAc 1/1 v/v) gave 2,6-trans **10** and 2,6-cis **10** as colorless oils: total yield 77 mg, 74%, 2 steps (cis/trans ≈3/5). 2,6-trans **10**: ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.00 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.6 Hz), 4.78 (bs, 1H, NHBoc), 4.61 (m, 1H, H$_\alpha$), 3.95 (m, 1H, H$_6$), 3.90 (bs, 1H, H$_2$), 3.82 (m, 1H, H$_3$), 3.75 (s, 3H, OCH$_3$), 3.53 and 3.18 (2×m, 2H, H$_7$), 1.94, 1.79-1.45 (2×m, 16H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$, *t*Bu), 0.96 (d, 6H, 2×CH$_3^{Leu}$, *J*= 5.4 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.1, 171.7 (C=O$^{amide\&ester}$), 155.9 (C=O^{Boc}), 79.5 (C$_q^{tBu}$), 74.0 (C$_6$), 72.7 (C$_2$), 66.9 (C$_3$), 52.4 (C$_\alpha^{Leu}$), 50.1 (OCH$_3$), 41.4 (C$_7$), 41.1 (C$_\beta^{Leu}$), 28.3 (*t*Bu), 26.4 (C$_4$), 24.9 (C$_\gamma^{Leu}$), 23.9 (C$_5$), 22.7, 21.8 (2×CH$_3^{Leu}$); MS (ESI): *m/z* 403.5 (M+H)$^+$; 425.3 (M+Na)$^+$; 2,6-cis **10**: ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.05 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 6.9 Hz), 4.84 (bs, 1H, NHBoc), 4.61 (m, 1H, H$_\alpha^{Leu}$), 3.76 (s, 3H, OCH$_3$), 3.71-3.51 (m, 3H, H$_6$, H$_3$, H$_2$), 3.36-3.17 (m, 2H, H$_7$), 2.17, 1.76-1.36 (m, 7H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.46 (s, 9H, *t*Bu), 0.96 (t, 6H, 2×CH$_3^{Leu}$, *J*= 1.9 and 3.9 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.0, 172.4 (C=O$^{amide\&ester}$), 155.2 (C=O^{Boc}), 79.5 (C$_q^{tBu}$), 77.4 (C$_6$), 76.4 (C$_2$), 68.6 (C$_3$), 51.5 (C$_\alpha^{Leu}$), 49.3 (OCH$_3$), 43.8 (C$_7$), 40.2 (C$_\beta^{Leu}$), 29.8 (C$_4$), 27.5 (*t*Bu), 26.1 (C$_5$), 24.0 (C$_\gamma^{Leu}$), 21.9, 21.0 (2×CH$_3^{Leu}$); MS (ESI): *m/z* 403.5 (M+H)$^+$; 425.3 (M+Na)$^+$.

N-(6-*N*-[(*N*-(10-fluorenyl methoxycarbonyl))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-L-leucine methyl ester (2,6-trans 8).
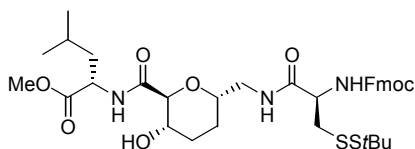
From 2,6-trans **7** (29 mg, 79 μmol) and Fmoc-L-Cys(S*t*Bu)-OH (37 mg, 86 μmol) following *GP 4* and *GP 5*. Silica gel chromatography (EtOAc) gave 2,6-trans **8** as a colorless oil (45 mg, 87%, 2 steps). ^{1}H-NMR (750 MHz, CDCl$_3$): δ 7.76 (dd, 2H, CH^{Fmoc}, *J*= 2.3 and 7.2 Hz), 7.58 (dd, 2H, CH^{Fmoc}, *J*= 7.2 and 16.3 Hz), 7.40 (dd, 2H, CH^{Fmoc}, *J*= 4.8 and 7.3 Hz), 7.30 (t, 2H, CH^{Fmoc}, *J*= 7.2 and 7.3 Hz), 7.12 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 7.4 Hz), 6.79 (bs, 1H, C$_7$NH), 6.07 (d, 1H, NHFmoc, *J*= 5.3 Hz), 4.61 (m, 1H, H$_\alpha^{Leu}$), 4.55 (m, 1H, H$_\alpha^{Cys}$), 4.44 (m, 2H, CH$_2^{Fmoc}$), 4.21 (t, 1H, CH^{Fmoc}), 4.02 (m, 1H, H$_6$), 3.96 (d, 1H, H$_2$, *J*= 7.7 Hz), 3.81 (m, 1H, H$_3$), 3.68 (s, 3H, OCH$_3$), 3.55, 3.39 (2×m, 2H, H$_7$), 3.10 (m, 2H, H$_\beta^{Cys}$), 1.93, 1.79 (2×m, 2H, H$_{5a}$, H$_{4a}$), 1.68-1.58 (m, 5H, H$_{5b}$, H$_{4b}$, H$_{\beta\gamma}^{Leu}$), 1.46 (s, 9H, *t*Bu), 0.92 (dd, 6H, 2×CH$_3^{Leu}$, *J*= 2.4 and 5.7 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.3, 171.7, 170.7 (C=O$^{amide\&ester}$), 156.7 (C=O^{Fmoc}), 144.7, 141.8 (2×C$_q^{Fmoc}$), 128.3, 127.7, 125.9, 120.5 (CH^{Fmoc}), 76.5 (C$_2$), 72.5 (C$_6$), 67.2 (CH$_2^{Fmoc}$), 66.3 (C$_3$), 55.5 (C$_\alpha^{Cys}$), 52.2 (OCH$_3$), 50.6 (C$_\alpha^{Leu}$), 48.1 (C$_q^{tBu}$), 47.7 (CH^{Fmoc}), 43.3 (C$_7$), 41.8, 40.9 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 29.8 (*t*Bu), 27.2 (C$_4$), 25.3 (C$_\gamma^{Leu}$), 23.8 (C$_5$), 23.0, 21.5 (2×CH$_3^{Leu}$); MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.
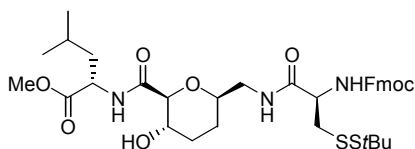
N-(6-*N*-[(*N*-(10-fluorenyl methoxycarbonyl))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-L-leucine methyl ester (2,6-cis 8).

From 2,6-cis **7** (42 mg, 104 μmol) and Fmoc-L-Cys(S*t*Bu)-OH (54 mg, 125 μmol) according to *GP 4* and *GP 5*. Silica gel chromatography (EtOAc) afforded the title compound as a colorless oil (64 mg, 86%, 2 steps). ^{1}H-NMR (750 MHz, CDCl$_3$): δ 7.76 (d, 2H, CH^{Fmoc}, *J*= 7.4 Hz), 7.57 (d, 2H, CH^{Fmoc}, *J*= 7.4 Hz), 7.40 (dd, 2H, CH^{Fmoc}, *J*= 7.4 and 7.5 Hz), 7.30 (t, 2H, CH^{Fmoc}, *J*= 3.7 and 7.0 Hz), 7.25 (bs, 1H, NHC$_\alpha^{Leu}$, *J*= 7.4 Hz), 6.82 (bs, 1H, C$_7$NH), 6.24 (bs, 1H, NHFmoc), 4.66 (m, 1H, H$_\alpha^{Leu}$), 4.48 (m, 1H, H$_\alpha^{Cys}$), 4.41, 4.30 and 4.20 (m, 3H, CH$_2$ and CH^{Fmoc}), 3.71 (s, 3H, OCH$_3$), 3.54 (m, 3H, H$_6$, H$_3$, H$_2$), 3.47 and 3.30 (2×m, 2H, H$_7$), 3.07 (d, 2H, H$_\beta^{Cys}$, *J*= 5.8 Hz), 2.15 (m, 1H, H$_4$), 1.69-1.36 (m, 6H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.33 (s, 9H, *t*Bu), 0.94 (dd, 6H, 2×CH$_3^{Leu}$, *J*= 5.9 and 10.8 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.2, 171.1, 170.7 (C=O$^{amide\&ester}$), 156.6 (C=O^{Fmoc}), 144.6

and 141.7 (2×C_q^{Fmoc}), 128.4, 127.6, 125.9, 120.5 (CH^{Fmoc}), 78.9 (C$_2$), 77.2 (C$_6$), 68.9 (C$_3$), 67.2 (CH$_2^{Fmoc}$), 55.4 (C$_\alpha^{Cys}$), 52.2 (OCH$_3$), 50.6 (C$_\alpha^{Leu}$), 48.0 (C$_q^{tBu}$), 47.6 (CH^{Fmoc}), 43.8 (C$_7$), 43.0 and 40.9 (C$_\beta^{Leu}$ and C$_\beta^{Cys}$), 31.4 (C$_4$), 29.8 (*t*Bu), 27.5 (C$_5$), 25.2 (C$_\gamma^{Leu}$), 22.9, 21.6 (2×CH$_3^{Leu}$); MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.

N-(6-*N*-[(*N*-(10-fluorenyl methoxycarbonyl))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-D-leucine methyl ester (2,6-trans 11).
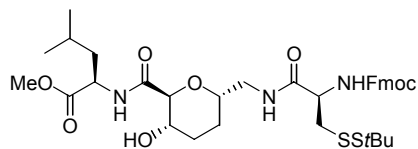
From 2,6-trans **10** (100 mg, 0.25 mmol) and Fmoc-L-Cys(S*t*Bu)-OH (116 mg, 0.27 mmol) according to *GP 4* and *GP 5*. Silica gel chromatography (EtOAc) gave 2,6-trans **11** as a colorless oil (137 mg, 77%, 2 steps). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.80 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.60 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.41 (t, 2H, CH^{Fmoc}, *J*= 7.4 and 7.5 Hz), 7.31 (t, 2H, CH^{Fmoc}, *J*= 7.4 Hz), 7.10 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.8 Hz), 6.74 (bs, 1H, C$_7$NH), 5.78 (bs, 1H, NHFmoc), 4.66 (m, 1H, H$_\alpha^{Leu}$), 4.45 (dd, 2H, CH$_2^{Fmoc}$, *J*= 7.2 and 10.5 Hz), 4.39 (m, 1H, H$_\alpha^{Cys}$), 4.22 (t, 2H, CH^{Fmoc}, *J*= 7.0 Hz), 4.08 (d, 1H, H$_2$, *J*= 6.4), 3.98 (m, 1H, H$_3$), 3.89 (m, 1H, H$_6$), 3.71 (s, 3H, OCH$_3$), 3.31 and 3.11 (2×m, 2H, H$_7$), 3.05 (dd, 2H, H$_\beta^{Cys}$, *J*= 6.9 and 13.6 Hz), 1.91 (m, 1H, H$_4$), 1.72-1.61 (m, 6H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.34 (s, 9H, *t*Bu), 0.93 (t, 6H, 2×CH$_3^{Leu}$, *J*= 4.2 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.4, 171.1, 170.1 (C=O$^{amide\&ester}$), 156.0 (C=O^{Fmoc}), 143.6 and 141.3 (2×C_q^{Fmoc}), 127.8, 127.1, 125.0, 120.0 (CH^{Fmoc}), 75.0 (C$_2$), 72.1 (C$_6$), 67.3 (CH$_2^{Fmoc}$), 66.1 (C$_3$), 54.8 (C$_\alpha^{Cys}$), 52.5 (OCH$_3$), 50.1 (C$_\alpha^{Leu}$), 48.6 (C$_q^{tBu}$), 47.0 (CH^{Fmoc}), 42.1 (C$_7$), 41.1 and 41.0 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 29.8 (*t*Bu), 26.3 (C$_4$), 24.8 (C$_\gamma^{Leu}$), 23.6 (C$_5$), 22.8 and 21.7 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.
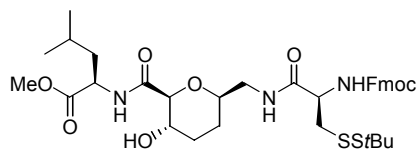
N-(6-*N*-[(*N*-(10-fluorenyl methoxycarbonyl))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-D-leucine methyl ester (2,6-cis 11).

From 2,6-cis **10** (53 mg, 132 μmol) and Fmoc-L-Cys(S*t*Bu)-OH (68 mg, 158 μmol) according to *GP 4* and *GP 5*. Silica gel chromatography (EtOAc) gave 2,6-cis **11** as a colorless oil (75 mg, 79%, 2 steps). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.77 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.58 (d, 2H, CH^{Fmoc}, *J*= 7.4 Hz), 7.39 (t, 2H, CH^{Fmoc}, *J*= 7.4 and 7.5 Hz), 7.30 (m, 2H, CH^{Fmoc}), 7.20 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.4 Hz), 6.97 (bs, 1H, C$_7$NH), 5.89 (bs, 1H, NHFmoc), 4.60 (m, 1H, H$_\alpha^{Leu}$), 4.45-4.25 (m, 3H, H$_\alpha^{Cys}$, CH$_2^{Fmoc}$), 4.21 (t, 1H, CH$_2^{Fmoc}$, *J*= 6.9 and 7.1 Hz), 3.68 (s, 3H, OCH$_3$), 3.68-3.53 (m, 4H, H$_6$, H$_3$, H$_2$ and H$_7$), 3.15 (m, 1H, H$_7$), 3.02 (dd, 2H, H$_\beta^{Cys}$, *J*= 7.4 Hz), 2.15 (m, 1H, H$_{4a}$), 1.70-1.35 (m, 6H, H$_5$, H$_{4b}$, H$_{\beta\gamma}^{Leu}$), 1.34 (s, 9H, *t*Bu), 0.93 (t, 6H, 2×CH$_3^{Leu}$, *J*= 6.5 and 6.6 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.0, 172.1, 170.3 (C=O$^{amide\&ester}$), 156.0 (C=O^{Fmoc}), 143.5, 141.1 (2×C_q^{Fmoc}), 127.6, 127.0, 125.0, 119.9 (CH^{Fmoc}), 78.0 (C$_2$), 76.3 (C$_6$), 67.8 (C$_3$), 67.2 (CH$_2^{Fmoc}$), 54.7 (C$_\alpha^{Cys}$), 52.3 (OCH$_3$), 50.1 (C$_\alpha^{Leu}$), 48.3 (C$_q^{tBu}$), 46.9 (CH^{Fmoc}), 43.4 (C$_7$), 42.1, 41.0 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 30.5 (C$_4$), 26.7 (*t*Bu), 27.0 (C$_5$), 26.2 (C$_\gamma^{Leu}$), 21.7, 20.9 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.
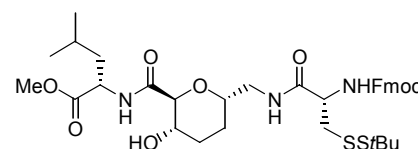
N-(6-*N*-[(*N*-(10-fluorenyl methoxycarbonyl))-*S*-tert-butylthio-D-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-L-leucine methyl ester (2,6-trans 13).

From 2,6-trans **7** (100 mg, 0.25 mmol) and Fmoc-D-Cys(S*t*Bu)-OH (116 mg, 0.27 mmol) according to *GP 4* and *GP 5*. Silica gel chromatography (EtOAc) gave 2,6-trans **13** as a colorless oil (129 mg, 72%). ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.77 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.60 (dd, 2H, CH^{Fmoc}, *J*= 2.2 and 2.3 Hz), 7.41 (t, 2H, CH^{Fmoc}, *J*= 7.4 and 7.5 Hz), 7.30 (m, 2H, CH^{Fmoc}), 7.00 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.8 Hz), 6.70 (bs, 1H, C$_7$NH), 6.20 (d, 1H, NHFmoc, *J*= 5.8 Hz), 4.64 (m, 1H, H$_\alpha^{Leu}$), 4.51 (dd, 1H, H$_\alpha^{Cys}$, *J*= 6.7 and 6.8 Hz), 4.25 (m, 3H, CH$_2^{Fmoc}$, CH^{Fmoc}), 4.00 (m, 2H, H$_2$, H$_6$), 3.87-3.72 (m, 2H, H$_3$, H$_{7a}$ and s, 3H, OCH$_3$), 3.11 (m, 3H, H$_{7b}$, H$_\beta^{Cys}$), 1.97-1.50 (m, 7H, H$_4$, H$_5$, H$_{\beta\gamma}^{Leu}$), 1.34 (s, 9H, *t*Bu), 0.90 (m, 6H, 2×CH$_3^{Leu}$); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.6, 171.6, 170.1 (C=O$^{amide\&ester}$), 157.0 (C=O^{Fmoc}), 144.7, 141.9 (2×C_q^{Fmoc}), 128.3, 127.8, 126.0, 120.6 (CH^{Fmoc}), 77.1 (C$_2$), 72.5 (C$_6$), 67.3 (CH$_2^{Fmoc}$), 65.8 (C$_3$), 55.6

(C_{α}^{Cys}), 52.2 (OCH_3), 50.7 (C_{α}^{Leu}), 48.1 (C_q^{tBu}), 47.7 (CH^{Fmoc}), 42.7 (C_7), 42.3, 40.8 (C_{β}^{Leu}, C_{β}^{Cys}), 29.9 (*t*Bu), 27.1 (C_4), 25.4 (C_{γ}^{Leu}), 23.6 (C_5), 23.2, 21.4 ($2 \times CH_3^{Leu}$). MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.

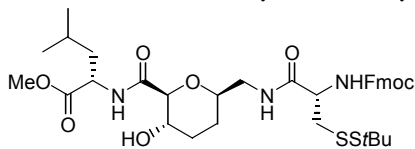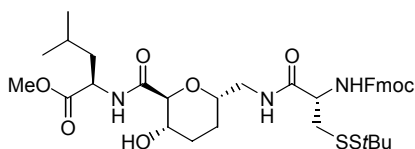**N-(6-N-[(N-(10-fluorenyl methoxycarbonyl))-S-tert-butylthio-D-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-L-leuci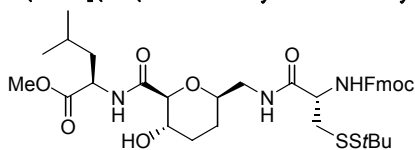ne methyl ester (2,6-cis 13).** From 2,6-cis **7** (195 mg, 0.48 mmol) and Fmoc-L-Cys(S*t*Bu)-OH (228 mg, 0.53 mmol) according to *GP 4* and *GP 5*. Purification by silica gel chromatography (EtOAc) gave 2,6-cis **13** as a colorless oil (262 mg, 77%, 2 steps). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.76 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.59 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.40 (t, 2H, CH^{Fmoc}, *J*= 7.3 and 7.6 Hz), 7.31 (m, 2H, CH^{Fmoc}), 7.19 (d, 1H, NHC$_{\alpha}^{Leu}$, *J*= 8.3 Hz), 6.82 (bs, 1H, C$_7$NH), 5.89 (d, 1H, NHFmoc, *J*= 7.1 Hz), 4.60 (m, 1H, H$_{\alpha}^{Leu}$), 4.45 (m, 3H, H$_{\alpha}^{Cys}$ and CH^{Fmoc}), 4.22 (t, 1H, CH$_2^{Fmoc}$, *J*= 7.0 and 7.1 Hz), 3.69 (s, 3H, OCH$_3$), 3.61-3.50 (m, 3H, H$_6$, H$_3$, H$_2$), 3.47 and 3.35 (2×m, 2H, H$_7$), 3.10 (m, 2H, H$_{\beta}^{Cys}$), 2.16 (m, 1H, H$_4$), 1.73-1.37 (2×m, 6H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.34 (s, 9H, *t*Bu), 0.95 (t, 6H, 2×CH$_3^{Leu}$, *J*= 6.2 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.1, 172.0, 170.4 (C=O$^{amide\&ester}$), 156.0 (C=O^{Fmoc}), 143.6 and 141.2 (2×C$_q^{Fmoc}$), 127.7, 127.0, 125.0, 120.0 (CH^{Fmoc}), 78.1 (C$_2$), 76.6 (C$_6$), 68.1 (C$_3$), 67.3 (CH$_2^{Fmoc}$), 54.6 (C$_{\alpha}^{Cys}$), 52.4 (OCH$_3$), 50.2 (C$_{\alpha}^{Leu}$), 48.5 (C$_q^{tBu}$), 47.0 (CH^{Fmoc}), 43.7 (C$_7$), 42.0, 41.1 (C$_{\beta}^{Leu}$, C$_{\beta}^{Cys}$), 30.6 (C$_4$), 29.8 (*t*Bu), 27.2 (C$_5$), 24.9 (C$_{\gamma}^{Leu}$), 22.7, 21.8 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.

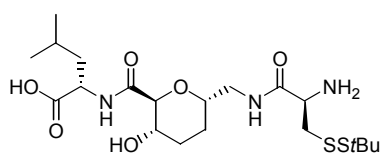**N-(6-N-[(N-(10-fluorenyl methoxycarbonyl))-S-tert-butylthio-D-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-D-leucine methyl ester (2,6-trans 15).** From 2,6-trans **10** (26 mg, 65 μmol) and Fmoc-D-Cys(S*t*Bu)-OH (33 mg, 78 μmol) according to *GP 4* and *GP 5*. Silica gel chromatography (EtOAc) gave the title compound as a colorless oil (46 mg, 99%, 2 steps). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.75 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.57 (d, 2H, CH^{Fmoc}, *J*= 7.4 Hz), 7.39 (t, 2H, CH^{Fmoc}, *J*= 7.3 and 7.5 Hz), 7.32-7.27 (m, 3H, CH^{Fmoc} and NHC$_{\alpha}^{Leu}$), 7.03 (bs, 1H, C$_7$NH), 5.97 (d, 1H, NHFmoc, *J*= 7.3 Hz), 4.70 (m, 1H, H$_{\alpha}^{Leu}$), 4.43-4.30 (m, 3H, H$_{\alpha}^{Cys}$ and CH$_2^{Fmoc}$), 4.20 (t, 1H, CH^{Fmoc}, *J*= 7.1 Hz), 4.15-4.05 (m, 3H, H$_6$, H$_3$ and H$_2$), 3.81 (m, 4H, H$_7$ and s, 3H, OCH$_3$), 3.22-3.09 (m, 3H, H$_7$ and H$_{\beta}^{Cys}$), 1.90 and 1.73-1.50 (m, 7H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.34 (s, 9H, *t*Bu), 0.91 and 0.85 (2×d, 6H, 2×Me Leu, *J*= 6.0 and 3.9 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 174.1, 170.7, 170.6 (3×C=O$^{amide\&ester}$), 156.2 (C=O^{Fmoc}), 143.6 and 141.3 (2×C$_q^{Fmoc}$), 127.8, 127.1, 125.1, 120.0 (CH^{Fmoc}), 76.1 (C$_2$), 72.3 (C$_6$), 67.4 (CH$_2^{Fmoc}$), 65.4 (C$_3$), 55.0 (C$_{\alpha}^{Cys}$), 52.5 (OCH$_3$), 50.0 (C$_{\alpha}^{Leu}$), 48.4 (C$_q^{tBu}$), 47.0 (CH^{Fmoc}), 41.8 and 41.3 (C$_7$, C$_{\beta}^{Leu}$ and C$_{\beta}^{Cys}$), 29.8 (*t*Bu), 26.4 (C$_4$), 24.8 (C$_{\gamma}^{Leu}$), 23.3 (C$_5$), 22.8 and 21.6 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.

N-(6-N-[(N-(10-fluorenyl methoxycarbonyl))-S-tert-butylthio-D-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-aminoglucurono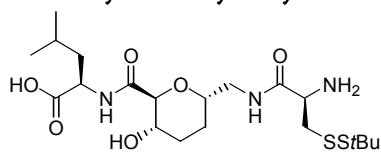pyranosyl)-D-leucine methyl ester (2,6-cis 15). From 2,6-cis **10** (39 mg, 97 μmol) and Fmoc-D-Cys(S*t*Bu)-OH (50 mg, 116 μmol) according to *GP 4* and *GP 5*. Purification by silica gel chromatography (EtOAc) afforded 2,6-cis **15** as a colorless oil (63 mg, 91%, 2 steps). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.76 (d, 2H, CH^{Fmoc}, *J*= 7.6 Hz), 7.58 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.40 (t, 2H, CH^{Fmoc}, *J*= 7.2 and 7.4 Hz), 7.30 (m, 3H, CH^{Fmoc} and NHC$_{\alpha}^{Leu}$), 6.87 (bs, 1H, C$_7$NH), 6.37 (bs, 1H, NHFmoc), 4.62 (m, 1H, H$_{\alpha}^{Leu}$), 4.49-4.40 (m, 2H, H$_{\alpha}^{Cys}$ and CH^{Fmoc}), 4.29-4.18 (m, 2H, CH$_2^{Fmoc}$), 3.73 (s, 3H, OCH$_3$), 3.67 (m, 1H, H$_7$), 3.63-3.50 (m, 2H, H$_6$ and H$_2$), 3.45 (m, 1H, H$_3$), 3.18-3.12 (dt, 1H, H$_7$, *J*= 5.3 Hz), 3.11 (d, 2H, H$_{\beta}^{Cys}$, *J*= 6.7 Hz), 2.07 (m, 1H, H$_4$), 1.67-1.37 (2×m, 6H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.34 (s, 9H, *t*Bu), 0.95 (t, 6H, 2×Me Leu, *J*= 6.2 and 7.3 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 174.5, 172.9, 171.5 (3×C=O$^{amide\&ester}$), 157.0 (C=O^{Fmoc}), 144.2 and 141.8 (2×C$_q^{Fmoc}$), 128.4, 127.7, 125.7, 120.7 (CH^{Fmoc}), 78.3 (C$_2$), 76.4 (C$_6$), 68.4 (C$_3$), 68.1 (CH$_2^{Fmoc}$), 55.1 (C$_{\alpha}^{Cys}$), 53.1 (OCH$_3$), 50.7 (C$_{\alpha}^{Leu}$), 49.0 (C$_q^{tBu}$), 47.5 (CH^{Fmoc}), 43.7 (C$_7$), 42.0 and 41.7 (C$_{\beta}^{Leu}$ and C$_{\beta}^{Cys}$), 30.9 (C$_4$), 30.4 (*t*Bu), 27.3 (C$_5$), 25.4 (C$_{\gamma}^{Leu}$), 23.4, 22.4 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 716.4 (M+H)$^+$; 738.8 (M+Na)$^+$.

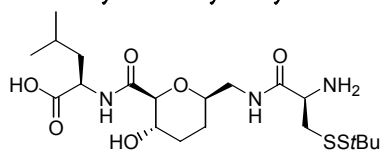**S-tert-butylthio-L-cysteinyl-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-L-leucine** (2,6-trans **9**). Treatment of 2,6-trans **8** (37 mg, 52 µmol) according to *GP 6* afforded the title compound as a white foam (8.7 mg, 45%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.63 (t, 1H, C$_7$NH, *J*= 5.6 Hz), 7.95 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.0 Hz), 4.76 (bs, 2H, NH$_2$), 4.23 (m, 1H, H$_\alpha^{Leu}$), 4.08 (d, 1H, H$_2$, *J*= 3.1 Hz), 3.97 (m, 1H, H$_3$), 3.93 (t, 1H, H$_\alpha^{Cys}$, *J*= 6.2 and 6.4 Hz), 3.75 (m, 1H, H$_6$), 3.27 (m, 2H, H$_7$), 3.08-3.17 (m, 2H, H$_\beta^{Cys}$), 1.51-1.66 (m, 6H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.40 (m, 1H, H$_5$), 1.31 (s, 9H, *t*Bu), 0.90 and 0.85 (2×d, 6H, 2×Me Leu, *J*= 6.3 and 6.4 Hz); ^{13}C-NMR (100 MHz, DMSO-*d6*): δ 173.8, 169.9, 166.7 (3×C=O), 77.5 (C$_2$), 70.9 (C$_6$), 63.3 (C$_3$), 51.8 (C$_\alpha^{Leu}$), 50.0 (C$_\alpha^{Cys}$), 48.0 (C$_q$ t-butyl S*t*Bu), 42.6 (C$_7$), 40.1 and 39.9 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 29.4 (*t*Bu), 26.2 (C$_4$), 26.2 (C$_\gamma^{Leu}$), 22.3 (C$_5$), 22.8 and 21.2 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 480.1 (M+H)$^+$; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)$^+$, found 480.2177.

**S-tert-butylthio-L-cysteinyl-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-L-leucine (2,6-cis 9).**
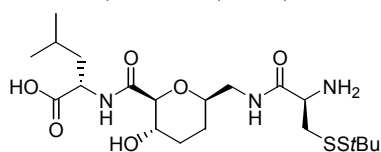
Treatment of 2,6-cis **8** (20 mg, 28 µmol) according to *GP 6* gave 2,6-cis **9** as a foam (5.3 mg, 40%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.66 (t, 1H, C$_7$NH, *J*= 5.0 Hz and 5.6 Hz), 7.95 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 7.9 Hz), 4.75 (bs, 2H, NH$_2$), 4.28 (m, 1H, H$_\alpha^{Leu}$), 3.97 (m, 1H, H$_\alpha^{Cys}$), 3.55 (d, 1H , H$_2$, *J*= 9.2 Hz), 3.44-3.19 (m, 4H, H$_6$, H$_3$, H$_7$), 3.12 (m, 2H, H$_\beta^{Cys}$), 1.97 (m, 1H, H$_4$), 1.51-1.75 (m, 4H, H$_5$, H$_{\beta\gamma}^{Leu}$), 1.31 (m, 11H, *t*Bu, H$_4$ and H$_5$), 0.90 and 0.87 (2×d, 6H, 2×Me Leu, *J*= 6.2 and 6.0 Hz); ^{13}C-NMR (100 MHz, DMSO-*d6*): δ 173.8, 170.1, 166.7 (3×C=O), 80.6 (C$_2$), 75.2 (C$_6$), 67.1 (C$_3$), 51.7 (C$_\alpha^{Leu}$), 50.0 (C$_\alpha^{Cys}$), 48.0 (C$_q$^{tBu}), 43.2 (C$_7$), 40.9, 40.0 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 31.4 (C$_4$), 29.4 (*t*Bu), 27.4 (C$_5$), 24.3 (C$_\gamma^{Leu}$), 22.7 and 21.5 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 480.1 (M+H)$^+$; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)$^+$, found 480.2145.

**S-tert-butylthio-L-cysteinyl-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-D-leucine** (2,6-trans **12**). Treatment of 2,6-trans **11** (9 mg, 13 µmol) according to *GP 6* gave 2,6-trans **12** as a foam (1.6 mg, 26%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.70 (d, 1H, C$_7$NH, *J*= 4.4 Hz), 8.00 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.2 Hz), 4.75 (bs, 2H, NH$_2$), 4.20 (m, 1H, H$_\alpha^{Leu}$), 4.10 (d, 1H, H$_2$, *J*= 3.2 Hz), 3.95 (m, 2H, H$_\alpha^{Cys}$, H$_3$), 3.80 (m, 1H, H$_1$), 3.06-2.90 (m, 4H, H$_7$, H$_\beta^{Cys}$), 1.65-1.30 (2×m, 7H, H$_5$, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.27 (s, 9H, *t*Bu), 0.87 (2×d, 6H, 2×CH$_3^{Leu}$, *J*= 6.6 and 6.5 Hz); MS (ESI): *m/z* 480.1 (M+H)$^+$; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)$^+$, found 480.2036.

S-tert-butylthio-L-cysteinyl-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-D-leucine (2,6-cis **12**). Treatment of 2,6-cis **11** (43 mg, 60 µmol) according to *GP 6* gave 2,6-cis **12** as a foam (10 mg, 36%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.77 (t, 1H, C$_7$NH, *J*= 5.9 Hz), 8.08 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.2 Hz), 4.80 (bs, 2H, NH$_2$), 4.32 (m, 1H, H$_\alpha^{Leu}$), 3.97 (t, 1H, H$_\alpha^{Cys}$ *J*= 6.3 Hz), 3.53 (d, 1H, H$_2$, *J*= 9.3 Hz), 3.43-3.30 (m, 3H, H$_2$, H$_3$, H$_7$), 3.11 (m, 3H, H$_7$, H$_\beta^{Cys}$), 1.95 (m, 1H, H$_4$), 1.70-1.50 (m, 4H, H$_5$, H$_{\beta\gamma}^{Leu}$), 1.35 (m, 2H, H$_4$, H$_5$) 1.30 (s, 9H, *t*Bu), 0.87 (2×d, 6H, 2×CH$_3^{Leu}$, *J*= 6.6 and 6.5 Hz); MS (ESI): *m/z* 480.1 (M+H)$^+$; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)$^+$, found 480.1982.

S-tert-butylthio-D-cysteinyl-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-L-leucine (2,6-trans 14).
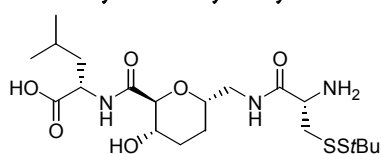
Treatment of 2,6-trans **13** (10 mg, 14 µmol) according to *GP 6* gave 2,6-trans **14** as a foam (2.0 mg, 30%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.62 (dd, 1H, C$_7$NH, *J*= 4.6 and 4.6 Hz), 7.99 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 8.2 Hz), 4.77 (bs, 2H, NH$_2$), 4.26 (m, 1H, H$_\alpha^{Leu}$), 4.09 (d, 1H, H$_2$, *J*= 2.9 Hz), 4.00 (m, 1H, H$_3$), 3.95 (m, 1H, H$_\alpha^{Cys}$), 3.78 (m, 1H, H$_6$), 3.36 and 3.16-3.06 (2×m, 4H, H$_7$ and H$_\beta^{Cys}$), 1.70-1.55 (m, 6H, H$_4$, H$_{\beta\gamma}^{Leu}$), 1.40 (m, 1H, H$_5$), 1.30 (s, 9H, *t*Bu), 0.87 (2×d, 6H, 2×CH$_3^{Leu}$, *J*= 6.6 and 6.5 Hz); MS (ESI): *m/z* 480.1 (M+H)$^+$; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)$^+$, found 480.2097.

### *S*-tert-butylthio-D-cysteinyl-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-L-leucine (2,6-cis **14**).
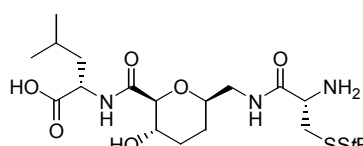
Treatment of 2,6-cis **13** (17 mg, 24 μmol) according to *GP 6* afforded the title compound as a foam (5.6 mg, 49%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.71 (d, 1H, C$_7$NH, *J*= 6.0 Hz), 8.08 (d, 1H, NHC$_\alpha$^{Leu}, *J*= 8.2 Hz), 4.80 (bs, 2H, NH$_2$), 4.30 (m, 1H, H$_\alpha$^{Leu}), 3.97 (t, 1H, H$_\alpha$^{Cys} *J*= 6.3 Hz), 3.54 (d, 1H, H$_2$, *J*= 9.2 Hz), 3.44-3.35 (m, 3H, H$_6$, H$_3$, H$_7$), 3.13 (m, 3H, H$_7$ and H$_\beta$^{Cys}), 1.95 (m, 1H, H$_4$), 1.70-1.50 (m, 4H, H$_5$, H$_{\beta\gamma}$^{Leu}), 1.35 (m, 2H, H$_4$ and H$_5$) 1.30 (s, 9H, *t*Bu), 0.87 (2×d, 6H, 2×CH$_3$^{Leu}, *J*= 6.3 and 6.4 Hz); MS (ESI): *m/z* 480.1 (M+H)[+]; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)[+], found 480.2159.

S-tert-butylthio-D-cysteinyl-aminomethyl-4,5-dideoxy-α-D-aminoglucuronopyranosyl)-D-leucine (2,6-trans **16**).
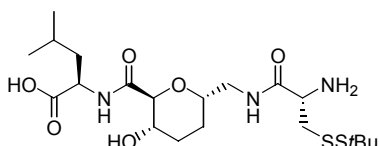
Treatment of 2,6-trans **15** (20 mg, 28 μmol) according to *GP 6* furnished the title compound as a foam (6.7 mg, 50%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.82 (d, 1H, C$_7$NH, *J*= 4.4 Hz), 8.00 (d, 1H, NHC$_\alpha$^{Leu}, *J*= 8.2 Hz), 4.60 (bs, 2H, NH$_2$), 4.28 (m, 1H, H$_\alpha$^{Leu}), 4.08 (d, 1H, H$_2$, *J*= 3.2 Hz), 4.01 (m, 1H, H$_\alpha$^{Cys}), 3.95 (m, 1H, H$_3$), 3.75 (m, 1H, H$_6$), 3.37-3.14 (m, 4H, H$_7$ and H$_\beta$^{Cys}), 1.70-1.50 (m, 6H, H$_5$, H$_4$, H$_{\beta\gamma}$^{Leu}), 1.39 (m, 1H, H$_5$), 1.30 (s, 9H, *t*Bu), 0.90 and 0.86 (2×d, 6H, 2×CH$_3$^{Leu}, *J*= 6.6 and 6.5 Hz); MS (ESI): *m/z* 480.1 (M+H)[+]; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)[+], found 480.2168.

S-tert-butylthio-D-cysteinyl-aminomethyl-4,5-dideoxy-β-D-aminoglucuronopyranosyl)-D-leucine (2,6-cis **16**).
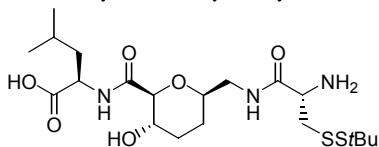
Treatment of 2,6-cis **15** (20 mg, 28 μmol) according to *GP 6* afforded the title compound as a foam (12 mg, 60%). ^{1}H-NMR (750 MHz, DMSO-*d6*): δ 8.84 (s, 1H, C$_7$NH), 8.08 (d, 1H, NHC$_\alpha$^{Leu}, *J*= 8.1 Hz), 4.83 (bs, 2H, NH$_2$), 4.27 (m, 1H, H$_\alpha$^{Leu}), 3.93 (m, 1H, H$_\alpha$^{Cys}), 3.54 (d, 1H, H$_2$, *J*= 9.2 Hz), 3.44-3.15 (m, 6H, H$_6$, H$_3$, H$_7$, H$_\beta$^{Cys}), 1.95 (m, 1H, H$_4$), 1.75-1.50 (m, 4H, H$_5$, H$_{\beta\gamma}$^{Leu}), 1.35-1.31 (m, 11H, *t*Bu, H$_4$ and H$_5$), 0.87 (2×d, 6H, 2×CH$_3$^{Leu}, *J*= 6.4 and 6.5 Hz); MS (ESI): *m/z* 480.1 (M+H)[+]; HR-MS: calc. 480.2202 (C$_{20}$H$_{37}$N$_3$O$_6$S$_2$+H)[+], found 480.2199.

2.5 References and Notes

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Chapter 3

Design, Synthesis and Evaluation of Sugar Amino Acid based
Inhibitors of Protein:farnesyl Transferase and
Protein:geranylgeranyl Transferase-1

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G. A. van der Marel, M. Overhand *J. Med. Chem.* **2004**, *47*, 3920 – 3923.

Abstract. Eleven peptidomimetic analogs of the *C*-terminal Ca_1a_2X motif found in natural substrates of the prenyl transferases PFT and PGGT-1 were synthesised and evaluated for their inhibition potency and selectivity against PFT and PGGT-1. Replacement of the central dipeptide part a_1a_2 by a benzylated sugar amino acid resulted in a good and selective PFT inhibitor (2,6-cis **21**, $IC_{50}= 250 \pm 20$ nM). The methyl ester of 2,6-cis **21** (2,6-cis **25**) selectively inhibited protein farnesylation in cultured cells.

3.1 Introduction

Tetrapeptides based on the C-terminal Ca_1a_2X motif can function as substrate analog inhibitors of protein:farnesyl transferase (PFT) and protein:geranylgeranyl transferase-1 (PGGT-1; see Chapter 1). Chapter 2 presented a new type of Ca_1a_2X analogs, in which a sugar amino acid (SAA) is incorporated as replacement of the central dipeptide a_1a_2. On the basis of this finding, it was envisaged that compounds with improved inhibitory potency can be obtained by modification of the SAA moieties. From literature data it is known that the introduction of hydrophobic aromatic residues at the a_2 position has a beneficial effect on the inhibitory potency against both PFT and PGGT-1.[1] For PFT this is exemplified by the potent and competitive inhibitor CVFM,[2] in which the presence of phenylalanine at the a_2 position is of prime importance. In line with this, it is reported that a series of Ca_1a_2L analogs, in which the a_1a_2 portion was replaced by dipeptide isosteric 2-aryl-4-aminobenzoic acid moieties, were effective inhibitors of PGGT-1.[1de,3] The replacement of amide linkages by amine connections in certain Ca_1a_2X analogs not only has a beneficial effect on the stability against proteolytic degradation but also influences the selectivity of inhibition.[4] The recently developed[5] synthesis of amino acid/carbohydrate conjugates *via* the Fukuyama/Mitsunobu glycosylation of amino acid derived *ortho*-nitrobenzenesulfonamides offered the opportunity to evaluate the inhibitory potency and selectivity of Ca_1a_2X analogs having an amine bond between the peptidomimetic and the C-terminal residue.

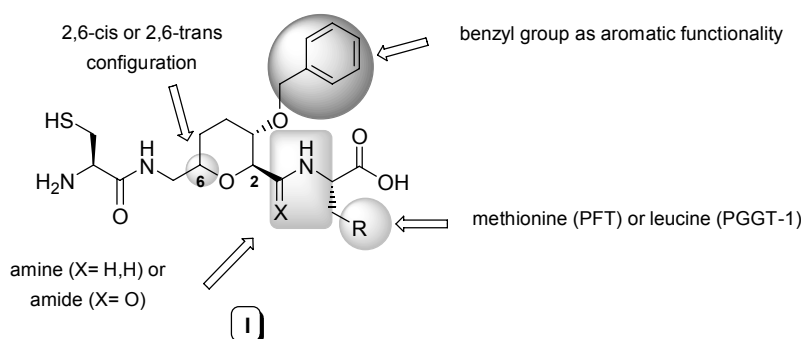**Figure 3.1** General representation SAA based Ca_1a_2X analogs presented in this chapter.

This chapter describes the synthesis of novel inhibitors of the prenylating enzymes PFT and PGGT-1 containing a benzylated SAA having a 2,6-trans or a 2,6-cis substituted sugar core (**I**, Figure 3.1). The Ca_1a_2L analogs **18** and **19** and the controls 2,6-cis **23** and 2,6-cis **24**[*] (Scheme 3.5) were designed to inhibit PGGT-1, while the corresponding Ca_1a_2M analogs **20** and **21** were projected to inhibit PFT. Sulfoxides 2,6-trans **22** and 2,6-cis **22** were obtained as side products in a deprotection step. All compounds were evaluated on their inhibitory potency and selectivity in both a PFT and PGGT-1 enzyme bio-assay.[6] The effect of compound 2,6-cis **25**, (*i.e.* methyl ester of 2,6-cis **21**, Figure 3.2), on the prenylation of proteins was investigated in cultured cells.
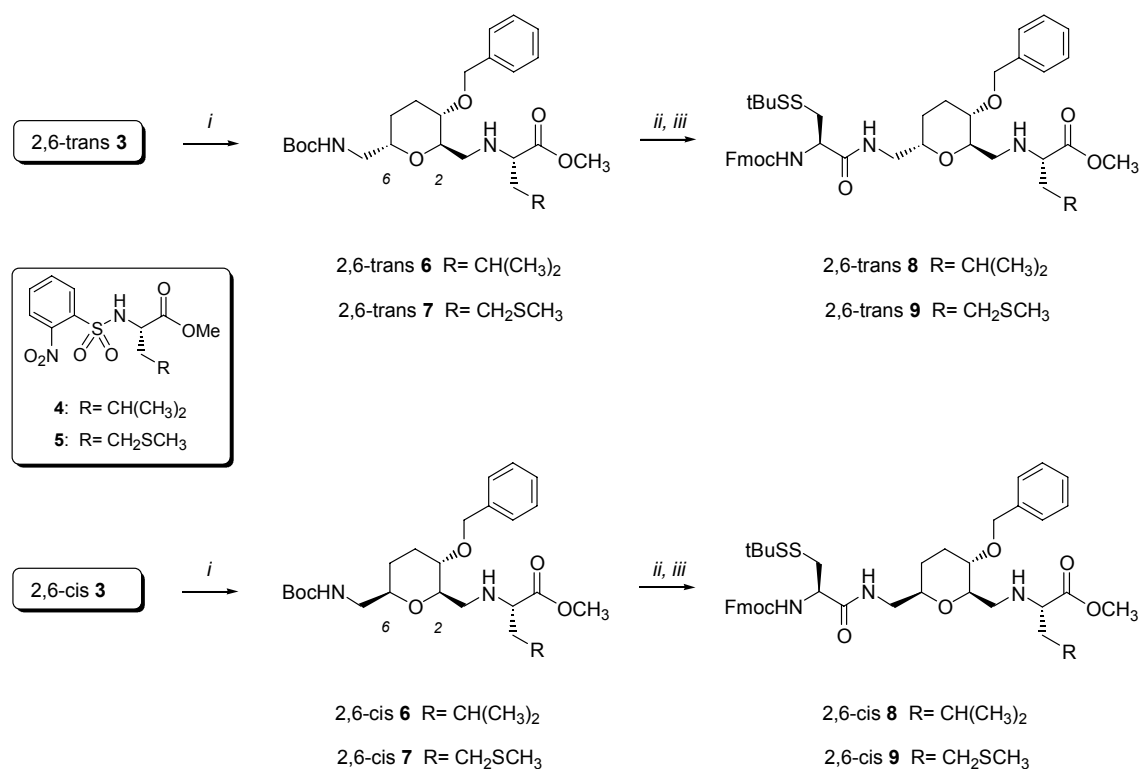
3.2 Results and Discussion

3.2.1 Synthesis – The assembly of all target Ca_1a_2X analogs started with the synthesis of epimeric alcohols 2,6-trans **3** and 2,6-cis **3** (Scheme 3.1). Known[7] cyanides 2,6-trans **1** and 2,6-cis **1** were hydrogenated (45 *psi* H_2) over Pd/C (10% Pd). Treatment of the resulting ammonium salts with Boc_2O followed by base catalysed deacetylation gave diols 2,6-trans **2** and 2,6-cis **2**, respectively in good overall yields. The introduction of the benzyl group at C_3 was achieved by protection of the primary hydroxyl with a trityl group and phase transfer catalysed benzylation of the C_3 hydroxyl[8] whereafter acidic removal of the triphenylmethyl group furnished the desired key building blocks 2,6-trans **3** and 2,6-cis **3**.

The route of synthesis to the fully protected amine precursors (2,6-trans/cis **8** and 2,6-trans/cis **9**) is exemplified by the conversion of 2,6-trans **3** into compounds 2,6-trans **8** and 2,6-trans **9** (Scheme 3.2). Thus, treatment of 2,6-trans **3** with either *o*-Ns-Leu-OMe (**4**)[9] or *o*-Ns-Met-OMe (**5**)[10] and PPh_3/DEAD gave, after removal of the nosyl group, the dimers 2,6-trans **6** and 2,6-trans **7**, respectively in good yield. The Boc group was removed and the corresponding ammonium salt was condensed with Fmoc-Cys(S*t*Bu)-OH furnishing the desired Ca_1a_2X analogs. Starting from 2,6-cis **3**, the corresponding 2,6-cis amine precursors (2,6-cis **8** and 2,6-cis **9**, Scheme 3.2) were obtained.

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[*] The synthesis of compound 2,6-cis **24** is described in Chapter 2.

Scheme 3.1 Synthesis of 2,6-trans and 2,6-cis **3**.[a]

2,6-trans **1**   2,6-trans **2**   2,6-trans **3**

2,6-cis **1**   2,6-cis **2**   2,6-cis **3**
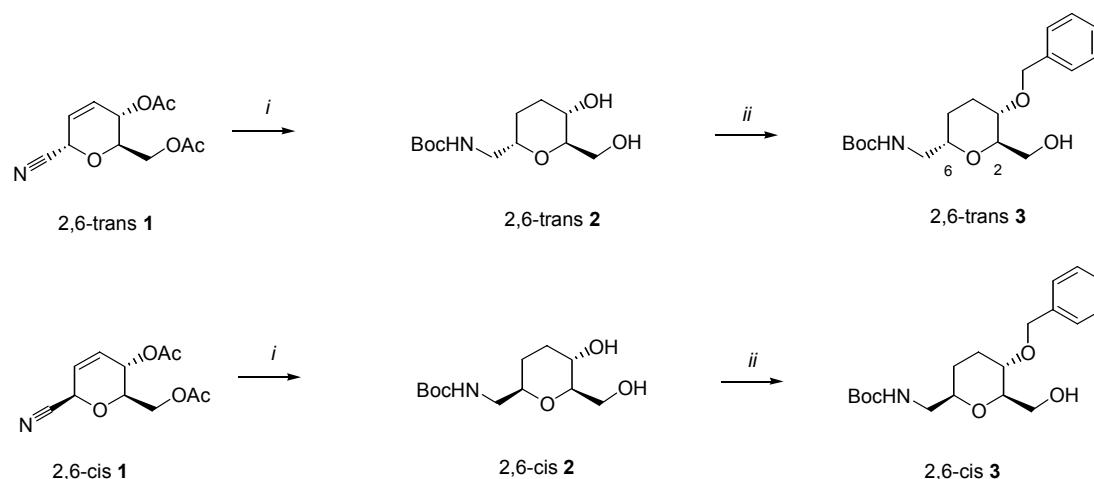
[a]Reagents and conditions (*i*) (a) 10% Pd/C, H$_2$, EtOH, MeOH, CHCl$_3$ (b) Boc$_2$O, Et$_3$N, DCM (c) NaOMe, MeOH (2,6-trans **2**: 95%, 2,6-cis **2**: 70%); (*ii*) (a) TrCl, pyridine, 60°C (b) BnBr, Bu$_4$NHSO$_4$, 50% aq. NaOH, DCM (c) *p*-TsOH·H$_2$O, DCM, MeOH (2,6-trans **3**: 73%, 2,6-cis **3**: 75%).

Scheme 3.2 Synthesis of 2,6-trans **8**, 2,6-cis **8**, 2,6-trans **9** and 2,6-cis **9**.[a]

2,6-trans **3**

2,6-trans **6** R= CH(CH$_3$)$_2$
2,6-trans **7** R= CH$_2$SCH$_3$

2,6-trans **8** R= CH(CH$_3$)$_2$
2,6-trans **9** R= CH$_2$SCH$_3$

4: R= CH(CH$_3$)$_2$
5: R= CH$_2$SCH$_3$

2,6-cis **3**

2,6-cis **6** R= CH(CH$_3$)$_2$
2,6-cis **7** R= CH$_2$SCH$_3$

2,6-cis **8** R= CH(CH$_3$)$_2$
2,6-cis **9** R= CH$_2$SCH$_3$

[a]Reagents and conditions (*i*) (a) *o*-Ns-Leu-OMe (**4**) or *o*-Ns-Met-OMe (**5**), PPh$_3$, DEAD, THF (b) K$_2$CO$_3$, PhSH, CH$_3$CN, 50°C (2,6-trans **6**: 65%, 2,6-cis **6**: 92%; 2,6-trans **7**: 81%, 2,6-cis **7**: 99%); (*ii*) TFA, DCM, *i*Pr$_3$SiH or Et$_3$SiH; (*iii*) Fmoc-Cys(S*t*Bu)-OH, BOP, *N*-ethylmorpholine, DMF (2,6-trans **8**: 40%, 2,6-cis **8**: 66%; 2,6-trans **9**: 47%, 2,6-cis **9**: 44%).

The fully protected precursor of debenzylated control compound 2,6-cis **11** (Scheme 3.5) was obtained by the procedure depicted in Scheme 3.3. Removal of the benzyl group in 2,6-cis **6** furnished 2,6-cis **10** (HCl salt) which was subjected to the same sequence of reactions as described for the synthesis of **8**, thereby affording 2,6-cis **11**.

Scheme 3.3 Synthesis of 2,6-cis **11**.[a]
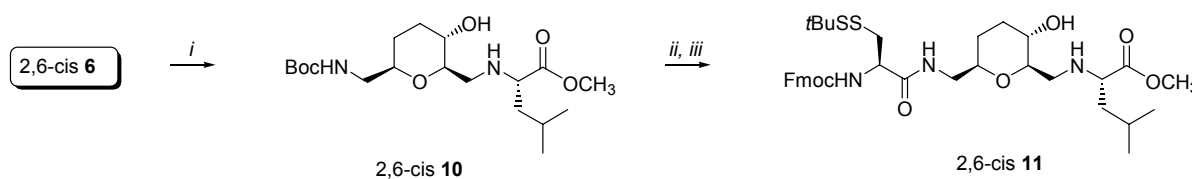
[a]Reagents and conditions: (*i*) 10% Pd/C, H$_2$, EtOH, CHCl$_3$ (100%); (*ii*) TFA, DCM, *i*Pr$_3$SiH or Et$_3$SiH; (*iii*) Fmoc-Cys(S*t*Bu)-OH, BOP, *N*-ethylmorpholine, DMF (52%, 2 steps).

The route of synthesis to the fully protected amide precursors is exemplified by the conversion of 2,6-cis **3** into compounds 2,6-cis **15** and 2,6-cis **16** (Scheme 3.4). Oxidation of 2,6-cis **3** with RuCl$_3$·3H$_2$O[11] or TEMPO/[bis(acetoxy)iodo]benzene[12] gave the carboxylic acid 2,6-cis **12**. In an alternative route to the SAAs 2,6-cis **12** and 2,6-trans **12**, diol **2** was processed as exemplified for the 2,6-cis isomer (Scheme 3.4). Treatment of 2,6-cis **2** with benzylidene dimethyl acetal afforded 2,6-cis **17** in good yield. Next, opening of the acetal by subjection of 2,6-cis **17** to excess diisobutylaluminium hydride gave 2,6-cis **3** along with the corresponding C$_1$–OBn regio-isomer (C$_3$–OBn/C$_1$–OBn: ≈7/1).[13] As separation of the two regio-isomers was found to be troublesome at this stage, the oxidation step was carried out on the mixture and the desired SAA building block 2,6-cis **12** could be isolated in pure form. Subsequent condensation of 2,6-cis **12** with H-Leu-OMe (HCl salt) or H-Met-OMe (HCl salt) yielded 2,6-cis **13** and 2,6-cis **14**, respectively. Deprotection of the Boc group and condensation with Fmoc-Cys(S*t*Bu)-OH afforded 2,6-cis **15** and 2,6-cis **16**.[14] The corresponding 2,6-trans Ca$_1$a$_2$X analogs (2,6-trans **15** and 2,6-trans **16**, Scheme 3.4) were obtained by executing the same sequence of reactions as described for the 2,6-cis analogs.

Scheme 3.4 Synthesis of 2,6-cis **15**, 2,6-trans **15**, 2,6-trans **16** and 2,6-cis **16**.[a]
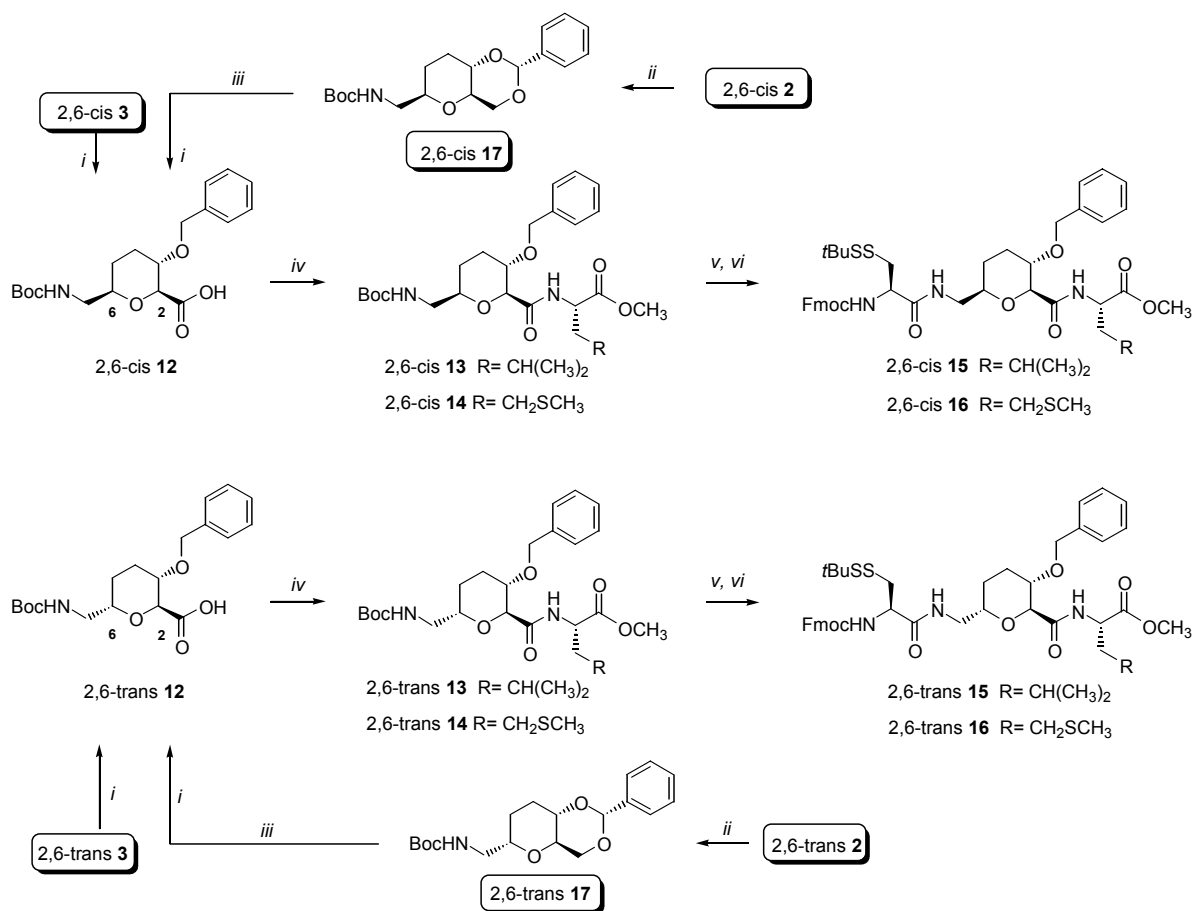
[a]Reagents and conditions (*i*) RuCl₃·3H₂O, K₂S₂O₈, 1M KOH or TEMPO, [bis(acetoxy)iodo]benzene, DCM, H₂O (2,6-trans **12**: 85%, 2,6-cis **12**: 100%); (*ii*) benzaldehyde dimethyl acetal, *p*-TsOH·H₂O, CH₃CN (2,6-trans **17**: 71%, 2,6-cis **17**: 77%); (*iii*) DIBAL-H, PhCH₃, 0°C; (*iv*) HCl·H-Leu-OMe, BOP, DIPEA (2,6-trans **13**: 98%, 2,6-cis **13**: 98%) or HCl·H-Met-OMe, EDC, HOBt, DMF (2,6-trans **14**: 73%, 2,6-cis **14**: 83%); (*v*) TFA, DCM, *i*Pr₃SiH or Et₃SiH; (*vi*) Fmoc-Cys(S*t*Bu)-OH, for **15**: BOP, HOBt, DIPEA (2,6-trans **15**: 80%, 2,6-cis **15**: 85%), for **16**: EDC, HOBt, DMF (2,6-trans **16**: 79%, 2,6-cis **16**: 79%).

Target compounds **18–22** and 2,6-cis **23** (Scheme 3.5) were obtained by treatment of the fully protected precursors with Tesser's base mixture (MeOH/1,4-dioxane/4M aq. NaOH, 15/4/1 v/v/v) to effect simultaneous hydrolysis of the ester and removal of the Fmoc group. The crude compounds were subsequently characterised by LC-MS and purified by RP-HPLC. In the case of 2,6-trans **21** and 2,6-cis **21**, the applied deprotection condition gave oxidised side-products which were identified as sulfoxides 2,6-trans **22** and 2,6-cis **22** (Scheme 3.5) by NMR and MS.[14] Isolation of these compounds allowed

evaluation of their biological activity. Fortunately, base mediated deprotection under stringent non-aerobic conditions furnished the desired 2,6-trans **21** and 2,6-cis **21**.

Scheme 3.5 Synthesis of **18 – 22** and 2,6-cis **23**.[a]
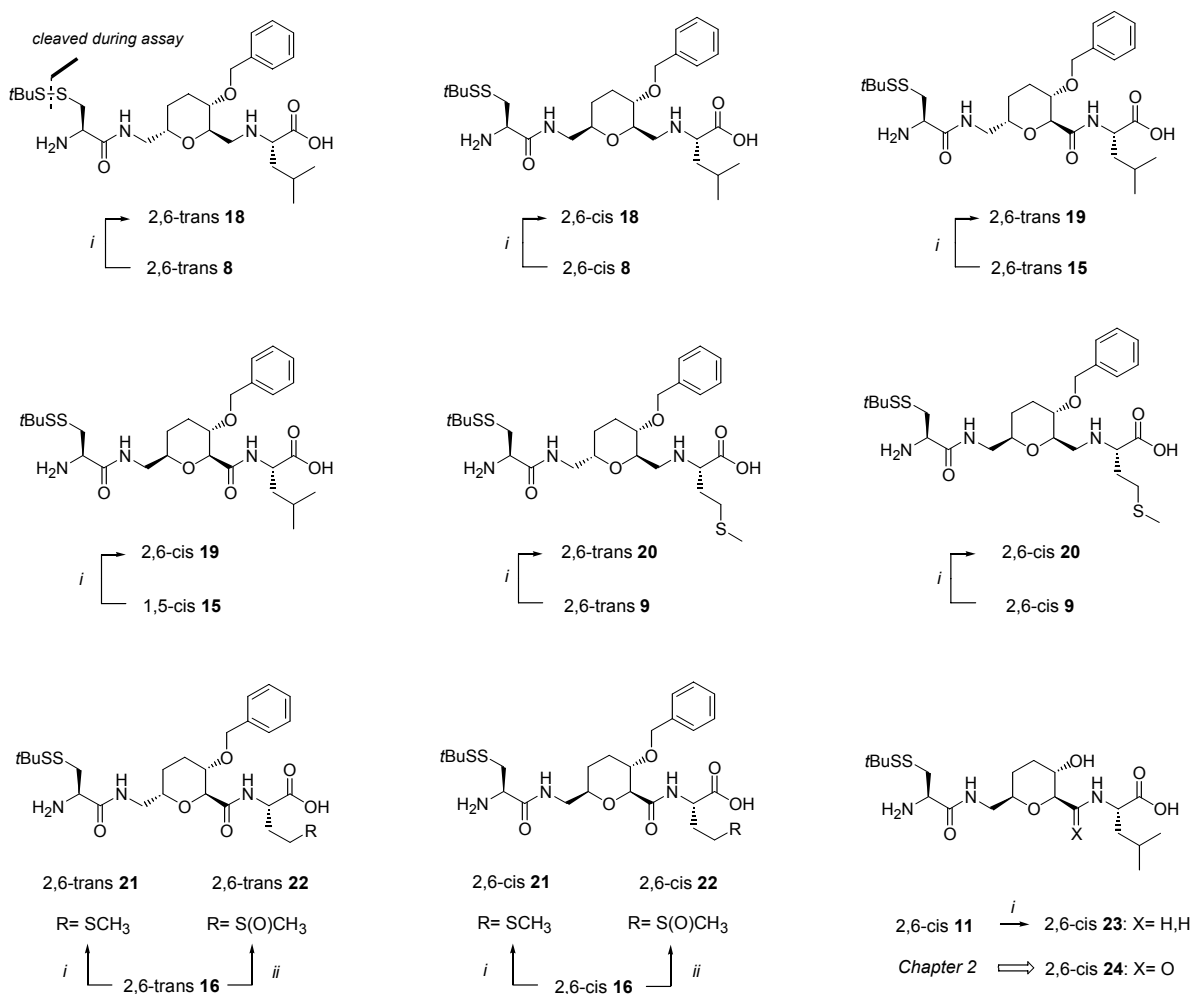
[a]Reagents and conditions (*i*) (a): MeOH/1,4-dioxane/4M NaOH (b) RP-HPLC purification; (*ii*) (a) MeOH/1,4-dioxane/4M NaOH: reaction was performed under argon with freshly distilled 1,4-dioxane (benzophenone ketyl) (b) RP-HPLC purification.

3.2.2 Biological Evaluation – Compounds **18–22** and 2,6-cis **23** (Scheme 3.5) were evaluated for their *in vitro* inhibitory activity against PFT and PGGT-1 following previously described procedures (Table 3.1). The S*t*Bu protective group on the cysteine residue is cleaved under the conditions of the assay (pH 7.4, DTT). As a reference the known tetrapeptides CVIM and CVIL[4,15] were also evaluated.

Table 3.1 Determined IC_{50} values of **18–22** and 2,6-cis **23**, reference tetrapeptides CVIM and CVIL.

compound	IC$_{50}$ (μM)[a]		compound	IC$_{50}$ (μM)[a]	
	PFT	PGGT-1		PFT	PGGT-1
2,6-trans **18**	~1000	270 ± 122	2,6-cis **21**	0.25 ± 0.02	>1000
2,6-cis **18**	>1000	464 ± 147	2,6-trans **22**	91 ± 14	521 ± 75
2,6-trans **19**	321 ± 18	206 ± 34	2,6-cis **22**	2.2 ± 0.6	>1000
2,6-cis **19**	57 ± 18	14 ± 6	2,6-cis **23**	>1000	261 ± 55
2,6-trans **20**	~1000	311 ± 94	2,6-cis **24**	–[b]	~1000
2,6-cis **20**	>1000	500 ± 81	**CVIM**	0.42 ± 0.05	–[b]
2,6-trans **21**	42 ± 6	48 ± 11	**CVIL**	–[b]	4.4 ± 0.2

[a]IC_{50}: concentration of compound required to inhibit for 50% the PGGT-1 or PFT catalysed incorporation of [³H]-GGPP or [³H]-PFT, respectively. All IC_{50} values are means of three determinations: one determination involves performing the assay at 5 concentrations of compound. By using a mathematical function fitting to the concentration/inhibition curve, the IC_{50} value was determined. [b]not determined.

Compound 2,6-cis **21** proved to be a good (IC_{50}= 250 ± 20 nM) and selective PFT inhibitor. Furthermore, 2,6-cis **21** has a 1000-4000 fold improved inhibitory potency compared to the SAA based PFT inhibitors reported previously[16] and was found to be slightly more active than the lead tetrapeptide inhibitor CVIM. When 2,6-trans **21** and 2,6-cis **21** are compared, it becomes clear that the stereochemical identity of the SAA is important with respect to both potency and selectivity.

For PGGT-1, 2,6-cis **19** was found to be the most active inhibitor (IC_{50}= 14 ± 6 μM). Compound 2,6-cis **19**, which only differs from 2,6-cis **21** in its X amino acid, shows no high selectivity for either enzyme.[15] Comparing 2,6-cis **24** with 2,6-cis **19** shows that enhanced hydrophobicity at the a$_2$ position has a positive effect on the inhibitory potency. Having an amine linkage between the dipeptide isostere and the X amino acid (**18** and **20**) proves to be detrimental for the inhibition of both enzymes, in particular for PFT.[17] However, compound 2,6-cis **23** is ~4 times more active against PGGT-1 than its amide analog 2,6-cis **24**. Although introduction of a sulfoxide functionality (**22**) leads to a decrease in inhibitory potency, this modification is better tolerated by PFT than PGGT-1.[18a] This is in analogy with results reported by Manne and co-workers[18b] who observed that replacement of the methionine by polar residues (*e.g.* carboxamide or sulfone) retained inhibitory potency against PFT and to a lesser extent against PGGT-1.

Figure 3.2 Synthesis[a] of 2,6-cis **25** and *in vivo* evaluation[b] of **TR006**, 2,6-cis **21** and 2,6-cis **25**.
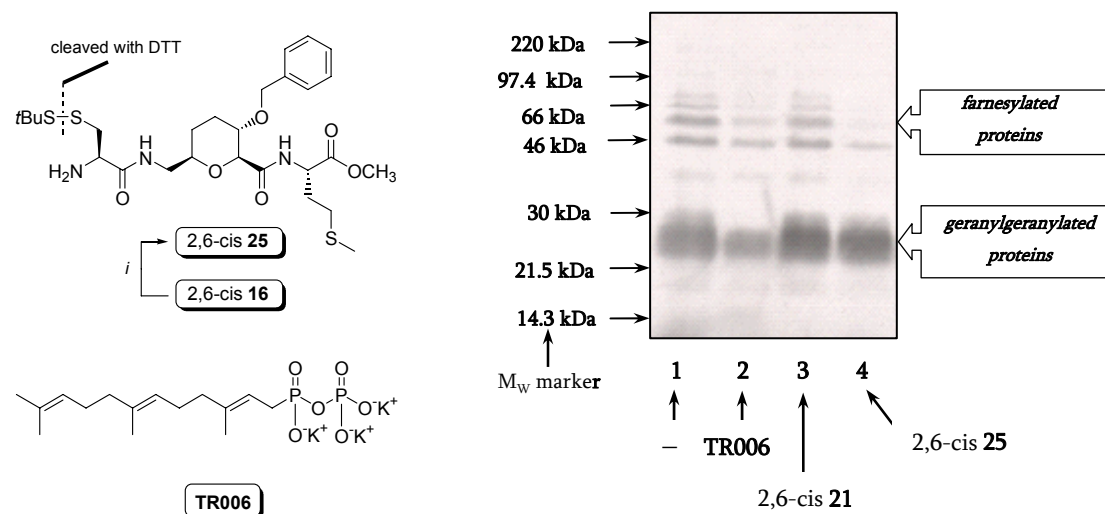
[a]Reagents and conditions (*i*) 20% piperidine in DMF (81%). [b]Met-18b-2 cells were treated with [³H]-mevalonate, simvastatin and in the absence of compound (lane 1); with **TR006** (lane 2, *c* = 100 µM); 2,6-cis **21** (lane 3, *c* = 100 µM); 2,6-cis **25** (lane 4, *c* = 100 µM). Monolayers of cells were dissolved in detergent solution and subjected to electrophoresis and autoradiography.

In order to evaluate whether compound 2,6-cis **21** is able to inhibit protein farnesylation in cultured cells, *in vivo* experiments were conducted using CHO (Chinese Hamster Ovary) Met-18b-2 cells.[19] The corresponding methylester 2,6-cis **25** was synthesised in order to facilitate cellular uptake by enhancing the hydrophobicity. Once having entered the cell, the methylester is believed to be hydrolysed by hydrolases to the corresponding acid. As illustrated in Figure 3.2, incubation with [³H]-mevalonate resulted in several radio-labelled prenylated proteins which can be roughly divided into farnesylated proteins at molecular weights of about 46 – 80 kDa and geranylgeranylated proteins at about 22 – 28 kDa.[20] While incubation with 2,6-cis **21** (lane 3) did not influence the prenylation pattern, 2,6-cis **25** (lane 4) inhibited the incorporation of labelled mevalonate into the higher molecular weight bands, indicating that protein farnesylation was strongly decreased. The 21 – 28 kDa bands did not change supporting the specificity of the inhibition. As a positive control, **TR006** (Figure 3.2), a potent inhibitor of PFT and GGPP synthase,[6b,21] was shown to decrease the prenylation of both the higher and lower molecular weight proteins (lane 2).

3.3 Conclusions

In summary, the synthesis and biological evaluation of novel Ca_1a_2X analogs, in which the a_1a_2 part is replaced by benzylated SAA dipeptide isosters is described. The stereochemistry of the SAA residue has a pronounced effect on inhibition potency and selectivity. Compound 2,6-cis **21**, having a 2,6-cis configuration in the SAA core and X= methionine, appeared to be the most potent and selective PFT inhibitor in this series. The corresponding diastereoisomer 2,6-trans **21** is a modestly active inhibitor of both enzymes. Compound 2,6-cis **19**, having a 2,6-cis SAA configuration and X= leucine, was also found to be a modest active dual inhibitor.[22] In addition, while 2,6-cis **21** was not active in intact cells, the corresponding methyl ester 2,6-cis **25** was shown to inhibit protein farnesylation in intact Met-18b-2 cells.

3.4 Experimental Section

3.4.1 General – AcOH, CH_3CN, $CHCl_3$, DCE, DCM, DMF, 1,4-dioxane, MeOH, pyridine and toluene were all of p.a. quality (Baker) and stored on molecular sieves (4Å). Methanol (p.a. Baker) was stored on molecular sieves (3Å). PE (40-60 fraction) and EtOAc were of technical grade and distilled before use. Et_3N (99%, Acros) was used as received or distilled, when necessary, over CaH_2 and stored over KOH pellets. DIPEA (peptide grade) and TFA were purchased from Biosolve. HCl·H-Leu-OMe, HCl·H-Met-OMe, Fmoc-Cys-(S*t*Bu)-OH and BOP were obtained from Novabiochem and used as received. Benzaldehyde dimethyl acetal (99%, Acros), BF_3·OEt_2 (Aldrich), CSA (Aldrich), Boc_2O (Fluka), DIBAL-H (1M in cyclohexane or toluene, Aldrich), DOWEX® (50WX4-H$^+$-form, Fluka), *N*-ethylmorpholine (Acros), [bis(acetoxy)iodo]benzene (Acros), KOH (Boom), $K_2S_2O_8$ (Acros), 10% Pd/C (Aldrich), PhSH (Aldrich), $RuCl_3$·$3H_2O$ (Acros), TEMPO (Acros), *p*-TsOH·H_2O (Acros), Et_3SiH (Fluka), iPr_3SiH (Fluka), TMSCN (Fluka) and TrCl (Aldrich) were used as received. RP-HPLC analysis and purification were performed on a Jasco HPLC system equiped with a Merck Lichrosphere C18 100Å column (4×250 mm). ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AC-200 (^{1}H-NMR 200 MHz, ^{13}C-NMR 50 MHz), Bruker DPX-300 (^{1}H-NMR 300 MHz; ^{13}C-NMR 75 MHz), Bruker AV-400 (^{1}H-NMR 400 MHz, ^{13}C-NMR 100 MHz), Bruker DMX-600 (^{1}H-NMR 600 MHz, ^{13}C-NMR 150 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. ^{1}H-NMR: resonance of $CDCl_3$ at 0.00 ppm as internal standard. ^{13}C-NMR: $CDCl_3$ soln.; middle resonance of $CDCl_3$ (77.0 ppm) as internal standard. MS (ESI): Perkin Elmer SCIEX API 165 quadrupole mass spectrometer, HR-MS: API QSTAR™ Pulsar (Applied Biosystems). ATR-IR spectra were recorded on a Shimadzu 8300 FT-IR Spectrometer. Reactions were followed by TLC analysis on silica gel (Schleicher & Schuell, F 1500 LS 254) or HPTLC aluminium sheets (Merck, silica gel 60, F254), with detection by UV-absorption (254 nm) where applicable and charring at 150°C with 20% H_2SO_4 in EtOH (25 g L^{-1}), ninhydrin (3 g L^{-1}) in EtOH/AcOH (100/3 v/v), $NH_4(Mo)_7O_{24}$·$4H_2O$ (25 g L^{-1}) and $NH_4Ce(SO_4)_4$·$2H_2O$ (10 g L^{-1}) in 10% aq. H_2SO_4 or 2% $KMnO_4$ in 1% aq. K_2CO_3. Column chromatography was performed with silica gel (Baker; 0.063-0.200 mm).

3.4.2 General procedures – *General procedure 1a (GP 1a); Fukuyama/Mitsunobu with o-Ns amino acids:* One equiv. of the amino alcohol and 1.1 equiv. of the *o*-Ns protected amino acid were coevaporated with toluene (3×) and then dissolved in freshly distilled THF (~0.01 M). At 0°C, 2.0 equiv. PPh$_3$ and 2.0 equiv. DEAD (dropwise) were added. The mixture was stirred at room temperature until TLC analysis showed completion and subsequently concentrated *in vacuo*.

General procedure 1b (GP 1b); deprotection o-Ns protective group: the crude product obtained from general procedure 1a is dissolved in CH$_3$CN (*c* ~0.1 M) and treated with 4 equiv. of K$_2$CO$_3$ and 3 equiv. of PhSH. The reaction mixture is stirred at 50°C for 30-60 min after which it is washed with water and brine (saturated aq. NaCl soln.) and extracted with EtOAc (3×).

General procedure 2 (GP 2); coupling of HCl·Leu-OMe with SAA Building block: to a ~0.1M soln. of the sugar amino acid in DMF were added 1.2 equiv. HCl·Leu-OMe, 1.2 equiv. BOP, 1.2 equiv. HOBt and 4 equiv. DIPEA. For the synthesis of 2,6-trans **14** and 2,6-cis **14**, 1.2 equiv. EDC and 1.2 equiv. HOBt were employed. After TLC analysis (PE/EtOAc 1/1 v/v) showed consumption of the starting material, DMF was removed *in vacuo*. The residue was dissolved in EtOAc and washed with water (2×), sat. NaHCO$_3$ (2×), water (2×), 5% aq. KHSO$_4$ (2×) and brine. The organic phase was dried (MgSO$_4$) and concentrated *in vacuo*.

General procedure 3 (GP 3); removal of Boc: to a ~0.05M soln. of dimer in DCM were added 1.3 equiv. *i*Pr$_3$SiH or Et$_3$SiH and TFA (→50% TFA in DCM). After TLC analysis (PE/EtOAc 1/1 v/v) showed total consumption of starting material (±½ h), the reaction mixture was coevaporated with anh. toluene (5×).

General procedure 4 (GP 4); coupling with Fmoc-Cys-(StBu)-OH: to a ~0.1M soln. of the deprotected dimer in DMF were added 4 equiv. DIPEA or 4 equiv. *N*-ethylmorpholine, 1.2 equiv. Fmoc-Cys-(S*t*Bu)-OH, 1.2 equiv. BOP and 1.2 equiv. HOBt. After TLC analysis (Et$_2$O/EtOH/25% ammonia 6/3/1 v/v/v) showed consumption of the starting material, the reaction was worked up as described in general procedure 2. For the synthesis of 2,6-trans **16** and 2,6-cis **16**, 1.2 equiv. EDC and 1.2 equiv. HOBt were employed.

General procedure 5 (GP 5); deprotection using Tesser's base: a ~0.01M solution of the trimer in MeOH/1,4-dioxane/4M aq. NaOH (15/4/1 v/v/v) is stirred for 45 min at rt, after which the reaction mixture is diluted with water and washed with DCM (3×). The combined aqueous layers are neutralised with AcOH (pH ~7) and evaporation gives the crude product, which is then subjected to RP-HPLC purification using a linear gradient of CH$_3$CN (= eluent B) in H$_2$O (= eluent A) and 1% aq. TFA (= eluent C, effective 0.1%).

(2R,3S,6S)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-2-hydroxymethyl-tetrahydropyran-3-ol (2,6-trans
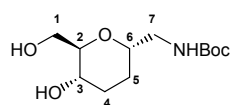

2). Compound 2,6-trans **1** (4.00 g, 16.7 mmol) was dissolved in EtOH/MeOH/CHCl$_3$ (50 mL, 5/5/1 v/v/v) and 10% Pd/C (1.00 g). After hydrogenation for 24 h under 45 *psi* H$_2$-atmosphere, the Pd/C was filtered off and the crude amine (HCl salt) was obtained as a foam after concentration *in vacuo*. To a ~0.1M soln. of the product in DCM were added 1.2 equiv. of Boc$_2$O and 2.2 equiv. of Et$_3$N and the reaction mixture was stirred until TLC analysis showed total consumption of the starting material (PE/EtOAc 1/1 v/v), water was added. The aq. layer was extracted 2× with EtOAc and the combined organic layers were washed with brine, dried (MgSO$_4$) and evaporated. Treatment of the crude diacetate with 0.3 equiv. NaOMe in MeOH (~0.1M) gave after silica gel chromatography (5% MeOH/EtOAc) 2,6-trans **2** (4.16 g, 15.9 mmol, 95%, 3 steps) as an oil. (R$_f$= 0.5, 10% MeOH in EtOAc). [α]$_D^{25}$= +31.2 (*c*= 0.25, CHCl$_3$). ^{1}H-NMR (200 MHz, CDCl$_3$/MeOD): δ 5.04 (bs, NH), 3.89-3.80 (m, 1H, H$_6$), 3.75-3.71 (m, 2H, H$_{1ab}$), 3.54-3.34, 3.21-3.10 (m, 4H, H$_3$, H$_2$, H$_{7ab}$), 1.92-1.81, 1.78-1.68 (m, 4H, H$_{5ab}$, H$_{4ab}$), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, MeOD): δ 158.2 (C=O^{Boc}), 79.8 (C$_q^{tBu}$), 77.5, 71.6, 66.5 (C$_2$, C$_3$, C$_6$), 62.4 (C$_1$), 42.3 (C$_7$), 28.7 (*t*Bu), 28.1, 25.3 (C$_5$, C$_4$). IR *ν* (cm^{-1}, film): 3323, 2936,

1688 (C=O), 1522, 1454, 1393, 1366, 1252, 1170, 1109, 1043. MS (ESI): *m/z* 262.1 (M+H)$^+$, 284.1 (M+Na)$^+$. HR-MS: calc. for $[C_{12}H_{23}NO_5+Na]^+$ 284.1468, found 284.1489.

(2R,3S,6R)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-2-hydroxymethyl-tetrahydropyran-3-ol (2,6-cis 2).
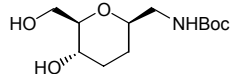
From 2,6-cis **1** (0.79 g, 3.29 mmol) as described for the 2,6-trans isomer. Silica gel chromatography (5% MeOH/EtOAc) gave the title compound (0.60 g, 2.31 mmol, 70% over three steps) as an oil (R_f 0.5, 10% MeOH in EtOAc). $[\alpha]_D^{25}$= −8.8 (*c*= 0.25, CHCl$_3$). ^{1}H-NMR (200 MHz, MeOD): δ 4.86 (s, 1H, NH), 3.85, 3.82 (2×d, 2H, H_{1ab}, *J*= 4.4 Hz), 3.62-3.47 (m, 2H, H_2, H_6), 3.41-3.27 (m, 1H, H_{7a}), 3.27-3.17 (m, 1H, H_3), 3.09-2.96 (m, 1H, H_{7b}), 2.17-2.10 (m, 1H, H_{4a}), 1.73-1.53 (m, 3H, H_{4b}, H_{5ab}), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, MeOD): δ 158.2 (C=O^{Boc}), 83.5 (C_2), 79.8 ($C_q{}^{tBu}$), 77.4 (C_3), 67.1 (C_6), 63.4 (C_1), 45.8 (C_7), 33.0 (C_4), 29.1 (C_5), 28.8 (*t*Bu). IR ν (cm^{-1}, film): 3340, 2936, 1690 (C=O), 1522, 1454, 1393, 1367, 1252, 1171, 1097, 1043. MS (ESI): *m/z* 262.1 (M+H)$^+$, 284.1 (M+Na)$^+$. HR-MS: calc. for $[C_{12}H_{23}NO_5+Na]^+$ 284.1468, found 284.1474.

(2R,3S,6S)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-2-trityloxymethyl-tetrahydropyran-3-ol (2,6-trans 2^{Tr}).
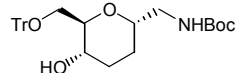
Diol 2,6-trans **2** (2.32 g, 8.8 mmol) was coevaporated with pyridine (3×10 mL) and dissolved in pyridine (60 mL). TrCl (2.69 g, 9.7 mmol) was added and the reaction mixture was stirred at 60°C overnight. The pyridine was removed *in vacuo* and the residue was partitioned between an aq. HCl solution (20 mL, 0.1 M) and EtOAc (20 mL). The aq. layer was extracted with EtOAc (2×) and the combined organic layers were washed with brine, dried (MgSO$_4$) and concentrated *in vacuo*. Silica gel chromatography (Et$_2$O) gave the title compound (4.1 g, 8.2 mmol, 93%) as a foam (R_f 0.3, Et$_2$O). $[\alpha]_D^{25}$= +2.8 (*c*= 0.5, CHCl$_3$). ^{1}H-NMR (200 MHz, CDCl$_3$): δ 7.47-7.20 (m, 15H, H^{Tr}), 4.92 (bs, 1H, NH), 3.74-3.18 (m, 7H, H_1, H_2, H_3, H_6, H_7), 1.71-1.58 (m, 4H, H_4, H_5), 1.42 (s, 9H, *t*Bu); ^{13}C-NMR: δ 155.3 (C=O^{Boc}), 143.1 ($C_q{}^{Tr}$), 127.8-126.0 (C^{Tr}), 85.8 ($C_q{}^{Tr}$), 77.6 ($C_q{}^{tBu}$), 75.1, 69.3, 64.9 (C_2, C_3, C_6), 62.4 (C_1), 42.0 (C_7), 27.4 (*t*Bu), 26.1 (C_4), 22.7 (C_5). IR ν (cm^{-1}, film): 3416, 2935, 1695, 1502, 1448, 1364, 1250, 1171, 1053, 760, 706. MS (ESI): *m/z* 526.5 (M+Na)$^+$. HR-MS: calc. for $[C_{31}H_{37}NO_5+Na]^+$ 526.2563, found 526.2550.

(2R,3S,6R)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-2-trityloxymethyl-tetrahydropyran-3-ol (2,6-cis 2^{Tr}).
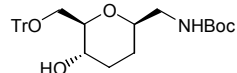
From 2,6-cis **2** (1.00 g, 3.83 mmol) as described for 2,6-trans **2^{Tr}** Silica gel chromatography (Et$_2$O) afforded the title compound (1.73 g, 3.44 mmol, 90%) as a foam (R_f 0.6, Et$_2$O). $[\alpha]_D^{25}$= −37.2 (*c*= 0.5, CHCl$_3$). ^{1}H-NMR (200 MHz, CDCl$_3$): δ 7.48-7.18 (m, 15H, H^{Tr}), 4.82 (bs, 1H, NH), 3.50 (m, 2H, H_{1ab}), 3.34 (m, 3H, H_6, H_2, H_{7a}), 3.21 (m, 1H, H_3), 2.95 (ddd, 1H, H_{7b}), 2.09 (m, 1H, H_{4a}), 1.67-1.31 (m, 3H, H_{5ab}, H_{4b}), 1.44 (s, 9H, *t*Bu); ^{13}C-NMR: δ 155.5 (C=O^{Boc}), 143.4 ($C_q{}^{Tr}$), 128.5-126.6 (C^{Tr}), 86.5 ($C_q{}^{Tr}$), 78.4 ($C_q{}^{tBu}$), 79.7, 75.4, 67.7 (C_2, C_3, C_6), 65.0 (C_1), 44.4 (C_7), 31.3 (C_4), 27.9 (*t*Bu), 27.3 (C_5). IR ν (cm^{-1}, film): 3427, 2930, 1695, 1493, 1448, 1365, 1169, 1099, 760, 706. MS (ESI): *m/z* 526.5 (M+Na)$^+$. HR-MS: calc. for $[C_{31}H_{37}NO_5+Na]^+$ 526.2563, found 526.2552.

(2R,3S,6S)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-3-benzyloxy-2-trityloxymethyl-tetrahydropyran (2,6-trans 2$^{Tr}{}_{Bn}$).
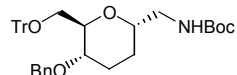
Compound 2,6-trans **2^{Tr}** (2.1 g, 4.3 mmol) was dissolved in DCM (50 mL), Bu$_4$NHSO$_4$ (4.3 g, 12.7 mmol) and benzyl bromide (1.3 mL, 10.6 mmol) were added. A 50% aq. solution of NaOH (50 mL) was added and the reaction mixture was stirred vigorously until TLC analysis showed completion (24–48 h). Ice-water was added and the layers were separated. The aqueous layer was extracted with EtOAc (2×). The combined organics were subsequently washed with water (2×) and brine (2×), dried (MgSO$_4$) and concentrated *in vacuo*. Silica gel chromatography (PE/Et$_2$O 1/1 v/v) yielded the title compound (2.3 g, 3.9 mmol, 91%) as an oil (R_f 0.8, Et$_2$O). $[\alpha]_D^{25}$= +22.2 (*c*= 1, CHCl$_3$). ^{1}H-NMR (200 MHz, CDCl$_3$): δ 7.46-7.21 (m, 20H, H$^{Tr\&Bn}$), 4.97 (bs, 1H, NHBoc), 4.59, 4.46 (2×d, 2H, CH$_2{}^{Bn}$, *J*= 11.7 Hz), 3.61-3.08 (m, 7H, H_1, H_2, H_3, H_6, H_7), 1.73-1.52 (m, 4H, H_5 and H_4), 1.42 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 156.0 (C=O^{Boc}), 143.9 ($C_q{}^{Tr}$), 138.4 ($C_q{}^{Bn}$), 129.5-

126.9 ($C^{Tr\&Bn}$), 86.5 (C_q^{Tr}), 78.8 (C_q^{tBu}), 74.1 (C_6), 71.8 (C_3), 70.3 (C_2), 70.2 (CH_2^{Bn}), 62.6 (C_1), 43.2 (C_7), 29.2 (*t*Bu), 27.9 (C_4), 23.6 (C_5). IR ν (cm^{-1}, film): 2934, 1713, 1493, 1448, 1366, 1169, 1101, 748, 704. MS (ESI): *m/z* 616.3 (M+Na)$^+$. HR-MS: calc. for [$C_{38}H_{43}NO_5$+Na]$^+$ 616.3033, found 616.3017.

(2R,3S,6R)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-3-benzyloxy-2-trityloxymethyl-tetrahydropyran
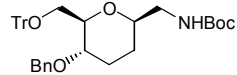
(2,6-cis 2$^{Tr}_{Bn}$). From 2,6-cis 2^{Tr} (2.69 g, 5.34 mmol) as described for 2,6-trans 2$^{Tr}_{Bn}$ Silica gel chromatography (PE/Et$_2$O 1/1 v/v) afforded the title compound (2.57 g, 4.33 mmol, 81%) as a foam (R$_f$ 0.9, Et$_2$O). [α]$_D^{25}$= +1.6 (*c*= 0.25, CHCl$_3$). ^{1}H-NMR (200 MHz, CDCl$_3$): δ 7.48-7.01 (m, 20H$^{Tr\&Bn}$), 5.01 (bs, 1H, NHBoc), 4.50, 4.30 (2×d, 2H, CH$_2^{Bn}$, *J*= 11.4 Hz), 3.56-3.03 (m, 7H, H$_1$, H$_2$, H$_3$, H$_6$, H$_7$), 2.24 (m, 1H, H$_{4a}$), 1.75-1.46 (m, 3H, H$_{4b}$, H$_{5ab}$), 1.46 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 156.0 (C=O^{Boc}), 143.3 (C$_q^{Tr}$), 138.0 (C$_q^{Bn}$), 128.5-127.2 (C$^{Tr\&Bn}$), 87.3 (C$_q^{Tr}$), 79.0 (C$_q^{tBu}$), 78.9, 76.3, 69.5 (C$_2$, C$_3$, C$_6$), 66.1 (CH$_2^{Bn}$), 60.6 (C$_1$), 44.7 (C$_7$), 28.3 (*t*Bu), 27.5, 31.1 (C$_4$, C$_5$). IR ν (cm^{-1}, film): 2930, 1716, 1493, 1448, 1366, 1171, 1099, 1028, 748, 698. MS (ESI): *m/z* 616.4 (M+Na)$^+$. HR-MS: calc. for [$C_{38}H_{43}NO_5$+Na]$^+$ 616.3033, found 616.3033.

### (2R,3S,6S)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-2-phenyl-hexahydro-pyrano[3,2-d][1,3]-dioxine
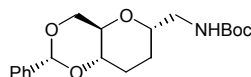
(2,6-trans 17). To a solution of 2,6-trans 2 (4.16 g, 15.9 mmol) in CH$_3$CN (75 mL) was added benzaldehyde dimethyl acetal (2.9 mL, 2.91 mmol) and CSA (0.74 g, 3.12 mmol). After stirring for 2 h at 65°C, TLC analysis (EtOAc) showed total consumption of starting material and the mixture was neutralised with Et$_3$N (3.12 mmol) and concentrated *in vacuo*. After extraction with EtOAc and washing with 10% aq. NaHCO$_3$ and brine, the organic layer was dried over MgSO$_4$. Silica gel chromatography (PE/EtOAc 4/1 v/v) yielded the title compound (3.95 g, 11.3 mmol, 71%) as a white crystalline compound (R$_f$ 0.7, EtOAc). [α]$_D^{25}$= +50.4 (*c*= 1, CHCl$_3$). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.49-7.33 (m, 5H, H^{Ph}), 5.55 (s, 1H, PhCH), 4.79 (s, 1H, NH), 4.19 (dd, 1H, H$_{1a}$, *J*= 5.9 and 10.2 Hz), 3.93 (t, 1H, H$_{1b}$), 3.70-3.55 (m, 4H, H$_6$, H$_3$, H$_2$, H$_{7a}$), 3.21 (m, 1H, H$_{7b}$), 2.00, 1.75 (2×m, 4H, H$_{4ab}$, H$_{5ab}$), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (75 MHz, CDCl$_3$): δ 155.6, (C=O^{Boc}), 137.4 (C$_q^{Ph}$), 128.70, 127.8, 125.91 (C^{Ph}), 101.4 (PhCH), 78.8 (C$_q^{Boc}$), 78.3 (C$_6$), 71.6 (C$_3$), 69.3 (C$_2$), 66.0 (C$_1$), 39.4 (C$_7$), 28.1 (*t*Bu), 25.3, 24.3 (C$_5$, C$_4$). IR ν (cm^{-1}, film): 3352, 2935, 1697, 1510, 1456, 1366, 1238, 1169, 1099, 1001, 750, 698. MS (ESI): *m/z* 350.2 (M+H)$^+$, 372.1 (M+Na)$^+$. HR-MS: calc. for [$C_{19}H_{27}NO_5$+Na]$^+$ 372.1781, found 372.1833.

(2R,3S,6R)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-2-phenyl-hexahydro-pyrano[3,2-d][1,3]-dioxine
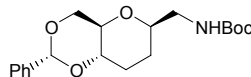
(2,6-cis 17). From 2,6-cis 2 (706 mg, 2.70 mmol) as described for 2,6-trans 17. Silica gel chromatography (PE/EtOAc 4/1 v/v) gave 2,6-cis 17 (726 mg, 2.08 mmol, 77%), white crystals (R$_f$ 0.9, EtOAc). [α]$_D^{25}$= −24.0 (*c*= 0.25, CHCl$_3$). ^{1}H-NMR (200 MHz, CDCl$_3$): δ 7.52-7.34 (m, 5H, H^{Ph}), 5.55 (s, 1H, PhCH), 4.88 (s, 1H, NH), 4.27 (dd, 1H, H$_{1a}$, *J*= 5.9 and 10.2 Hz), 3.69 (m, 1H, H$_{1b}$), 3.59-3.47 (m, 2H, H$_2$, H$_6$), 3.46-3.34 (m, 2H, H$_3$, H$_{7a}$), 3.08-2.95 (m, 1H, H$_{7b}$, *J*= 5.1 and 4.4 Hz), 2.15-2.05 (m, 1H, H$_{5a}$), 1.84-1.53 (m, 3H, H$_{5b}$, H$_{4ab}$), 1.46 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 155.7, (C=O^{Boc}), 137.4 (C$_q^{Ph}$), 128.7, 128.0, 125.9 (C^{Ph}), 101.4 (PhCH), 79.0 (C$_q^{Boc}$), 77.9 (C$_2$), 76.4 (C$_3$), 73.0 (C$_6$), 69.0 (C$_1$), 44.6 (C$_7$), 28.2 (*t*Bu), 27.9, 27.7 (C$_5$, C$_4$). IR ν (cm^{-1}, film): 3375, 2933, 1715, 1510, 1456, 1393, 1366, 1246, 1171, 1099. MS (ESI): 350.2 (M+H)$^+$, 372.2 (M+Na)$^+$. HR-MS: calc. for [$C_{19}H_{27}NO_5$+Na]$^+$ 372.1781, found 372.1801.

(2R,3S,6S)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-3-benzyloxy-tetrahydropyran-2-ol (2,6-trans 3).
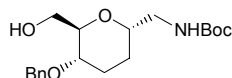
Method 1: A solution of 2,6-trans 2$^{Tr}_{Bn}$ (2.3 g, 3.9 mmol) and 0.3 equiv. of *p*-TsOH·H$_2$O (0.22 g) in MeOH (50 mL) and DCM (50 mL) was stirred at rt until TLC analysis (PE/EtOAc 1/3 v/v) showed completion. The reaction was quenched with a half-saturated NaHCO$_3$ solution and extracted with EtOAc. The combined organics were washed with brine, dried (MgSO$_4$) and concentrated to dryness. Silica gel chromatography (R$_f$ 0.7, EtOAc) yielded the title compound (1.21 g, 89%) as an oil which crystallised on standing. **Method 2**: A solution of 2,6-trans 17

(283 mg, 0.81 mmol) in toluene (12 mL) under a blanket of argon was cooled to 0°C. Subsequently, 8.1 mL of a 1M soln. of DIBAL-H in toluene (8.1 mmol, 10 equiv.) was added and after ½h the reaction mixture was allowed to warm up to rt. When TLC analysis showed total consumption of starting material (12–24 h), the temperature was lowered to 0°C and the reaction was quenched carefully with MeOH (5 mL). After ½h the temperature was again brought to 0°C and 10% aq. NaOH was added. The water layer was extracted with EtOAc (3×) and the combined organic layers were washed with brine and dried with MgSO$_4$. Silica gel chromatography yielded the title compound (198 mg, 70%) and the 1-OBn regio-isomer (39 mg, 14%, R_f= 0.6, EtOAc). [α]$_D^{25}$= +68.4 (*c*= 1, CHCl$_3$). ^{1}H-NMR (200 MHz, CDCl$_3$) δ 7.31-7.28 (m, 5H^{Bn}), 5.10 (bs, 1H, NHBoc), 4.59 and 4.46 (2×d, CH$_2^{Bn}$, *J*= 11.7 Hz), 3.85-3.20 (m, 7H, H$_6$, H$_3$, H$_2$, H$_1$, H$_7$), 1.93-1.67 (m, 4H, H$_{5ab}$ and H$_{4ab}$), 1.42 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$) δ 156.1 (C=O^{Boc}), 138.2 (C$_q^{Bn}$), 128.2, 127.5, 127.2 (C^{Bn}), 79.1 (C$_q^{Boc}$), 74.3 (C$_2$), 72.5 (C$_6$), 70.7 (C$_3$), 70.3 (CH$_2^{Bn}$), 61.6 (C$_1$), 41.6 (C$_7$), 28.2 (*t*Bu), 24.2, 23.9 (C$_4$, C$_5$). IR v (cm^{-1}, film): 3340, 2976, 1693, 1520, 1454, 1366, 1250, 1169, 1092. 750, 698. MS (ESI): *m/z* 352.1 (M+H)$^+$, 374.1 (M+Na)$^+$. HR-MS: calc. for [C$_{19}$H$_{29}$NO$_5$+Na]$^+$ 374.1943, found 372.1935.

(2R,3S,6R)-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-3-benzyloxy-tetrahydropyran-2-ol (2,6-cis 3).
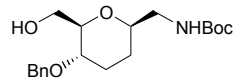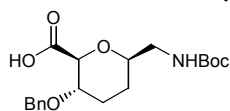
Following method 1 as described for the synthesis of 2,6-trans **3** using 2,6-cis **2**$^{Tr}_{Bn}$ (800 mg, 1.35 mmol) gave after silica gel chromatography (PE/EtOAc 1/1 v/v) the title compound (479 mg, 1.35 mmol, 100%) as an oil. Employing method 2 using 2,6-cis **17** (365 mg, 4.93 mmol) gave an unseparable mixture of the title compound (R$_f$= 0.7, EtOAc) and the 1-OBn regio-isomer (R$_f$= 0.4, EtOAc/PE 2/1 v/v) in a ~7/1 ratio as determined by ^{13}C-NMR (det. limit <5%), total yield: 292 mg, 0.83 mmol, 80%. [α]$_D^{25}$= +60.8 (*c*=0.5, CHCl$_3$). ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.36-7.24 (m, 5H, H^{Bn}), 5.34 (s, 1H, NH), 4.62, 4.46 (2×d, 2H, CH$_2^{Bn}$, *J*= 11.6 Hz), 3.88 and 3.68 (2×dd, 2H, H$_{1ab}$, *J*= 4.4 and 11.5 Hz), 3.50-3.41 (m, 1H, H$_6$, *J*= 3.4 and 2.6 Hz), 3.37-3.30 (m, 3H, H$_3$, H$_2$, H$_{7a}$), 3.06-2.97 (m, 1H, H$_{7b}$, *J*= 4.8 Hz), 2.29-2.24 (m, 1H, H$_{5a}$), 1.71-1.50 (m, 1H, H$_{4a}$), 1.43 (s, 9H, *t*Bu), 1.39-1.30 (m, 2H, H$_{5b}$, H$_{4b}$); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 156.0 (C=O^{Boc}), 138.1 (C$_q^{Bn}$), 128.3, 127.6, 127.2 (C^{Bn}), 80.4 (C$_2$), 79.1 (C$_q^{Boc}$), 76.5 (C$_6$), 73.4 (C$_3$), 70.7 (CH$_2^{Bn}$), 62.7 (C$_1$), 44.7 (C$_7$), 28.3 (*t*Bu), 28.3, 27.5 (C$_5$, C$_4$). IR v (cm^{-1}, film): 3340, 2976, 1693, 1520, 1454, 1366, 1250, 1169, 1092, 750, 698. MS (ESI): *m/z* 352.1 (M+H)$^+$, 374.1 (M+Na)$^+$. HR-MS: calc. for [C$_{19}$H$_{29}$NO$_5$+Na]$^+$ 374.1943, found 372.1938.

(2S,3S,6S)-3-*O*-Benzyl-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-tetrahydropyran-1-carboxylic acid
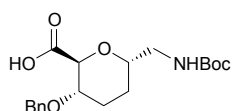
(**2,6-trans 12**). Method 1: To an aq. soln. of KOH (1M, 14.2 mL) and K$_2$S$_2$O$_8$ (1.69 g, 6.25 mmol) was added 2,6-trans **3** (400 mg, 1.42 mmol) and RuCl$_3$·3H$_2$O (166 mg). The solution immediately turned black and after TLC analysis (EtOAc) showed completion, the brown mixture was quenched with MeOH (10 mL). Salts were filtered off and excess K$_2$S$_2$O$_8$ was destroyed with NaSO$_3$·H$_2$O. After neutralization with AcOH (pH ≈7), the crude product was extracted with EtOAc, dried (MgSO$_4$) and concentrated *in vacuo*. Silica gel chromatography (R$_f$= 0.7, 1% AcOH in EtOAc) gave 2,6-trans **12** (345 mg, 0.94 mmol, 66%) as an oil which solidified on standing. Method 2: to a solution of 2,6-trans **3** (134 mg, 381 µmol) in DCM (2 mL) was added water (2 mL), 2.2 equiv. [bis(acetoxy)iodo]benzene (839 µmol, 270 mg) and a cat. amount of TEMPO (10 mg). After stirring for 12–16 h, Na$_2$S$_2$O$_3$ (0.5 g) was added and the reaction was washed with 1N HCl and brine and extracted with EtOAc, dried (MgSO$_4$) and concentrated *in vacuo*. Yield 2,6-trans **12**: 118 mg, 85%. [α]$_D^{25}$= +21.6 (*c*= 0.25, CHCl$_3$). ^{1}H-NMR (300 MHz, MeOD): δ 7.35 (m, 5H, H^{Bn}), 5.73 (s, 1H, NH), 4.57 (m, 3H, CH$_2^{Bn}$, H$_2$), 3.97 (m, 2H, H$_3$, H$_6$), 3.13 (m, 2H, H$_7$), 2.05 (m, 2H, H$_5$), 1.70 (m, 2H, H$_4$), 1.42 (s, 9H, *t*Bu); ^{13}C-NMR (75 MHz, aceton-*d6*): δ 173.5 (C=O^{carboxyl}), 158.2 (C=O^{Boc}), 139.5 (C$_q^{Bn}$), 129.3, 128.7, 128.6 (C^{Bn}), 81.1 (C$_3$), 79.9 (C$_q^{Boc}$), 76.2, 73.1 (C$_2$, C$_6$), 71.5 (CH$_2^{Bn}$), 45.9 (C$_7$), 28.7 (*t*Bu), 25.1 (C$_4$), 23.4 (C$_5$). IR v (cm^{-1}, film): 3363, 2926, 1773, 1709, 1522, 1452, 1367, 1271, 1250, 1165. MS (ESI): *m/z* 366.0 (M+H)$^+$, 388.3 (M+Na)$^+$. HR-MS: calc. for [C$_{19}$H$_{27}$NO$_6$+Na]$^+$ 388.1736, found 388.1784.

(2S,3S,6R)-3-*O*-Benzyl-6-{*N*-[(tert-butyloxycarbonyl)amino]methyl}-tetrahydropyran-1-carboxylic acid (2,6-cis 12).

From 2,6-cis **3** (339 mg, 0.96 mmol) as described for 2,6-trans **12** employing method 1. Silica gel chromatography (R_f 0.8, 1% AcOH/EtOAc) gave 2,6-cis **12** (351 mg, 0.96 mmol, 100%) as an oil. Following method 2 as described for 2,6-trans **12**, employing TEMPO and [bis(acetoxy)iodo]benzene, furnished 2,6-cis **12** in 100% yield. $[\alpha]_D^{25}$= +8.0 (*c*= 0.5, CHCl$_3$). ^{1}H-NMR (200 MHz, MeOD): δ 7.25-7.05 (m, 5H, H^{Bn}), 5.12 (s, 1H, NH), 4.54, 4.44 (2×d, 2H, CH$_2^{Bn}$, *J*= 12.3 Hz), 4.05 (d, 1H, H$_2$, *J*= 9.5 Hz), 3.58-3.29 (m, 2H, H$_3$, H$_{7a}$, *J*= 10.2 Hz), 3.16-3.08 (m, 2H, H$_6$, H$_{7b}$), 2.25-2.04 (m, 1H, H$_{4a}$), 1.70-1.55 (m, 1H, H$_{5a}$), 1.37-1.25 (m, 2H, H$_{5b}$, H$_{4b}$), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (75 MHz, MeOD): δ 173.9 (C=O^{carboxyl}), 158.3 (C=O^{Boc}), 139.4 (C$_q^{Bn}$), 129.2, 128.7, 125.2 (C^{Bn}), 81.1 (C$_3$), 80.0 (C$_q^{tBu}$), 77.7 (C$_2$), 76.1 (C$_6$), 72.1 (CH$_2^{Bn}$), 45.2 (C$_7$), 28.7 (*t*Bu), 29.7, 28.0 (C$_4$, C$_5$). MS (ESI): *m/z* 366.0 (M+H)$^+$, 388.3 (M+Na)$^+$.

(2R,3S,6S)-3-*O*-Benzyl-6-{*N*-[(*t*-butyloxycarbonyl)amino]methyl}tetrahydropyran-1-*N*-L-leucine methyl ester (2,6-trans 6).
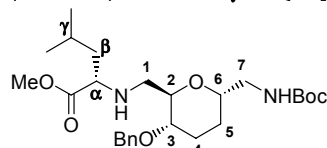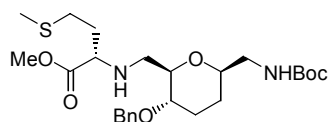
Following *GP 1a* and *GP 1b* employing 2,6-trans **3** (44 mg, 0.13 mmol) and *o*-Ns-Leu-OMe (**4**, 56 mg, 0.17 mmol) gave after silica gel chromatography (PE/EtOAc 1/1 v/v) the title compound (39 mg, 0.08 mmol, 65%) as an oil. ^{1}H-NMR (600 MHz, CDCl$_3$): δ 7.34-7.10 (m, 6H, H^{Bn} and NHC$_\alpha$), 5.00 (bs, 1H, NHBoc), 4.60, 4.49 (2×d, 2H, CH$_2^{Bn}$, *J*= 11.7 and 12.4 Hz), 4.22 (m, 1H, H$_\alpha$), 3.71 (s, 3H, OCH$_3$), 3.38-3.23 (m, 5H, H$_2$, H$_3$, H$_6$, H$_7$), 2.70 (m, 2H, H$_{1ab}$), 1.88 -1.22 (m, 7H, H$_{4ab}$, H$_{5ab}$, H$_{\beta\gamma}$), 1.38 (s, 9H, *t*Bu), 0.89 (m, 6H, 2×CH$_3^{Leu}$); ^{13}C-NMR (150 MHz, CDCl$_3$): δ 172.6 (C=O^{ester}), 155.6 (C=O^{Boc}), 138.0 (C$_q^{Bn}$), 128.2, 127.5 (C^{Bn}), 79.0 (C$_q^{tBu}$), 73.6, 73.4, 70.1 (C$_2$, C$_3$, C$_6$), 70.3 (CH$_2^{Bn}$), 59.9 (C$_\alpha$), 51.5 (OCH$_3$), 47.8 (C$_1$), 42.4 (C$_7$), 42.2 (C$_\beta$), 28.7 (*t*Bu), 24.8 (C$_\gamma$), 24.2, 23.8 (C$_4$ and C$_5$), 22.6 (CH$_3^{Leu}$), 22.1 (CH$_3^{Leu}$). MS (ESI): *m/z* 497.2 (M+H)$^+$, 510.4 (M+Na)$^+$.

(2R,3S,6R)-3-*O*-Benzyl-6-{*N*-[(*t*-butyloxycarbonyl)amino]methyl} tetrahydropyran-1-*N*-L-leucine methyl ester (2,6-cis 6).
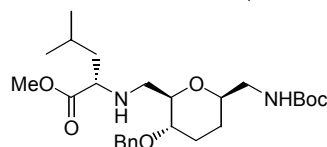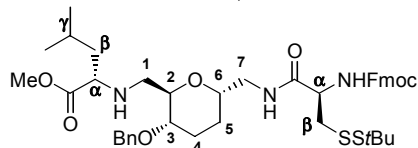
Following *GP 1a* employing 2,6-cis **3** (347 mg, 1.0 mmol) and *o*-Ns-Leu-OMe (**4**, 367 mg, 1.1 mmol) and following *GP 1b*, gave after silica gel chromatography (PE/EtOAc 1/1 v/v) the title compound (439 mg, 0.9 mmol, 92%) as an oil. ^{1}H-NMR (200 MHz, CDCl$_3$): δ 7.32-7.26 (m, 5H^{Bn}), 7.05 (d, 1H, NHC$_\alpha$, *J*= 8.2 Hz), 5.09 (bs, 1H, NHBoc), 4.54 (m, 3H, H$_\alpha$ and CH$_2^{Bn}$), 4.50 (d, 1H, H$_2$, *J*= 3.9 Hz), 4.11 (bs, 1H, H$_3$), 3.67 (s, 3H, OCH$_3$), 3.56 (m, 1H, H$_6$), 3.30 and 3.06 (2×m, 2H, H$_7$), 1.71 and 1.63-1.49 (m, 7H, H$_{5a}$, H$_{4ab}$, H$_{\beta\gamma}^{Leu}$), 1.38 (s, 9H, *t*Bu), 1.34 (m, 1H, H$_{5b}$), 0.96 (2×d, 6H, 2×CH$_3^{Leu}$, *J*= 5.9 and 6.0 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.6 (C=O^{ester}), 155.9 (C=O^{Boc}), 138.2 (C$_q^{Bn}$), 128.2, 127.5 (C^{Bn}), 78.9 (C$_q^{tBu}$), 80.1, 76.4, 74.5 (C$_2$, C$_3$, C$_6$), 70.7 (CH$_2^{Bn}$), 59.9 (OCH$_3$), 51.4 (C$_\alpha$), 47.9 (C$_1$), 44.7 (C$_7$), 42.7 (C$_\beta$), 28.5 (C$_4$), 28.2 (*t*Bu), 27.6 (C$_5$), 24.7 (C$_\gamma$), 22.6, 22.1 (2×CH$_3^{Leu}$).

(2R,3S,6S)-3-*O*-Benzyl-6-{*N*-[(*t*-butyloxycarbonyl)amino]methyl}tetrahydropyran-1-*N*-L-methionine methyl ester (2,6-trans 7).
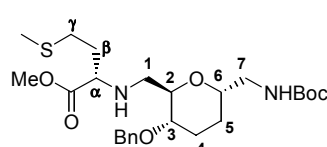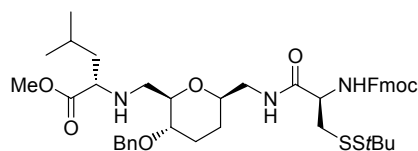
Following *GP 1a* and *GP 1b* using 2,6-trans **3** (171 mg, 0.49 mmol) and *o*-Ns-Met-OMe (**5**, 184 mg, 0.53 mmol) yielded after silica gel chromatography (PE/EtOAc 1/1 v/v) the title compound (196 mg, 0.40 mmol, 81%) as an oil. ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.36-7.24 (m, 6H, H^{Bn} and NHC$_\alpha$), 5.05 (bs, 1H, NHBoc), 4.63 and 4.49 (2×d, 2H, CH$_2^{Bn}$, *J*= 11.5 Hz), 3.73 (m, 5H, OCH$_3$, H$_3$, H$_2$), 3.40 (m, 2H, H$_6$ and H$_\alpha$), 3.25 (m, 2H, H$_7$), 2.75 (m, 2H, H$_{1ab}$), 2.59 (m, 1H, H$_\gamma$), 2.10 (s, 3H, SCH$_3$), 2.00-1.55 (m, 6H, H$_4$, H$_5$ and H$_\beta$), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 175.5 (C=O^{ester}), 156.0 (C=O^{Boc}), 138.3 (C$_q^{Bn}$), 128.3, 127.6 (C^{Bn}), 79.2 (C$_q^{tBu}$), 73.5, 73.3, 70.3 (C$_2$, C$_3$, C$_6$), 70.5 (CH$_2^{Bn}$), 60.1 (C$_\alpha$), 51.9 (OCH$_3$), 47.8 (C$_1$), 42.0 (C$_7$), 32.3 (C$_\beta$), 30.4 (C$_\gamma$), 28.3 (*t*Bu), 24.3, 23.9 (C$_4$ and C$_5$), 15.3 (SCH$_3$). MS (ESI): *m/z* 497.4 (M+H)$^+$, 519.4 (M+Na)$^+$.

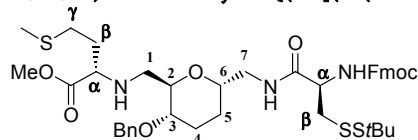**(2R,3S,6R)-3-*O*-Benzyl-6-{*N*-[(*t*-butyloxycarbonyl)amino]methyl}tetrahydropyran-1-*N*-L-methionine   methyl ester (2,6-cis 7).** Following *GP 1a* and *GP 1b* using 2,6-cis **3** (354 mg, 1.0 mmol) and *o*-Ns-Met-OMe (**5**, 383 mg, 1.1 mmol) gave after silica gel chromatography (R$_f$ 0.2, PE/EtOAc 2/1 v/v) 2,6-cis **7** (494 mg, 1.0 mmol, 99%) as an oil. ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.37-7.25 (m, 6H, 5H^{Bn} and NHC$_\alpha$), 5.00 (bs, 1H, NHBoc), 4.61, 4.47 (2×d, 2H, CH$_2$^{Bn}, *J*= 11.5 Hz), 3.73 (s, 3H, OCH$_3$), 3.71-3.27 (m, 5H, H$_{7a}$, H$_6$, H$_3$, H$_2$ and H$_\alpha$), 3.02-2.88 (m, 2H, H$_{7b}$, H$_{1a}$), 2.67 (2×d, 1H, H$_{1b}$, *J*= 5.0 and 5.1 Hz), 2.59 (t, 1H, H$_\gamma$, *J*= 7.3 and 7.4 Hz), 2.20 (m, 1H, H$_{5a}$), 2.07 (s, 3H, SCH$_3$), 2.00-1.66 (m, 5H, H$_{5b}$, H$_{4ab}$, H$_\beta$), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (75 MHz, CDCl$_3$): δ 175.6 (C=O^{ester}), 156.0 (C=O^{Boc}), 138.3 (C$_q$^{Bn}), 128.3, 127.7 (C^{Bn}), 80.0, 76.6, 74.5 (C$_2$, C$_3$ and C$_6$), 79.2 (C$_q$^{tBu}), 70.9 (CH$_2$^{Bn}), 60.3 (C$_\alpha$), 51.8 (OCH$_3$), 49.0 (C$_1$), 44.8 (C$_7$), 32.7 (C$_\beta$), 30.5 (C$_\gamma$), 28.6 (C$_4$), 28.4 (*t*Bu), 27.8 (C$_5$), 15.3 (SCH$_3$). MS (ESI): *m/z* 497.4 (M+H)$^+$, 519.4 (M+Na)$^+$.

(2R,3S,6S)-3-*O*-Benzyl-6-{(*N*-[(*S*-(tert-butyl)sulfanyl]-*N*-{[9H-fluoren-9-yl]methoxy]carbonyl}-L-cysteinyl) amino]methyl}-tetrahydropyran-1-*N*-L-leucine methyl ester (2,6-trans 8). From 2,6-trans **6** (39 mg, 0.08 mmol) and *N*-ethylmorpholine (42 µL, 0.33 mmol) following *GP 3* and *GP4*. Silica gel chromatography (PE/EtOAc 1/1 v/v) gave 2,6-trans **8** (26 mg, 0.03 mmol, 40%) as an oil. ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.75 (d, 2H, H^{Fmoc}, *J*= 7.5 Hz), 7.60 (d, 2H, H^{Fmoc}, *J*= 7.3 Hz), 7.42-7.25 (m, 9H, H$^{Bn\&Fmoc}$), 6.93 (bs, 1H, C$_7$NH), 5.95 (d, 1H, C$_\alpha$^{Cys}, NH^{Fmoc}, *J*= 7.4 Hz), 4.61-4.41 (m, 4H, H$_\alpha$^{Met}, CH$_2$^{Bn} and H^{Fmoc}), 4.33 (t, 1H, H^{Fmoc}, *J*= 7.2 and 10.4 Hz), 4.23 (t, 1H, H^{Fmoc}, *J*= 7.1 Hz), 3.80-3.68 (m, 5H, H$_2$, H$_6$ and OCH$_3$), 3.55 (m, 1H, H$_{7a}$), 3.25 (m, 2H, H$_\alpha$^{Met}, H$_{7b}$, H$_3$), 3.13 (m, 2H, H$_\beta$^{Cys}), 2.75 (m, 2H, H$_{1ab}$), 1.90-1.54 (m, 5H, H$_{4ab}$, H$_{5ab}$, H$_\gamma$^{Leu}), 1.33 (s, 9H, *t*Bu), 0.89 (t, 6H, 2×CH$_3$^{Leu}, *J*= 6.2 and 6.3 Hz); ^{13}C-NMR (75 MHz, CDCl$_3$): δ 176.5-169.9 (C=O$^{ester\&amide}$), 155.8 (C=O^{Fmoc}), 143.8, 141.2 (C$_q$^{Fmoc}), 138.3 (C$_q$^{Bn}), 128.4, 127.6, 127.1, 125.2, 119.9 (CH$^{Bn\&Fmoc}$), 73.4, 69.5 (C$_2$, C$_3$, C$_6$), 70.5 (CH$_2$^{Bn}), 67.1 (CH$_2$^{Fmoc}), 59.5 (C$_\alpha$^{Leu}), 54.7 (C$_\alpha$^{Cys}), 51.9 (OCH$_3$), 48.3 (C$_q$^{tBu}), 47.8 (C$_1$), 47.1 (CH^{Fmoc}), 43.0 (C$_\beta$^{Cys}), 41.9 (C$_\beta$^{Leu}), 41.4 (C$_7$), 29.8 (*t*Bu), 24.8 (C$_\gamma$^{Leu}), 24.3 (C$_4$), 23.8 (C$_5$), 22.7, 22.3 (2×CH$_3$^{Leu}). MS (ESI): *m/z* 792.6 (M+H)$^+$, 814.4 (M+Na)$^+$.

**(2S,3S,6R)-3-*O*-Benzyl-6-{(*N*-[(*S*-(tert-butyl)sulfanyl]-*N*-{[9H-fluoren-9-yl]methoxy]carbonyl}-L-cysteinyl) amino]methyl}-tetrahydropyran-1-*N*-L-leucine methyl ester (2,6-cis 8).** From 2,6-cis **6** (40 mg, 0.08 mmol) and *N*-ethylmorpholine (42 µL, 0.33 mmol) according to *GP 3* and *GP4*. Silica gel chromatography (PE/EtOAc 1/1 v/v) gave 2,6-cis **8** (44 mg, 0.05 mmol, 66%) as a foam. ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.75 (d, 2H, H^{Fmoc}, *J*= 7.5 Hz), 7.60 (d, 2H, CH^{Fmoc}, *J*= 7.5 Hz), 7.42-7.25 (m, 9H, H$^{Bn\&Fmoc}$), 6.93 (bs, 1H, C$_7$NH), 5.85 (d, 1H, NHFmoc, *J*= 7.8 Hz), 4.61-4.41 (m, 4H, H$_\alpha$^{Met}, CH$_2$^{Bn} and H^{Fmoc}), 4.34 (t, 1H, H^{Fmoc}, *J*= 7.1 and 10.2 Hz), 4.23 (t, 1H, H^{Fmoc}, *J*= 7.0 and 7.1 Hz), 3.68 (s, 3H, OCH$_3$), 3.55 (m, 1H, H$_{7a}$), 3.41 (m, 1H, H$_2$), 3.25 (m, 3H, H$_\alpha$^{Leu}, H$_6$ and H$_3$), 3.04-3.15 (m, 3H, H$_\beta$^{Cys} and H$_{7b}$), 2.95 (dd, 1H, H$_{1a}$, *J*= 2.4 and 2.7 Hz), 2.95 (dd, 1H, H$_{1b}$, *J*= 5.5 and 5.6 Hz), 2.22 (m, 1H, H$_{4a}$), 1.78-1.68 and 1.54-1.21 (2×m, 6H, H$_{5ab}$, H$_{4b}$, H$_{\beta\gamma}$^{Leu}), 1.33 (s, 9H, *t*Bu), 0.89 (dd, 6H, 2×CH$_3$^{Leu}, *J*= 6.7 and 6.8 Hz); ^{13}C-NMR (75 MHz, CDCl$_3$): δ 176.9 and 169.9 (C=O$^{ester\&amide}$), 155.8 (C=O^{Fmoc}), 143.7, 141.2 (C$_q$^{Fmoc}), 138.3 (C$_q$^{Bn}), 128.4, 127.7, 127.1, 125.6, 120.0 (CH$^{Bn\&Fmoc}$), 79.6, 75.6, 74.6 (C$_2$, C$_3$, C$_6$), 71.0 (CH$_2$^{Bn}), 67.2 (CH$_2$^{Fmoc}), 60.0 (C$_\alpha$^{Leu}), 54.5 (C$_\alpha$^{Cys}), 51.8 (OCH$_3$), 49.4 (C$_\beta$^{Cys}), 48.3 (C$_q$^{tBu}), 47.1 (CH^{Fmoc}), 43.9 (C$_7$), 42.8 (C$_\beta$^{Leu} and C$_1$), 29.8 (*t*Bu), 24.8 (C$_\gamma$^{Leu}), 28.6 (C$_4$), 28.0 (C$_5$), 22.7, 22.3 (2×CH$_3$^{Leu}).

(2R,3S,6S)-3-*O*-Benzyl-6-{(*N*-[(*S*-(tert-butyl)sulfanyl]-*N*-{[9H-fluoren-9-yl]methoxy]carbonyl}-L-cysteinyl) amino]methyl}-tetrahydropyran-1-*N*-L-methionine methyl ester (2,6-trans 9). From 2,6-trans **7** (160 mg, 0.32 mmol) *N*-ethylmorpholine (164 µL, 1.3 mmol) according to *GP 3* and *GP4*. Silica gel chromatography (PE/EtOAc 1/1 v/v) gave 2,6-trans **9**

(122 mg, 0.15 mmol, 47%) as an oil. ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.77 (d, 2H, H^{Fmoc}, J= 7.5 Hz), 7.62 (d, 2H, H^{Fmoc}, J= 7.4 Hz), 7.44-7.28 (m, 9H, H$^{Bn\&Fmoc}$), 7.01 (bs, 1H, C$_7$NH), 6.02 (d, 1H, NHFmoc, J= 8.0 Hz), 4.63-4.42 (m, 4H, H$_α^{Met}$, CH$_2^{Bn}$, H^{Fmoc}), 4.34 (t, 1H, H^{Fmoc}, J= 7.3 and 10.2 Hz), 4.27 (t, 1H, H^{Fmoc}, J= 6.9 and 7.1 Hz), 3.82-3.71 (m, 5H, H$_2$, H$_6$ and OCH$_3$), 3.60 (m, 1H, H$_{7a}$), 3.45 (dd, 1H, H$_α^{Met}$, J= 5.5, 5.6 and 7.7 Hz), 3.35-3.20 (m, 2H, H$_{7b}$ and H$_3$), 3.14 (m, 2H, H$_β^{Cys}$), 2.80 (m, 2H, H$_{1ab}$), 2.55 (m, 2H, H$_γ^{Met}$), 2.07 (s, 3H, SCH$_3$), 1.99-1.56 (m, 6H, H$_{4ab}$, H$_{5ab}$, H$_β^{Met}$), 1.35 (s, 9H, tBu); ^{13}C-NMR (75 MHz, CDCl$_3$): δ 175.9, 170.0 (C=O$^{ester\&amide}$), 155.8 (C=O^{Fmoc}), 143.7, 141.2 (C$_q^{Fmoc}$), 138.2 (C$_q^{Bn}$), 128.4, 127.6, 127.0, 125.1, 119.9 (CH$^{Bn\&Fmoc}$), 73.3, 69.5 (C$_2$, C$_3$, C$_6$), 70.9 (CH$_2^{Bn}$), 67.1 (CH$_2^{Fmoc}$), 59.5 (C$_α^{Met}$), 54.6 (C$_α^{Cys}$), 52.1 (OCH$_3$), 48.2 (C$_q^{tBu}$), 47.6 (C$_1$), 47.1 (CH^{Fmoc}), 42.9 (C$_β^{Cys}$), 41.3 (C$_7$), 32.2 (C$_β^{Met}$), 30.4 (C$_γ^{Met}$), 29.8 (tBu), 24.3 (C$_4$), 23.7 (C$_5$), 15.3 (SCH$_3$). MS (ESI): m/z 810.1 (M+H)$^+$, 832.3 (M+Na)$^+$.

(2R,3S,6R)-3-*O*-Benzyl-6-{(*N*-[(*S*-(tert-butyl)sulfanyl]-*N*-{[9H-fluoren-9-yl]methoxy]carbonyl}-L-cysteinyl)amino]methyl}-tetrahydropyran-1-*N*-L-methionine methyl ester (2,6-cis 9).
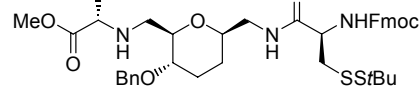
From 2,6-cis **7** (131 mg, 0.26 mmol) and *N*-ethylmorpholine (134 µL, 1.06 mmol) according to *GP 3* and *GP4*. Silica gel chromatography (PE/EtOAc 1/1 v/v) gave 2,6-cis **9** (92 mg, 0.11 mmol, 44%) as an oil. ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.75 (d, 2H, H^{Fmoc}, J= 7.5 Hz), 7.60 (d, 2H, H^{Fmoc}, J= 7.4 Hz), 7.44-7.25 (m, 9H, H$^{Bn\&Fmoc}$), 6.94 (bs, 1H, C$_7$NH), 5.85 (d, 1H, C$_α^{Cys}$NHFmoc, J= 8.0 Hz), 4.60-4.40 (m, 4H, H$_α^{Met}$, CH$_2^{Bn}$ and H^{Fmoc}), 4.34 (t, 1H, HFmoc, J= 7.1 and 10.6 Hz), 4.23 (t, 1H, H^{Fmoc}, J= 7.0 and 7.1 Hz), 3.69 (s, 3H, OCH$_3$), 3.55 (m, 1H, H$_7$), 3.40 (m, 2H, H$_α^{Met}$ and H$_2$), 3.25 (m, 2H, H$_6$ and H$_3$), 3.13 (m, 3H, H$_β^{Cys}$ and H$_{7b}$), 2.95 (dd, 1H, H$_{1a}$, J= 2.1 and 2.6 Hz), 2.72 (dd, 1H, H$_{1b}$, J= 5.4 and 5.5 Hz), 2.55 (t, 2H, H$_γ^{Met}$, J= 7.1 and 7.5 Hz), 2.24 (m, 1H, H$_{4a}$), 2.05 (s, 3H, SCH$_3$), 2.04-1.55 (m, 5H, H$_{5ab}$, H$_{4b}$, H$_β^{Met}$), 1.34 (s, 9H, tBu); ^{13}C-NMR (75 MHz, CDCl$_3$): δ 169.9 (C=O$^{ester\&amide}$), 155.8 (C=O^{Fmoc}), 143.7, 141.2 (C$_q^{Fmoc}$), 138.2 (C$_q^{Bn}$), 128.4, 127.7, 127.1, 125.1, 120.0 (C$^{Bn\&Fmoc}$), 79.5, 75.6, 74.4 (C$_2$, C$_3$, C$_6$), 71.0 (CH$_2^{Bn}$), 67.2 (CH$_2^{Fmoc}$), 60.1 (C$_α^{Met}$), 54.6 (C$_α^{Cys}$), 52.0 (OCH$_3$), 48.3 (C$_q^{tBu}$), 47.1 (CH^{Fmoc}), 49.3 (C$_1$), 43.9 (C$_7$), 42.8 (C$_β^{Cys}$), 32.8 (C$_β^{Met}$), 30.5 (C$_γ^{Met}$), 29.8 (tBu), 28.6 (C$_4$), 28.0 (C$_5$), 15.3 (SCH$_3$). MS (ESI): m/z 810.3 (M+H)$^+$, 832.4 (M+Na)$^+$.

(2R,3S,6R)-3-hydroxy-6-{*N*-[(*t*-butyloxycarbonyl)amino]methyl}-tetrahydropyran-1-*N*-L-leucine methyl ester hydrochloride (2,6-cis 10).[23]
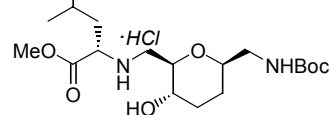
Compound 2,6-cis **6** (209 mg, 0.44 mmol) was dissolved in EtOH/CHCl$_3$ (5.5 mL, 10/1 v/v) and 10% Pd/C (20 mg) was added. The mixture was hydrogenated overnight using a balloon filled with hydrogen-gas. The catalyst was removed by filtration over Celite$^®$ and the filtrate was concentrated *in vacuo* to give crude 2,6-cis **10** (HCl salt, 185 mg, 0.44 mmol, 100%). ^{1}H-NMR (300 MHz, MeOD): δ 4.15 (m, 1H, H$_α$), 3.86 (s, 3H, OCH$_3$), 3.45 (m, 3H, H$_6$, H$_2$ and H$_{7a}$), 3.25 (m, 2H, H$_3$ and H$_{1a}$), 3.21 (m, 1H, H$_{7b}$), 3.08 (m, 1H, H$_{1b}$), 2.06, 1.71, 1.92-1.43 (2×m, 7H, H$_{5ab}$, H$_{4ab}$, H$_{βγ}^{Leu}$), 1.42 (s, 9H, tBu), 0.96 (2×d, 6H, 2×CH$_3^{Leu}$, J= 5.8 and 6.0 Hz); ^{13}C-NMR (150 MHz, MeOD): δ 170.6 (C=O^{ester}), 158.4 (C=O^{Boc}), 80.3 (C$_q^{tBu}$), 78.8, 78.0, 68.8 (C$_2$, C$_3$, C$_6$), 60.1 (C$_α^{Leu}$), 53.8 (OCH$_3$), 49.0 (C$_1$), 45.5 (C$_7$), 39.6 (C$_β$), 32.8 (C$_4$), 28.8 (tBu, C$_5$), 26.0 (C$_γ$), 23.5, 21.8 (2×CH$_3^{Leu}$). MS (ESI): m/z 389.3 (M+H)$^+$, 411.2 (M+Na)$^+$.

(2R,3S,6R)-3-hydroxy-6-{(*N*-[(*S*-(tert-butyl)sulfanyl]-*N*-{[9H-fluoren-9-yl)methoxy]carbonyl}-L-cysteinyl)amino]methyl}-tetrahydropyran-1-*N*-L-leucine methyl ester (2,6-cis 11).
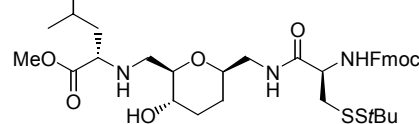
From 2,6-cis **10** (154 mg, 0.36 mmol) and *N*-ethylmorpholine (185 µL, 1.45 mmol) according to *GP 3* and *GP4*. Silica gel chromatography (PE/EtOAc 1/1 v/v) yielded the title compound (132 mg, 0.19 mmol, 52%). ^{1}H-NMR (750 MHz, CDCl$_3$): δ 7.76 (d, 2H, H^{Fmoc}, J= 7.5 Hz), 7.60 (m, 2H, H^{Fmoc}), 7.42-7.26 (m, 9H, H^{Fmoc}), 6.93 (bs, 1H, C$_7$NH), 6.28 (d, 1H, NHFmoc, J= 7.3 Hz), 4.65 (m, 1H, H$_3$), 4.47-4.32 (m, 3H, CH$_2^{Fmoc}$ and H$_α^{Cys}$), 4.25 (m, 1H, CH^{Fmoc}), 3.66 (s, 3H, OCH$_3$), 3.58 (m, 1H, H$_{7a}$), 3.49 (m, 2H, H$_2$ and H$_6$), 3.24 (m, 1H, H$_α^{Leu}$), 3.15 (m, 3H, H$_{7b}$ and H$_β^{Cys}$), 2.60

and 2.80 (2×m, 2H, H$_{1ab}$), 2.25 (m, 1H, H$_{4a}$), 1.73-1.41 (m, 6H, H$_{4b}$, H$_{5ab}$, H$_\beta^{Leu}$, H$_\gamma^{Leu}$), 1.33 (s, 9H, *t*Bu), 0.93 (m, 6H, 2×CH$_3^{Leu}$); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 176.7, 169.9 (C=O$^{amide\&ester}$), 155.8 (C=O^{Fmoc}), 143.7, 141.2 (C$_q^{Fmoc}$), 128.3, 127.7, 127.0, 119.9 (CH^{Fmoc}), 78.4, 75.9 and 70.8 (C$_2$, C$_3$, C$_6$), 67.2 (CH$_2^{Fmoc}$), 54.0 (C$_\alpha^{Cys}$), 51.8 (OCH$_3$), 48.7 (C$_1$), 48.3 (C$_q^{tBu}$), 47.0 (CH^{Fmoc}), 43.6 (C$_7$), 42.6 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 29.0 (*t*Bu), 24.8 (C$_\gamma^{Leu}$), 28.4, 27.7 (C$_4$, C$_5$), 23.5, 21.8 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 702.4 (M+H)$^+$.

N-[(6S)-6-({*N*-[(tert-Butoxy)carbonyl]amino}methyl-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-leucine methyl ester (2,6-trans 13).
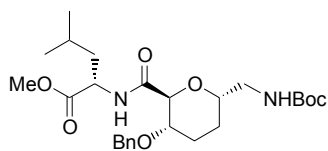
From 2,6-trans **12** (53 mg, 145 μmol) and HCl·H-Leu-OMe (27 mg, 0.15 mmol) according to *GP 2*. Silica gel chromatography (R$_f$ 0.7, EtOAc) gave 2,6-trans **13** as an oil (70 mg, 142 μmol, 98%). [α]$_D^{25}$= +16.8 (*c*= 0.25, CHCl$_3$). ^{1}H-NMR (600 MHz, CDCl$_3$): δ 7.29-7.19 (m, 5H^{Bn}), 7.00 (d, 1H, NHC$_\alpha$, *J*= 8.2 Hz), 5.13 (bs, 1H, NHBoc), 4.54 (m, 3H, H$_\alpha$ and CH$_2^{Bn}$), 4.50 (d, 1H, H$_2$, *J*= 3.9 Hz), 4.11 (bs, 1H, H$_3$), 3.67 (s, 3H, OCH$_3$), 3.56 (m, 1H, H$_6$), 3.30 and 3.06 (2×m, 2H, H$_7$), 1.71 and 1.63-1.49 (2×m, 7H+9H, H$_{5a}$, H$_{4ab}$, H$_{\beta\gamma}^{Leu}$), 1.38 (s, 9H, *t*Bu), 1.34 (m, 1H, H$_{5b}$), 0.96 (2×d, 6H, 2×CH$_3^{Leu}$, *J*= 5.9 and 6.0 Hz); ^{13}C-NMR (150 MHz, CDCl$_3$): δ 172.6, 169.3 (C=O$^{amide\&ester}$), 155.6 (C=O^{Boc}), 138.0 (C$_q^{Bn}$), 128.0, 127.2 (C^{Bn}), 78.7 (C$_q^{tBu}$), 76.2 (C$_2$), 72.9 (C$_6$), 70.4 (CH$_2^{Bn}$ and C$_3$), 50.9 (OCH$_3$), 50.1 (C$_\alpha$), 44.8 (C$_7$), 40.8 (C$_\beta$), 28.0 (*t*Bu), 24.7 (C$_\gamma$), 23.5 (C$_4$), 22.4 (CH$_3^{Leu}$), 22.0 (C$_5$), 21.4 (CH$_3^{Leu}$). MS (ESI): *m/z* 515.5 (M+Na)$^+$. HR-MS: calc. for [C$_{26}$H$_{40}$N$_2$O$_7$+Na]$^+$ 515.2727, found 515.2769.

N-[(6R)-6-({*N*-[(tert-Butoxy)carbonyl]amino}methyl-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-leucine methyl ester (2,6-cis 13).
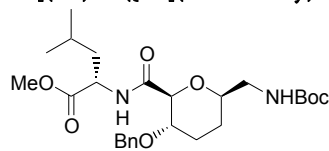
From 2,6-cis **12** (53 mg, 145 μmol) and HCl·H-Leu-OMe (27 mg, 0.15 mmol) according to *GP 2*. Silica gel chromatography (R$_f$ 0.7, EtOAc) gave 2,6-cis **13** as an oil (70 mg, 142 μmol, 98%). [α]$_D^{25}$= −22.4 (*c*= 0.5, CHCl$_3$). ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.33-7.24 (m, 5H^{Bn}), 6.83 (d, 1H, NHC$_\alpha$, *J*= 7.9 Hz), 5.11 (bs, 1H, NHBoc), 4.61 (m, 3H, H$_\alpha$, CH$_2^{Bn}$), 3.78 (d, 1H, H$_2$, *J*= 9.2 Hz), 3.77 (s, 3H, OCH$_3$), 3.53-3.40 (m, 3H, H$_6$, H$_3$, H$_{7a}$), 3.10 (m, 1H, H$_{7b}$), 2.27, 1.74-1.23 (2×m, 7H, H$_{5ab}$, H$_{4ab}$, H$_{\beta\gamma}$), 1.44 (s, 9H, *t*Bu), 0.90 (2×d, 6H, 2×CH$_3^{Leu}$, *J*= 6.2 and 6.4 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.2, 169.8 (C=O$^{amide\&ester}$), 156.0 (C=O^{Boc}), 137.8 (C$_q^{Bn}$), 128.3, 127.7 (C^{Bn}), 79.5 (C$_2$), 79.3 (C$_q^{tBu}$), 76.8 (C$_6$), 75.2 (C$_3$), 71.4 (CH$_2^{Bn}$), 52.1 (OCH$_3$), 50.4 (C$_\alpha$), 44.6 (C$_7$), 41.3 (C$_\beta$), 29.0 (C$_4$), 28.3 (*t*Bu), 26.9 (C$_5$), 24.8 (C$_\gamma$), 22.7, 21.8 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 515.5 (M+Na)$^+$. HR-MS: calc. for [C$_{26}$H$_{40}$N$_2$O$_7$+Na]$^+$ 515.2727, found 515.2728.

N-[(6S)-6-({*N*-[(tert-Butoxy)carbonyl]amino}methyl-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-methionine methyl ester (2,6-trans 14).
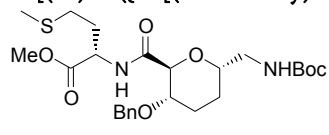
From 2,6-trans **12** (52 mg, 142 μmol) and HCl·H-Met-OMe (34 mg, 170 μmol) according to *GP 2*. Silica gel chromatography (R$_f$ 0.9, EtOAc/AcOH 98/2 v/v) gave 2,6-trans **14** as an oil (53 mg, 104 μmol, 73%). [α]$_D^{25}$= +28.2 (*c*= 1, CHCl$_3$). ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.61-7.10 (m, H^{Bn} and NHC$_\alpha$), 5.68 (bs, 1H, NHBoc), 5.05 (m, 1H, H$_\alpha$), 4.77, 4.70 (2×d, CH$_2^{Bn}$, *J*= 7.3 Hz), 3.77 (s, 3H, OCH$_3$), 3.46-3.06 (m, 5H, H$_6$, H$_3$, H$_2$ and H$_7$), 2.58-2.34 (m, 2H, H$_\gamma$), 2.29-2.01 (m, 2H, H$_\beta$), 2.11 (s, 3H, SCH$_3$), 197-1.51 (m, 4H, H$_{5ab}$ and H$_{4ab}$), 1.45 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 171.8, 169.9 (C=O$^{amide\&ester}$), 155.9 (C=O^{Boc}), 138.0 (C$_q^{Bn}$), 128.1, 127.3 (C^{Bn}), 79.1 (C$_q^{tBu}$) 76.4 (C$_2$), 73.1 (C$_6$), 70.8 (C$_3$), 70.7 (CH$_2^{Bn}$), 52.3 (OCH$_3$), 51.0 (C$_\alpha$), 44.9 (C$_7$), 31.2 (C$_\beta$), 30.0 (C$_\gamma$), 28.2 (*t*Bu), 23.6 (C$_4$), 22.1 (C$_5$), 15.2 (SCH$_3$). MS (ESI): *m/z* 511.5 (M+H)$^+$, 533.3 (M+Na)$^+$. HR-MS: calc. for [C$_{25}$H$_{38}$N$_2$O$_7$S +Na]$^+$ 533.2291, found 533.2331.

N-[(6R)-6-({*N*-[(tert-Butoxy)carbonyl]amino}methyl-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-methionine methyl ester (2,6-cis 14).
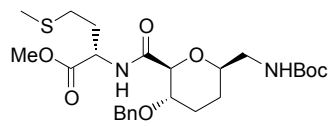
From 2,6-cis **12** (244 mg, 0.67 mmol) according to *GP 2*. Silica gel chromatography (R$_f$ 0.8, EtOAc/AcOH 98/2 v/v) gave 2,6-cis **14** as an oil which solidified on standing (284 mg, 0.56 mmol, 83%). [α]$_D^{25}$= +4.6 (*c*= 1, CHCl$_3$). ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.36-

7.27 (m, 5H^{Bn}), 7.09 (d, 1H, NHC$_\alpha$, J= 7.7 Hz), 5.10 (bs, 1H, NHBoc), 4.61 (ddd, 1H, H$_\alpha$, J= 5.3, 5.5, 7.4 and 7.5 Hz), 4.58 (s, CH$_2^{Bn}$), 3.79 (d, 1H, H$_2$, J= 9.2 Hz), 3.73 (s, 3H, OCH$_3$), 3.52-3.42 (m, 3H, H$_6$, H$_3$, H$_{7a}$), 3.08 (m, 1H, H$_{7b}$), 2.46 (t, 2H, H$_\gamma$, J= 7.4 and 7.6 Hz), 2.30-1.46 (2×m, 6H, H$_{5ab}$, H$_{4ab}$ and H$_\beta$), 2.03 (s, 3H, SCH$_3$), 1.44 (s, 9H, tBu); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 171.9, 170.4 (C=O$^{amide\&ester}$), 156.2 (C=O^{Boc}), 137.7 (C$_q^{Bn}$), 128.4, 127.9 (C^{Bn}), 79.5 (C$_q^{tBu}$) 79.3 (C$_2$), 77.5 (C$_6$), 74.8 (C$_3$), 71.2 (CH$_2^{Bn}$), 52.4 (OCH$_3$), 51.3 (C$_\alpha$), 44.5 (C$_7$), 31.3 (C$_\beta$), 29.9 (C$_\gamma$), 29.0 (C$_4$), 28.3 (tBu), 26.7 (C$_5$), 15.3 (SCH$_3$). MS (ESI): m/z 511.6 (M+H)$^+$, 533.1 (M+Na)$^+$. HR-MS: calc. for [C$_{25}$H$_{38}$N$_2$O$_7$S +Na]$^+$ 533.2291, found 533.2297.

N-[(6S)-6-({*N*-(S-[tert-Butyl)sulfanyl]-*N*-{[(9H-fluoren-9-yl)methoxy]carbonyl}-L-cysteinyl)amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-leucine methyl ester (2,6-trans 15).
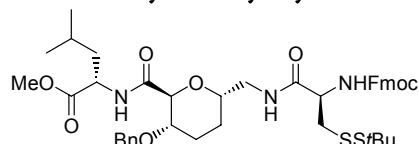
From 2,6-trans **13** (200 mg, 407 μmol) according to *GP 3* and *GP4*. Silica gel chromatography (R$_f$ 0.9, PE/EtOAc 1/3 v/v) afforded the title compound (263 mg, 326 μmol, 80%) as an oil. [α]$_D^{25}$= −20.0 (*c*= 0.5, CHCl$_3$). ^{1}H-NMR (750 MHz, CDCl$_3$): δ 7.76 (d, 2H, H^{Fmoc}, J= 7.6 Hz), 7.60 (d, 2H, H^{Fmoc}, J= 7.4), 7.41-7.26 (m, 9H, H$^{Fmoc\&Bn}$), 7.10 (d, 1H, NHFmoc, J= 8.2 Hz), 6.87 (bs, 1H, C$_7$NH), 6.28 (d, 1H, NHC$_\alpha^{Leu}$, J= 8.2 Hz), 4.66 (m, 1H, H$_\alpha^{Leu}$), 4.57-4.51 (m, 3H, CH$_2^{Bn}$ and H$_\alpha^{Cys}$), 4.49-4.42 (m, 1H, H^{Fmoc}), 4.40 (bs, 1H, H$_2$), 4.35 (t, 1H, H^{Fmoc}, J= 7.7 and 10.2 Hz), 4.23 (t, 1H, H^{Fmoc}, J= 7.1 and 7.2 Hz), 4.18 (m, 1H, H$_3$), 3.73 (s, 3H, OCH$_3$), 3.78-3.69 (m, 2H, H$_6$ and H$_{7a}$), 3.21-3.12 (m, 2H, H$_\beta^{Cys}$), 3.02 (ddd, 1H, H$_{7b}$, J= 4.3, 4.6, 9.4 Hz and J= 4.4, 4.8, 9.2 Hz), 2.00 (m, 1H, H$_{4a}$), 1.75 (m, 1H, H$_{5a}$), 1.69-1.54 (m, 3H, H$_{\beta\gamma}^{Leu}$), 1.51 (m, 1H, H$_{4b}$), 1.42 (m, 1H, H$_{5b}$), 1.33 (s, 9H, tBu), 0.93 (d, 6H, 2×CH$_3^{Leu}$, J= 5.1 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.9, 170.2, 169.0 (C=O$^{amide\&ester}$), 156.3 (C=O^{Fmoc}), 143.7, 143.6, 141.2 (C$_q^{Fmoc}$), 138.1 (C$_q^{Bn}$), 128.3, 127.7, 127.6, 127.0, 125.1, 125.0, 119.9 (C$^{Bn\&Fmoc}$), 76.6 (C$_2$), 72.2 (C$_6$), 70.7 (CH$_2^{Bn}$), 70.6 (C$_3$), 67.2 (CH$_2^{Fmoc}$), 54.4 (C$_\alpha^{Leu}$), 52.6 (OCH$_3$), 50.3 (C$_\alpha^{Cys}$), 48.1 (C$_q^{tBu}$), 47.0 (CH^{Fmoc}), 44.1 (C$_7$), 42.4, 41.6 (C$_\beta^{Leu}$ and C$_\beta^{Cys}$), 29.7 (tBu), 24.9 (C$_\gamma^{Leu}$), 23.7 (C$_4$), 22.6 (CH$_3^{Leu}$), 22.4 (C$_5$), 21.9 (CH$_3^{Leu}$). MS (ESI): m/z 828.4 (M+Na)$^+$. HR-MS: calc. for [C$_{43}$H$_{55}$N$_3$O$_8$S$_2$+H]$^+$ 806.3503, found 806.3479.
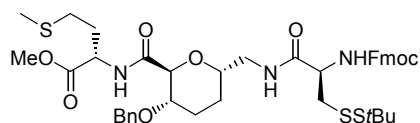
N-[(6R)-6-({*N*-(S-[tert-Butyl)sulfanyl]-*N*-{[(9H-fluoren-9-yl)methoxy]carbonyl}-L-cysteinyl)amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-leucine methyl ester (2,6-cis 15).
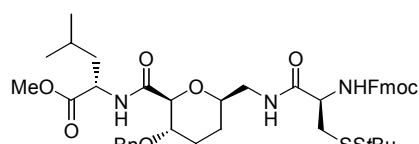
From 2,6-cis **13** (70 mg, 142 μmol) according to *GP 3* and *GP4*. Silica gel chromatography (R$_f$ 0.5, PE/EtOAc 1/3 v/v) gave 2,6-cis **15** (97 mg, 120 μmol, 85%) as an oil. [α]$_D^{25}$= −21.2 (*c*= 1, CHCl$_3$). ^{1}H-NMR (750 MHz, CDCl$_3$): δ 7.74 (d, 2H, H^{Fmoc}, J= 7.5 Hz), 7.58 (d, 2H, H^{Fmoc}, J= 7.4), 7.39-7.24 (m, 9H, H$^{Fmoc\&Bn}$), 7.12 (bs, 1H, C$_7$NH, 7.00 (d, 1H, NHC$_\alpha^{Leu}$, J= 7.4 Hz), 6.12 (d, 1H, NHC$_\alpha^{Cys}$, J= 7.3 Hz), 4.60 (m, 1H, H$_\alpha^{Leu}$), 4.50 (m, 3H, CH$_2^{Bn}$ and H$_\alpha^{Cys}$), 4.42-4.30 (m, 2H, CH$_2^{Fmoc}$), 4.21 (t, 1H, CH^{Fmoc}, J= 7.3 and 7.2 Hz), 3.77 (d, 1H, H$_2$, J= 8.7 Hz), 3.68 (s, 3H, OCH$_3$), 3.49 (m, 2H, H$_6$ and H$_{7a}$), 3.44 (ddd, 1H, H$_3$, J= 4.5, 4.6 and 10.1 Hz), 3.28 (m, 1H, H$_{7b}$), 3.11 (m, 2H, H$_\beta^{Cys}$), 2.18 (m, 1H, H$_{4a}$), 1.70 (m, 1H, H$_{5a}$), 1.59-1.39 (m, 4H, H$_{4b}$, H$_{\beta\gamma}^{Leu}$), 1.38 (m, 1H, H$_{5b}$), 1.32 (s, 9H, tBu), 0.88 (2×d, 6H, 2×CH$_3^{Leu}$, J= 5.9 and 6.0 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 173.6, 170.3, 169.9 (C=O$^{amide\&ester}$), 156.0 (C=O^{Fmoc}), 143.7, 141.2 (C$_q^{Fmoc}$), 137.8 (C$_q^{Bn}$), 129.7, 128.3, 127.8, 127.7, 127.6, 127.2, 125.1, 119.9 (CH$^{Bn\&Fmoc}$), 79.2 (C$_2$), 75.9 (C$_6$), 75.2 (C$_3$), 71.4 (CH$_2^{Bn}$), 67.2 (CH$_2^{Fmoc}$), 54.5 (C$_\alpha^{Leu}$), 52.2 (OCH$_3$), 50.3 (C$_\alpha^{Cys}$), 48.2 (C$_q^{tBu}$), 47.0 (CH^{Fmoc}), 43.3 (C$_7$), 42.3, 41.1 (C$_\beta^{Leu}$ and C$_\beta^{Cys}$), 29.7 (tBu), 29.0 (C$_4$), 27.1 (C$_5$), 24.8 (C$_\gamma^{Leu}$), 22.7, 21.8 (2×CH$_3^{Leu}$). MS (ESI): m/z 828.4 (M+Na)$^+$. HR-MS: calc. for [C$_{43}$H$_{55}$N$_3$O$_8$S$_2$+H]$^+$ 806.3503, found 806.3479.

N-[(6S)-6-({*N*-(S-[tert-Butyl)sulfanyl]-*N*-{[(9H-fluoren-9-yl)methoxy]carbonyl}-L-cysteinyl)amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-methionine methyl ester (2,6-trans 16).

From 2,6-trans **14** (367 mg, 0.72 mmol) according to *GP 3* and *GP4*. Silica gel chromatography (R$_f$ 0.7, PE/EtOAc 2/3 v/v) gave 2,6-trans **16** (468 mg, 0.57 mmol,

79%) as an oil. $[\alpha]_D^{25} = -8.0$ ($c = 0.5$, CHCl$_3$). ^{1}H-NMR (750 MHz, CDCl$_3$): δ 7.71 (d, 2H, H^{Fmoc}, $J = 7.5$ Hz), 7.61 (d, 2H, H^{Fmoc}, $J = 7.4$ Hz), 7.41-7.25 (m, 10H, H$^{Fmoc\&Bn}$, NHC$_\alpha^{Leu}$), 6.86 (bs, 1H, C$_7$NH), 6.26 (d, 1H, NHFmoc, $J = 8.3$ Hz), 4.61 (m, 1H, H$_\alpha^{Met}$), 4.62-4.45 (m, 4H, H$_\alpha^{Cys}$, CH$_2^{Bn}$, CH^{Fmoc}), 4.42 (s, 1H, H$_2$), 4.35 (t, 1H, CH^{Fmoc}, $J = 7.4$ and 10.1 Hz), 4.24 (t, 1H, CH^{Fmoc}, $J = 7.0$ Hz), 4.18 (m, 1H, H$_3$), 3.75 (m, 5H, H$_6$, H$_{7a}$, OCH$_3$), 3.17 (m, 2H, H$_\beta^{Met}$), 3.03 (m, 1H, H$_{7b}$), 2.49 (t, 2H, H$_\gamma^{Met}$, $J = 7.3$ and 7.7 Hz), 2.20 (m, 1H, H$_{4a}$), 2.07 (s, 3H, SCH$_3$), 2.10-1.99 (m, 4H, H$_{5a}$, H$_{\beta\gamma}^{Leu}$), 1.77 (m, 2H, H$_{5b}$, H$_{4b}$), 1.33 (s, 9H, tBu). ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.4, 169.9, 169.2 (C=O$^{amide\&ester}$), 155.9 (C=O^{Fmoc}), 143.4, 140.8 (C$_q^{Fmoc}$), 137.8 (C$_q^{Bn}$), 127.9-126.7, 124.7 and 119.6 (C$^{Fmoc\&Bn}$), 76.3, 72.0, 70.3 (C$_2$, C$_3$, C$_6$), 70.3 (CH$_2^{Bn}$), 66.8 (CH$_2^{Fmoc}$), 54.2 (C$_\alpha^{Met}$), 52.4 (OCH$_3$), 50.9 (C$_\alpha^{Cys}$), 47.7 (C$_q^{tBu}$), 46.7 (CH^{Fmoc}), 44.0 (C$_7$), 42.2 (C$_\beta^{Cys}$), 30.9, 29.9 (C$_{\beta\gamma}^{Met}$), 29.9 (tBu), 23.4 (C$_4$), 22.3 (C$_5$), 15.0 (SCH$_3$). MS (ESI): m/z 824.6 (M+H)$^+$. HR-MS: calc. for [C$_{42}$H$_{53}$N$_3$O$_8$S$_3$+H]$^+$ 824.3073, found 824.3097.

N-[(6R)-6-({*N*-(S-[tert-Butyl)sulfanyl]-*N*-{[(9H-fluoren-9-yl)methoxy]carbonyl}-L-cysteinyl)amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-methionine methyl ester (2,6-cis 16).

From 2,6-cis **14** (50 mg, 98 µmol) following *GP 3* and *GP4*. Silica gel chromatography (R$_f$ = 0.6, EtOAc) gave 2,6-cis **16** (64 mg, 77 µmol, 79%) as a white foam. $[\alpha]_D^{25} = +6.8$ ($c = 0.5$, CHCl$_3$). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.76, 7.52 (2×d, 4H, H^{Fmoc}, $J = 7.5$ Hz), 7.39 (t, 2H, H^{Fmoc}, $J = 7.2$ and 7.4 Hz), 7.36-7.25 (m, 9H, H$^{Fmoc\&Bn}$), 7.10 (t, 1H, NHC$_\alpha^{Met}$, $J = 6.2$ and 7.6 Hz), 6.77 (bs, 1H, C$_7$NH), 5.98 (m, 1H, NHFmoc, $J = \sim6$ Hz), 4.70 (ddd, 1H, H$_\alpha^{Leu}$, $J = 5.2$, 7.6 and 12.9 Hz), 4.60, 4.54 (2×d, 2H, CH$_2^{Bn}$, $J = 1.9$ and 2.1 Hz), 4.46 (m, 1H, H$_\alpha^{Cys}$), 4.42 (dd, 1H, CH^{Fmoc}, $J = 7.3$ Hz), 4.33 (t, 1H, CH^{Fmoc}, $J = 7.4$ and 10.1 Hz), 4.35 (t, 1H, CH^{Fmoc}, $J = 7.0$ and 7.1 Hz), 3.80 (m, 1H, H$_2$), 3.75 (s, 3H, OCH$_3$), 3.59-3.40 (m, 3H, H$_6$, H$_3$, H$_{7a}$), 3.25 (m, 1H, H$_{7b}$), 3.07 (m, 2H, H$_\beta^{Met}$), 2.47 (m, 2H, H$_\gamma^{Met}$), 2.21-2.04 (m, 2H, H$_{4a}$, H$_\beta^{Cys}$), 2.04 (s, 3H, SCH$_3$), 2.00-1.86 (m, 1H, H$_\beta^{Cys}$), 1.74 (m, 1H, H$_{5a}$), 1.58 (m, 1H, H$_{4b}$), 1.44 (m, 1H, H$_{5b}$), 1.33 (s, 9H, tBu). ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.4, 170.2, 169.9 (C=O$^{amide\&ester}$), 156.0 (C=O^{Fmoc}), 143.7, 141.2 (C$_q^{Fmoc}$), 137.8 (C$_q^{Bn}$), 129.0, 128.3, 128.1, 127.9, 127.7, 127.1, 125.1, 119.9 (C$^{Fmoc\&Bn}$), 79.4 (C$_2$), 76.0 (C$_6$), 75.0 (C$_3$), 71.3 (CH$_2^{Bn}$), 67.2 (CH$_2^{Fmoc}$), 54.6 (C$_\alpha^{Met}$), 52.5 (OCH$_3$), 51.1 (C$_\alpha^{Cys}$), 48.3 (C$_q^{tBu}$), 47.0 (CH^{Fmoc}), 43.3 (C$_7$), 42.4 (C$_\beta^{Cys}$), 29.9 (tBu), 31.5, 29.9 (C$_{\beta\gamma}^{Met}$), 29.0 (C$_4$), 27.1 (C$_5$), 15.3 (SCH$_3$). MS (ESI): m/z 824.6 (M+H)$^+$. HR-MS: calc. for [C$_{42}$H$_{53}$N$_3$O$_8$S$_3$+H]$^+$ 824.3073, found 824.3103.

(2R,3S,6S)-3-*O*-Benzyl-6-{(*N*-[(S-(tert-butyl)sulfanyl]-L-cysteinyl)amino]methyl}-tetrahydropyran-1-*N*-L-leucine (2,6-trans 18).

From 2,6-trans **8** (20 mg, 25 µmol) according to *GP 5*. RP-HPLC purification (linear gradient B) afforded the title compound (2.4 mg, 4.2 µmol, 17%). ^{1}H-NMR (600 MHz, MeOD): δ 7.35-7.29 (m, 5H, H^{Bn}), 4.65 and 4.48 (2×d, 2H, CH$_2^{Bn}$, $J = 11.6$ Hz), 4.08 (m, 2H, H$_\alpha^{Cys}$ and H$_2$), 3.91 (m, 2H, H$_3$ and H$_6$), 3.73 (m, 1H, H$_\alpha^{Leu}$), 3.60 (m, 1H, H$_{7a}$), 3.38 (m, 1H, H$_3$), 3.30 (m, 1H, H$_{7b}$), 3.25-3.13 (m, 4H, H$_{1ab}$ and H$_\beta^{Cys}$), 2.00 (m, 1H, H$_{4a}$), 1.79-1.70 (m, 4H, H$_{\beta\gamma}^{Leu}$ and H$_{5a}$ and H$_{4b}$), 1.58 (m, 1H, H$_{5b}$), 1.37 (s, 9H, tBu), 0.98 (t, 6H, 2×CH$_3^{Leu}$, $J = 5.7$ and 5.8 Hz). HR-MS: calc. for [C$_{26}$H$_{43}$N$_3$O$_5$S$_3$+H]$^+$ 556.28789, found 556.28408.

(2R,3S,6R)-3-*O*-Benzyl-6-{(*N*-[(S-(tert-butyl)sulfanyl]-L-cysteinyl)amino]methyl}-tetrahydropyran-1-*N*-L-leucine (2,6-cis 18).

From 2,6-cis **8** (10 mg, 13 µmol) according to *GP 5*. RP-HPLC purification (linear gradient B) afforded the title compound (4.9 mg, 8.5 µmol 67%). ^{1}H-NMR (600 MHz, MeOD): δ 7.34-7.29 (m, 5H, H^{Bn}), 4.68 and 4.45 (2×d, 2H, CH$_2^{Bn}$, $J = 11.5$ Hz), 4.15 (t, 1H, H$_\alpha^{Cys}$, $J = 6.5$ and 6.6 Hz), 3.55 (m, 1H, H$_\alpha^{Leu}$), 3.55 (m, 2H, H$_2$ and H$_6$), 3.39 (m, 1H, H$_{1a}$), 3.34-3.23 (m, 4H, H$_{7ab}$, H$_3$ and H$_\beta^{Cys}$), 3.15 (dd, 1H, H$_\beta^{Cys}$, $J = 7.2$, 7.3 and 14.1 Hz), 3.06 (dd, 1H, H$_{1b}$, $J = 9.2$, 9.3, 12.8 and 13.0 Hz), 2.42 (m, 1H, H$_{4a}$), 1.80 (m, 3H, H$_{\beta\gamma}^{Leu}$ and H$_{5a}$), 1.65 (m, 1H, H$_\beta^{Leu}$), 1.49-1.39 (m, 2H, H$_{5b}$ and H$_{4b}$), 1.36 (s, 9H, tBu), 1.00, 0.97 (2 d, 6H, 2×CH$_3^{Leu}$, $J = 6.1$ and 6.2 Hz); ^{13}C-NMR (100 MHz, MeOD): δ 168.9

(C=O$^{amide\&carboxyl}$), 137.5 (C$_q^{Bn}$), 77.6, 76.3, 75.9 (C$_2$, C$_3$, C$_6$), 71.5 (CH$_2^{Bn}$), 54.3 (C$_\alpha^{Leu}$), 49.6-48.4 (C$_\alpha^{Cys}$, C$_q^{tBu}$ and C$_7$, obscured by MeOD peak), 44.7 (C$_1$), 42.5, 40.5 (C$_\beta^{Leu}$ and C$_\beta^{Cys}$), 30.1 (*t*Bu), 29.2, 28.6 (C$_5$, C$_4$), 26.1 (C$_\gamma^{Leu}$), 23.1, 22.6 (2×CH$_3^{Leu}$). HR-MS: calc. for [C$_{26}$H$_{43}$N$_3$O$_5$S$_3$+H]$^+$ 556.28789, found 556.28440.

N-[(6S)-6-({*N*-(S-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexopyranos-1-yl]-L-leucine (2,6-trans 19).

Compound 2,6-trans **15** (20 mg, 25 μmol) was deprotected according to *GP 5*. The crude product was divided in 2 portions and 1 portion was purified by RP-HPLC (linear gradient B, 20→70%) affording 2,6-trans **15** (3.0 mg, 5.3 μmol, 42%) as a white foam. LC-MS R$_f$= 14.9 min (lin. gradient B, 26 min). ^{1}H-NMR (600 MHz, D$_2$O/CD$_3$CN): δ 7.62 (d, 1H, NHC$_\alpha^{Leu}$, *J*= 7.3 Hz), 7.32-7.27 (m, 6H, H^{Bn} and C$_7$NH), 4.50 (2×d, 2H, CH$_2^{Bn}$, *J*= 11.9 and 12.0 Hz), 4.27 (m, 1H, H$_\alpha^{Leu}$), 4.06 (m, 1H, H$_\alpha^{Cys}$), 3.94 (m, 3H, H$_2$, H$_3$ and H$_6$), 3.28 (m, 2H, H$_{7ab}$), 3.02 (d, 2H, H$_\beta^{Cys}$, *J*= 6.3 Hz), 1.88 (m, 1H, H$_{4a}$), 1.63-1.40 (m, 6H, H$_{4b}$, H$_{5ab}$, H$_{\beta\gamma}^{Leu}$), 1.25 (s, 9H, *t*Bu), 0.80 (2×d, 6H, 2×CH$_3^{Leu}$, *J*= 6.0 and 6.1 Hz); ^{13}C-NMR (50 MHz, D$_2$O/CD$_3$CN): δ 179.9–171.6 (C=O$^{amide\&carboxyl}$), 139.0 (C$_q^{Bn}$), 128.0-127.3 (C^{Bn}), 75.6, 71.1, 70.5 (C$_2$, C$_3$, C$_6$), 69.8 (CH$_2^{Bn}$), 52.6 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$), 47.4 (C$_q^{tBu}$), 43.0–40.8 (C$_7$, C$_\beta^{Leu}$ and C$_\beta^{Cys}$), 28.4 (*t*Bu), 24.2 (C$_\gamma^{Leu}$), 22.5, 21.0 (C$_4$, C$_5$), 22.0, 20.4 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 570.4 (M+H)$^+$. HR-MS: calc. for [C$_{27}$H$_{43}$N$_3$O$_6$S$_2$+H]$^+$ 570.2666, found 570.2631.
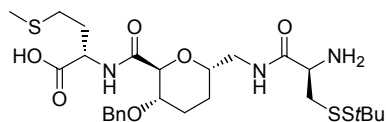
N-[(6R)-6-({*N*-(S-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexopyranos-1-yl]-L -leucine (2,6-cis 19).

Compound 2,6-cis **15** (37 mg, 46 μmol) was deprotected according to *GP 5*. RP-HPLC purification of 23 μmol crude product (linear gradient B, 20→70%) gave **2,6-cis 19** (5.6 mg, 9.8 μmol, 43%) as a white foam. LC-MS R$_f$= 14.9 min (lin. gradient B, 26 min). ^{1}H-NMR (600 MHz, D$_2$O/CD$_3$CN): δ 7.56-7.20 (m, 5H, C^{Bn}), 4.42 (t, 2H, CH$_2^{Bn}$, *J*= 12.4 Hz), 4.09 (m, 1H, H$_\alpha^{Leu}$), 3.85 (m, 1H, H$_\alpha^{Cys}$), 3.60 (d, 1H, H$_2$, *J*= 9.3 Hz), 3.47 (m, 1H, H$_6$), 3.44 (dt, 1H, H$_3$, *J*= 9.5, 10.3 and *J*= 9.6, 10.4 Hz), 3.23 (m, 2H, H$_{7ab}$), 3.02 (m, 2H, H$_\beta^{Cys}$), 2.11 (m, 1H, H$_{4a}$), 1.65 (m, 1H, H$_{5a}$), 1.49-1.27 (m, 5H, H$_{4b}$, H$_{5b}$, H$_{\beta\gamma}^{Leu}$), 1.22 (s, 9H, *t*Bu), 0.80 (d, 6H, 2×CH$_3^{Leu}$, *J*= 3.8 Hz); ^{13}C-NMR (50 MHz, D$_2$O/CD$_3$CN/MeOD): δ 181.0, 175.0, 171.6 (C=O$^{amide\&carboxyl}$), 139.1 (C$_q^{Bn}$), 129.7-127.3 (C^{Bn}), 81.2, 77.1, 76.8 (C$_2$, C$_3$, C$_6$), 72.2 (CH$_2^{Bn}$), 55.1, 54.2 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$), ~48.0 (C$_q^{tBu}$, obscured by MeOD signal), 45.7, 44.0, 42.9 (C$_7$, C$_\beta^{Leu}$ and C$_\beta^{Cys}$), 30.1 (*t*Bu), 30.0, 27.9 (C$_4$, C$_5$), 25.8 (C$_\gamma^{Leu}$), 23.8, 22.3 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 570.4 (M+H)$^+$. HR-MS: calc. for [C$_{27}$H$_{43}$N$_3$O$_6$S$_2$+H]$^+$ 570.2666, found 570.2646.

(2R,3S,6S)-3-*O*-Benzyl-6-{(*N*-[(S-(tert-butyl)sulfanyl]-L-cysteinyl)amino]methyl}-tetrahydropyran-1-*N*-L-methionine (2,6-trans 20).

From 2,6-trans **9** (20 mg, 12.3 μmol) according to *GP 5*. RP-HPLC purification (linear gradient B) gave the title compound (6.4 mg, 11.1 μmol, 90%) as a white foam. ^{1}H-NMR (600 MHz, MeOD): δ 7.35-7.29 (m, 5H, H^{Bn}), 4.65, 4.48 (2×d, 2H, CH$_2^{Bn}$, *J*= 11.6 Hz), 4.10 (m, 2H, H$_\alpha^{Met}$ and H$_2$), 3.91 (m, 2H, H$_\alpha^{Cys}$ and H$_6$), 3.54 (m, 1H, H$_{7a}$), 3.36 (m, 2H, H$_{1a}$ and H$_{7b}$), 3.26-3.15 (m, 4H, H$_\beta^{Cys}$ and H$_{3,1b}$), 2.68 (m, 2H, H$_\gamma^{Met}$), 2.20, 2.10 (m, 2H, H$_\beta^{Met}$), 2.10 (s, 3H, SCH$_3$), 2.06 (m, 1H, H$_{4a}$), 1.79-1.70 (m, 3H, H$_{5ab}$ and H$_{4b}$), 1.36 (s, 9H, *t*Bu); ^{13}C-NMR (100 MHz, MeOD): δ 173.0, 169.6 (C=O$^{amide\&carboxyl}$), 139.5 (C$_q^{Bn}$), 129.5-128.9 (C^{Bn}), 75.1 72.7, 71.7 (C$_2$, C$_3$, C$_6$), 71.4 (CH$_2^{Bn}$), 61.1 (C$_\alpha^{Met}$), 52.4 (C$_\alpha^{Cys}$), 46.8 (C$_q^{tBu}$), 42.3, 41.8 (C$_7$ and C$_\beta^{Cys}$), 30.9, 29.1 (C$_{\beta\gamma}^{Met}$), 30.6 (*t*Bu), 25.2, 24.6 (C$_4$, C$_5$), 15.0 (SCH$_3$). HR-MS calc. for [C$_{26}$H$_{43}$N$_3$O$_5$S$_3$+Na]$^+$ 596.22626, found 596.19714.

(2R,3S,6R)-3-*O*-Benzyl-6-{(*N*-[(S-(tert-butyl)sulfanyl]-L-cysteinyl)amino]methyl}-tetrahydropyran-1-*N*-L-methionine (2,6-cis 20).

From 2,6-cis **9** (20 mg, 12.3 μmol) according to *GP 5*. RP-HPLC purification (linear gradient B) gave 2,6-cis **20** (4.7 mg, 8.2 μmol 67%). ^{1}H-NMR (600 MHz, MeOD): δ 7.60-7.50 (m, 5H^{Bn}), 4.68, 4.44 (2×d, 2H, CH$_2^{Bn}$, *J*= 11.4 and 11.5 Hz), 4.71 (t, 1H, H$_\alpha^{Cys}$, *J*=

6.5 and 6.6 Hz), 3.79 (m, 1H, H$_\alpha^{Met}$), 3.54 (m, 2H, H$_2$ and H$_6$), 3.44 (m, 1H, H$_{1a}$), 3.34 (m, 2H, H$_{7ab}$), 3.26 (m, 1H, H$_3$), 3.23-3.13 (2×dd, 2H, H$_\beta^{Cys}$, J= 6.0, 6.1 Hz and 7.2, 7.3 Hz), 3.07 (m, 1H, H$_{1b}$), 2.66 (m, 2H, H$_\gamma^{Met}$), 2.43 (m, 1H, H$_{4a}$), 2.17-2.11 (m, 2H, H$_\beta^{Met}$), 2.09 (s, 3H, SCH$_3$), 1.80 (m, 1H, H$_{5a}$), 1.52-1.36(m, 2H, H$_{5b}$ and H$_{4b}$), 1.36 (s, 9H, *t*Bu). HR-MS calc. for [C$_{26}$H$_{43}$N$_3$O$_5$S$_3$+H]$^+$ 574.24431, found 574.21685.

N-[(6S)-1-({*N*-(S-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino -hexopyranos-1-yl]-L-methionine (2,6-trans 21).

Compound 2,6-trans **16** (121 mg, 146 μmol) was deprotected according to *GP 5*. The employed 1,4-dioxane was freshly distilled over Na/K and benzophenon. The crude product was dissolved in 13 mL H$_2$O/CH$_3$CN (10/3 v/v) and 3 mL (~34 μmol of crude compound) was used for RP-HPLC (linear gradient B, 20→70%) affording the title compound (7.1 mg, 12 μmol, 35%) as a white foam. LC-MS R$_t$= 15.4 min (lin. gradient B, 26 min). ^{1}H-NMR (600 MHz, D$_2$O/CD$_3$CN): δ 7.60-7.50 (m, 5H, H^{Bn}), 4.77 (partly obscured by solvent, 2H, CH$_2^{Bn}$), 4.71 (m, 1H, H$_\alpha^{Met}$), 4.59 (d, 1H, H$_2$, J= 2.8 Hz), 4.35 (t, 1H, H$_\alpha^{Cys}$, J= 6.2 Hz), 4.17 (m, 1H, H$_3$), 3.98 (m, 1H, H$_6$), 3.75, 3.40 (2×m, 2H, H$_{7ab}$), 3.08-3.17 (dd, 2H, H$_\beta^{Cys}$, J= 2.8 and 3.3 Hz), 2.80-2.64 (2×m, 2H, H$_\gamma^{Met}$), 2.45–2.14, 1.86–1.70 (2×m, 6H, H$_\beta^{Met}$, H$_{5ab}$, H$_{4ab}$), 2.25 (s, 3H, SCH$_3$), 1.47 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, D$_2$O/CD$_3$CN): δ 179.7, 176.2, 169.0 (C=O$^{amide\&carboxyl}$), 137.5 (C$_q^{Bn}$), 128.1, 127.6, 127.5 (C^{Bn}), 75.9 (C$_2$), 71.1, 70.7 (C$_6$ and C$_3$), 70.0 (CH$_2^{Bn}$), 53.5 (C$_\alpha^{Met}$), 52.5 (C$_\alpha^{Cys}$), 47.7 (C$_q^{tBu}$), 43.6 (C$_7$), 41.0 (C$_\beta^{Cys}$), 31.1, 28.9 (C$_{\beta\gamma}^{Met}$), 28.5 (*t*Bu), 22.8 (C$_4$), 21.5 (C$_5$), 13.9 (SCH$_3$). MS (ESI): *m/z* 588.2 (M+H)$^+$. HR-MS: calc. for [C$_{26}$H$_{41}$N$_3$O$_6$S$_3$+H]$^+$ 588.2230, found 588.2227.

N-[(6R)-1-({*N*-(S-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexopyranos-1-yl]-L-methionine (2,6-cis 21).
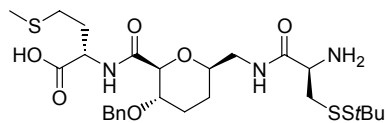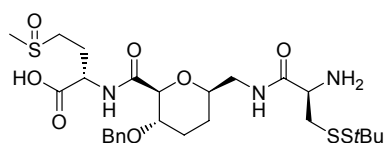
From 2,6-cis **16** (30 mg, 36 μmol) according to *GP 5*. The employed 1,4-dioxane was bubbled through with argon by sonification before use in the Tesser's base mixture. RP-HPLC purification (linear gradient B, 05→95%) afforded 2,6-cis **21** (4.6 mg, 7.8 μmol, 21%) as a white foam. LC-MS R$_t$= 13.4 min (lin. gradient B, 26 min). LC-MS still showed presence of 2,6-cis **22** (2,6-cis **22**/2,6-cis **21**: ~3/7). ^{1}H-NMR (600 MHz, D$_2$O/CD$_3$CN): δ 7.40-7.33 (m, 5H, H^{Bn}), 4.59 (d, 1H, PhCHH, J= 12.1 Hz), 4.51 (partly obscured by watersignal: 2H, PhCH and H$_\alpha^{Met}$), 4.35 (t, 1H, H$_\alpha^{Cys}$, J= 6.3 Hz), 3.78 (d, 1H, H$_2$, J= 9.4 Hz), 3.61-3.53 (m, 2H, H$_6$ and H$_{7a}$), 3.47 (ddd, 1H, H$_3$, J= 4.5, 6.0 and 10.3 Hz), 3.21 (2×d, 1H, H$_\beta^{Cys}$, J= 5.8 and 5.9 Hz), 3.14 (m, 2H, H$_\beta^{Cys}$ and H$_{7b}$), 2.52 (m, 2H, H$_\gamma^{Met}$), 2.30 (m, 1H, H$_{4a}$), 2.11, 1.94 (2m, 2H, H$_\beta^{Met}$), 2.05 (s, 3H, SCH$_3$), 1.76 (m, 1H, H$_{5a}$), 1.53 (m, 1H, H$_{4b}$), 1.40 (m, 1H, H$_{5b}$), 1.32 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, D$_2$O/CD$_3$CN): δ 179.7, 176.2, 169.0 (C=O$^{amide\&carboxyl}$), 137.5 (C$_q^{Bn}$), 128.1, 127.6, 127.5 (C^{Bn}), 75.9, 71.1, 70.7 (C$_2$, C$_3$, C$_6$), 70.0 (CH$_2^{Bn}$), 53.5 (C$_\alpha^{Met}$), 52.5 (C$_\alpha^{Cys}$), 47.7 (C$_q^{tBu}$), 43.6 (C$_7$), 41.0 (C$_\beta^{Cys}$), 31.1, 28.9 (C$_{\beta\gamma}^{Met}$), 28.5 (*t*Bu), 22.8, 21.5 (C$_4$, C$_5$), 13.9 (SCH$_3$). MS (ESI): *m/z* 588.2 (M+H)$^+$. HR-MS: calc. for [C$_{26}$H$_{41}$N$_3$O$_6$S$_3$+H]$^+$ 588.2230, found 588.2165.

N-[(6S)-1-({*N*-(S-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexopyranos-1-yl]-L-methionine sulfoxide (2,6-trans 22).
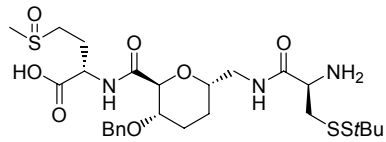
From 2,6-trans **16** (27 mg, 33 μmol) according to *GP 5*. RP-HPLC purification (linear gradient B, 20→70%) gave 2,6-trans **22** (3.6 mg, 6.0 μmol, 18%), white foam. LC-MS R$_t$= 11.8 min (lin. gradient B, 26 min). ^{1}H-NMR (600 MHz, D$_2$O/MeOD): δ 7.38-7.33 (m, 5H, H^{Bn}), 4.60 (dd, 2H, CH$_2^{Bn}$, J= 11.9 Hz), 4.71 (dd, 1H, H$_\alpha^{Met}$, J= 5.3 and 5.4 Hz), 4.45 (s, 1H, H$_2$), 4.18 (t, 1H, H$_\alpha^{Cys}$, J= 6.2 Hz), 4.02 (m, 1H, H$_3$), 3.98 (m, 1H, H$_6$), 3.70, 3.48 (2×m, 2H, H$_{7ab}$), 3.08-3.17 (d, 2H, H$_\beta^{Cys}$, J= 6.2 Hz), 2.97-2.87 (2×m, 2H, H$_\gamma^{Met}$), 2.70 (s, 3H, MeS=O), 2.36 (m, 1H, H$_{4a}$), 2.22 (m, 1H, H$_{5a}$), 1.99 (m, 1H, H$_{4b}$), 1.71 (m, 2H, H$_\beta^{Met}$), 1.55 (m, 1H, H$_{5b}$), 1.31 (s, 9H, *t*Bu). MS (ESI): *m/z* 604.5 (M+H)$^+$. HR-MS: calc. for [C$_{26}$H$_{41}$N$_3$O$_6$S$_3$+H]$^+$ 604.2179, found 604.2176.
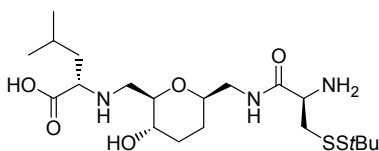
N-[(6R)-1-({*N*-(S-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexopyranos-1-yl]-L-methionine sulfoxide (2,6-cis 22).

From 2,6-cis **16** (17 mg, 21 μmol) according to *GP 5*. RP-HPLC purification (linear gradient B, 20→70%) afforded the title compound (3.0 mg, 5 μmol, 24%) as a white foam. LC-MS R_f = 11.6 min (lin. gradient B, 26 min). ^{1}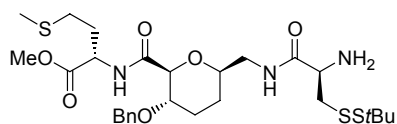H-NMR (600 MHz, D$_2$O/CD$_3$CN): δ 7.32-7.24 (m, 5H, H^{Bn}), 4.51 (partly obscured by solvent: 1H, PhC*H*), 4.42 (m, 2H, PhC*H* and H$_α^{Met}$), 4.09 (t, 1H, H$_α^{Cys}$, *J*= 6.2 and 6.3 Hz), 3.71 (d, 1H, H$_2$, *J*= 9.5 Hz), 3.51 (m, 1H, H$_6$), 3.43 (m, 2H, H$_3$ and H$_{7a}$), 3.16-3.04 (m, 3H, H$_β^{Cys}$ and H$_{7b}$), 2.75 (m, 2H, H$_γ^{Met}$), 2.52 (s, 3H, MeS=O), 2.20 (m, 2H, H$_{4a}$ and H$_β^{Met}$), 2.02 (m, 1H, H$_β^{Met}$), 1.68 (m, 1H, H$_{5a}$), 1.42 (m, 1H, H$_{4b}$), 1.33 (m, 1H, H$_{5b}$), 1.23 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, D$_2$O/CD$_3$CN, signals marked an ∗ appear double due to chiral sulfoxide): δ 176.8, 171.8, 168.7 (C=O$^{amide\&carboxyl}$), 138.5 (C$_q^{Bn}$), 129.4-128.9 (C^{Bn}), 81.5, 75.5, 74.0, 80.6, 76.4, 76.0 (C$_2$*, C$_3$* and C$_6$*), 71.7, 71.4 (CH$_2^{Bn*}$), 54.0, 53.3 (C$_α^{Met*}$, C$_α^{Cys*}$), 50.0 (C$_γ$S(O)CH$_3$), 49.2 (C$_q^{tBu}$), 44.0, 41.3 (C$_7$ and C$_β^{Cys}$), 37.5 (MeS=O). 29.8 (*t*Bu), 29.4 (C$_β^{Met}$), 27.6, 26.2 (C$_4$, C$_5$). MS (ESI): *m/z* 604.4 (M+H)$^+$. HR-MS: calc. for [C$_{26}$H$_{41}$N$_3$O$_6$S$_3$+H]$^+$ 604.2179, found 604.2180.

(2S,3S,6R)-3-hydroxy-6-{(*N*-[(S-(tert-butyl)sulfanyl]-L-cysteinyl)amino]methyl}-tetrahydropyran-1-*N*-L-leucine (2,6-cis 23).

From 2,6-cis **11** (20 mg, 28.4 μmol) according to *GP 5*. RP-HPLC purification (linear gradient B) gave the title compound (2.7 mg, 5.7 μmol, 20%). ^{1}H-NMR (600 MHz, MeOD): δ 4.85 (bs, 1H, H$_α^{Leu}$), 3.51 (m, 2H, H$_α^{Leu}$ and H$_2$), 3.41 (m, 1H, H$_{1a}$), 3.34–3.25 (m, 4H, H$_{7ab}$, H$_6$ and H$_3$), 3.25-3.15 (m, 3H, H$_β^{Cys}$ and H$_{1b}$), 2.12 (m, 1H, H$_{4a}$), 1.85 (m, 2H, H$_β^{Leu}$ and H$_γ^{Leu}$), 1.77 (m, 1H, H$_{5a}$), 1.68 (m, 1H, H$_β^{Leu}$), 1.52-1.41 (m, 2H, H$_{5b}$ and H$_{4b}$), 1.36 (s, 9H, *t*Bu), 1.02 (m, 6H, 2×CH$_3^{Leu}$). MS (ESI): *m/z* 466.4 (M+H)$^+$, 488.4 (M+Na)$^+$. HR-MS calc. for [C$_{26}$H$_{43}$N$_3$O$_5$S$_3$+Na]$^+$ 488.222886, found 488.222886.

N-[(6R)-6-({*N*-(S-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo pyranos-1-yl]-L-methionine methyl ester (2,6-cis 25).

A solution of 2,6-cis **16** (83 mg, 102 μmol) in DMF (4 mL) and 1 mL of piperidine was stirred for ½h, after which TLC analysis (PE/EtOAc 1/3 v/v) showed total consumption of 2,6-cis **16**. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in EtOAc, washed with 1N HCl and brine, the combined organic phases were dried (MgSO$_4$) and concentrated *in vacuo*. Silica gel chromatography (R$_f$= 0.5, EtOAc/MeOH 4/1 v/v) gave 2,6-cis **25** as an oil (50 mg, 81 μmol, 81%). ^{1}H-NMR (300 MHz, CDCl$_3$): δ 7.74 (t, C$_7$NH, 1H), 7.34-7.27 (m, 5H, H^{Bn}), 7.05 (d, 1H, NHC$_α^{Met}$, *J*= 7.8 Hz), 4.71 (dd, 1H, H$_α^{Met}$, *J*= 7.5 and 12.8 Hz), 4.59 (s, 2H, CH$_2^{Bn}$), 3.79 (d, 1H, H$_2$, *J*= 9.1 Hz), 3.74 (s, 3H, OCH$_3$), 3.66 (dd, 1H, H$_{7a}$, *J*= 3.6 and 3.7 Hz), 3.58-3.47 (m, 3H, H$_6$ and H$_3$ and H$_β^{Met}$), 3.27 (dd, 1H, H$_{7b}$, *J*= 3.7 Hz), 3.16 (m, 1H, H$_β^{Met}$), 2.49 (m, 2H, H$_γ^{Met}$), 2.25-1.96 (m, H$_{4a}$ and H$_β^{Cys}$), 2.06 (s, 3H, SCH$_3$), 1.75 (m, 1H, H$_{5a}$), 1.55 (m, 1H, H$_{4b}$), 1.39 (m, 1H, H$_{5b}$), 1.34 (s, 9H, *t*Bu); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 178.7, 173.4, 172.6 (C=O$^{amide\&ester}$), 139.6 (C$_q^{Bn}$), 129.3, 129.0, 128.6 (C^{Bn}), 81.4, 77.2, 76.1 (C$_2$, C$_3$, C$_6$), 72.2 (CH$_2^{Bn}$), 55.2 (C$_α^{Met}$), 52.9 (OCH$_3$), 52.5 (C$_α^{Cys}$), ~49 (C$_q^{tBu}$, obscured by solvent), 45.2, 44.3 (C$_7$ and C$_β^{Cys}$), 30.2 (*t*Bu), 31.9, 31.0, 30.0, 28.2 (C$_{βγ}^{Met}$, C$_4$, C$_5$), 15.2 (SCH$_3$). MS (ESI): *m/z* 602.4 (M+H)$^+$. HR-MS: calc. for [C$_{27}$H$_{43}$N$_3$O$_6$S$_3$+H]$^+$ 602.2392, found 602.2370.

Procedure Protein prenylation in Met-18b-2 CHO cells – Method according to Cohen *et al.*:[6] Met-18b-2 CHO cells[19] were grown in HamF12/DMEM (1:1) medium supplemented with 5% FCS. The cells were incubated in the same medium containing 5% dialysed FCS and 5 μM simvastatin in the presence or absence of 100 μM of 2,6-cis-**25** for 2 h at 37°C and the incubation was continued after addition of [^{3}H]-mevalonate (20 μCi/mL) for 18 h at 37°C. The monolayers were washed and lysed in SDS-sample buffer.

After the cell lysates had been sheared and heated for 5 min at 95°C, proteins were separated by electrophoresis on 12.5% polyacrylamide-SDS gels. Subsequently the gel was impregnated with EN³HANCE (NEN), dried and the radiolabeled bands were made visible by exposure to Kodak X-omat AR film for 6 days at –80°C.

3.5 References and Notes

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(21)   L. H. Cohen, E. Pieterman, R. E. W. van Leeuwen, B. E. A. Burm, G. A. van der Marel, J. H. van Boom *Biochem. Pharm.* **2000**, *60*, 1061.

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(23)   Subjection of diols 2,6-cis **2** and 2,6-trans **2** (Scheme 3.2) to the Fukuyama/Mitsunobu glycosylation conditions yielded no products. This may indicate that the additional presence of the secondary hydroxyl is not compatible with the applied Fukuyama/Mitsunobu chemistry. Subjection of the methionine derived 2,6-cis **7** and 2,6-trans **7** to the same reductive conditions used for the synthesis

of 2,6-cis **10** (Scheme 3.3), gave poor results. This may be accounted to poisoning of the Pd/C by the sulfur atom of the methionine residue.

Chapter 4

Synthesis and Biological Evaluation of Lipophilic Ca$_1$a$_2$L Analogs as Potential Bisubstrate Inhibitors of Protein:geranylgeranyl Transferase-1

Farid El Oualid, Jayand Baktawar, Ingrid M. Leroy, Hans van den Elst, Louis H. Cohen, Gijs A. van der Marel, Herman S. Overkleeft, Mark Overhand *Bioorg. Med. Chem.* **2005**, *13*, 1463 – 1475.

Abstract. The attachment of lipids, with or without a linker, to several of the SAA modified Ca$_1$a$_2$L analogs presented in Chapter 2 and 3 gave lipophilic Ca$_1$a$_2$L analogs as potential bisubstrate inhibitors of PGGT-1. The inhibitory potency toward PGGT-1 of these lipophilic Ca$_1$a$_2$L analogs was improved in comparison with the corresponding original Ca$_1$a$_2$L analogs (IC$_{50}$ values in the low micromolar range.

4.1 Introduction

The reported strategies aimed at the development of PFT and PGGT-1 inhibitors are generally based on either of the two substrates, that is, the isoprenyl pyrophosphate entity or the Ca_1a_2X tetrapeptide, as a lead structure. A relatively unexplored area of research entails the design of bisubstrate analog inhibitors,[1] containing elements from both the tetrapeptide and isoprenyl moiety. The idea that product analogs might be effective PFT/PGGT-1 inhibitors originates from the finding that isoprenyl transferases exhibit an unusually high affinity for their two substrates and especially for the turnover product. Isoprenylated proteins are removed from the active site only when a new isoprenyl pyrophosphate enters the active site.[2] Based on these considerations, several research groups have reported on the development of bisubstrate inhibitors against PFT.[3] In line with these studies, lipophilic Ca_1a_2L analogs were constructed as potential bisubstrate inhibitors of PGGT-1.

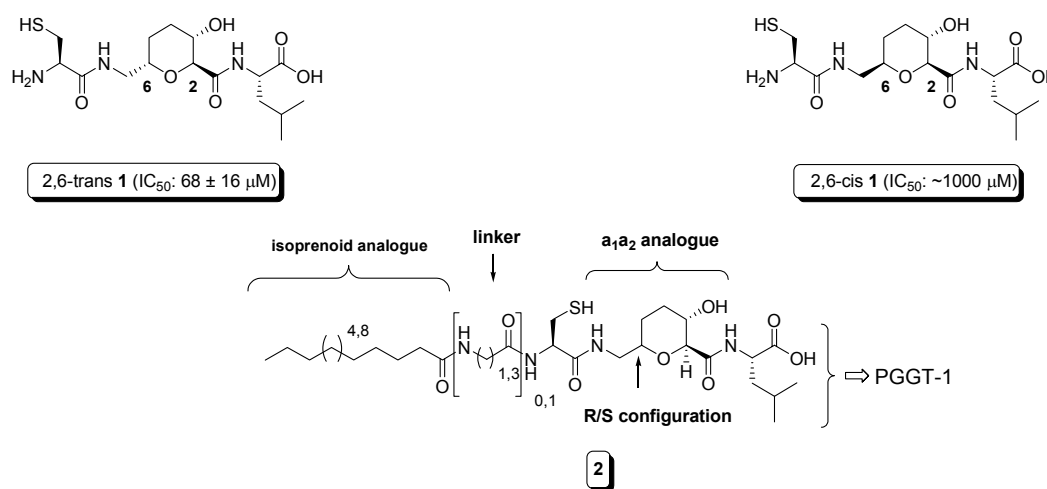**Figure 4.1** Lipophilic Ca_1a_2L analogs as potential bisubstrate inhibitors of PGGT-1.

The discovery of new PGGT-1 inhibitors[4] out of a series of Ca_1a_2L analogs featuring sugar amino acid (SAA) based dipeptide isosters as replacement of the central a_1a_2 dipeptide was presented in Chapters 2 and 3. The Ca_1a_2L analog 2,6-trans **1** (Figure 4.1), in which the amino acids cysteine and leucine are arranged in a 2,6-trans fashion on the pyranoid SAA core, was found to inhibit PGGT-1 with an IC_{50}-value of 68 ± 16 μM. In contrast, the corresponding 2,6-cis analog (2,6-cis **1**), with the stereochemistry at C_6

inverted, was found to be much less active against PGGT-1, with an IC_{50}-value of ≈ 1000 μM.[5] On the basis of these results, the preparation of a set of lipophilic Ca_1a_2L analogs with general structure **2** (Figure 4.1) was undertaken. The potential bisubstrate inhibitors are composed of Ca_1a_2L analogs 2,6-cis **1** or 2,6-trans **1** which are connected, either directly or *via* a linker (C_2: glycine or C_4: 4-aminobutyric acid), to lauric acid (C_{12}) or palmitic acid (C_{16}). It should be noted that a saturated fatty acid is known to be well tolerated by PFT and PGGT-1 as an isoprenyl analog.[6]
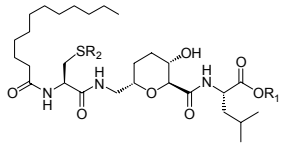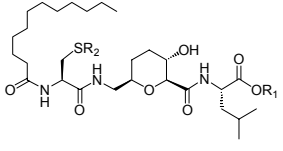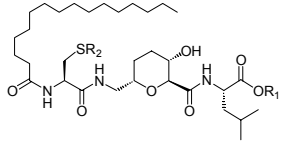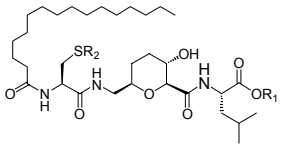
4.2 Results and Discussion
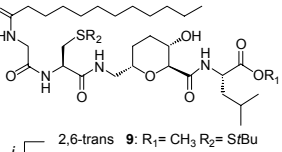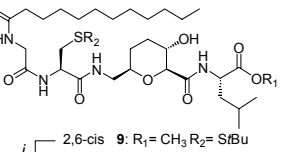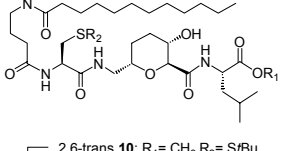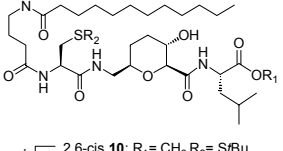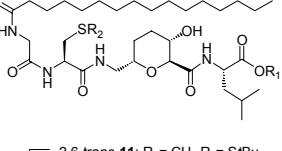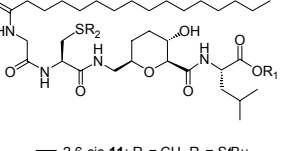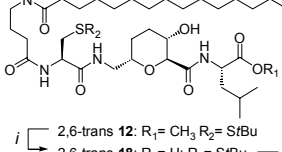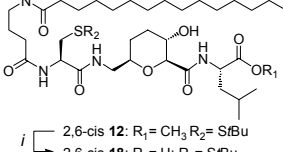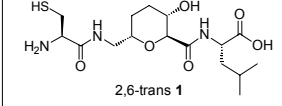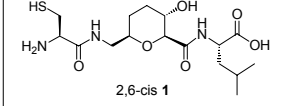
4.2.1 Synthesis – The synthesis of the partially protected precursors of the projected inhibitors, having a 2,6-trans or 2,6-cis relationship in the central SAA residue, is shown in Schemes 4.1 and 4.2, respectively. TFA/DCM mediated removal of the Boc group in compounds 2,6-trans **3** and 2,6-cis **3**, the syntheses of which are reported in Chapter 2, and condensation of the free amine with Boc-Cys(S*t*Bu)-OH (BOP, HOBt, DIPEA) furnished suitably protected Ca_1a_2L analogs 2,6-trans **4** and 2,6-cis **4**, respectively, both in 72% overall yield. Next, unmasking the amine in 2,6-trans **4** and 2,6-cis **4** followed by condensation with either lauric $[CH_3(CH_2)_{10}CO_2H]$ or palmitic acid $[(CH_3(CH_2)_{14}CO_2H]$ with BOP/HOBt gave 2,6-trans **7** and 2,6-trans **8** and 2,6-cis **7** and 2,6-cis **8**, respectively (84%–100%, 2 steps). The synthesis of lipophilic Ca_1a_2L analogs provided with a linker started with condensation of Boc-Gly-OH or Boc-4-aminobutyric acid with the ammonium salt of 2,6-trans **4** or 2,6-cis **4** to give the desired 2,6-trans **5** and 2,6-trans **6** and 2,6-cis **5** and **6**, respectively (70%–75%, 2 steps). Finally, these intermediates were elongated with lauric or palmitic acid according to the same procedure as described for **7** and **8**, furnishing 2,6-trans **9** – **12** (64–95%, 2 steps) and 2,6-cis **9** – **12** (78%–100%, 2 steps).

The partially protected precursors 2,6-trans **7** – **12** and 2,6-cis **7** – **12** were converted to the target bisubstrate analogs (2,6-trans **19** – **24** and 2,6-cis **19** – **24**, respectively) by a two step deprotection procedure (Scheme 4.3): aq. LiOH mediated saponification of the methyl ester released the acid (**13** – **18**) and treatment with DTT resulted in cleavage of the S*t*Bu group. The crude products were purified by RP-HPLC and characterised by LC-MS analysis.

Scheme 4.1 Synthesis of lipophilic Ca₁a₂L analogs based on 2,6-trans **1**.*ᵃ*
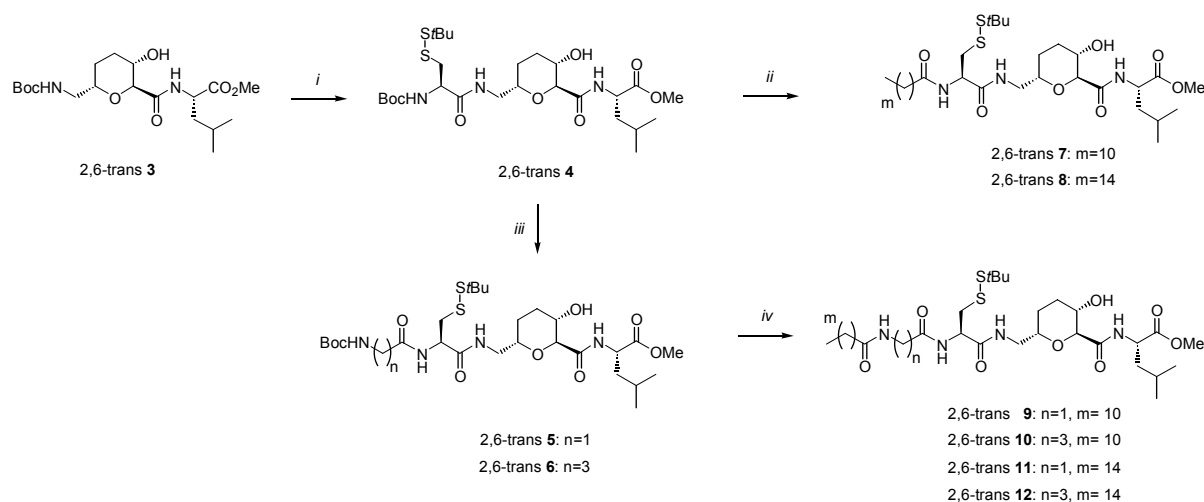
Scheme 4.2 Synthesis of lipophilic Ca₁a₂L analogs based on 2,6-cis **1**.*ᵃ*
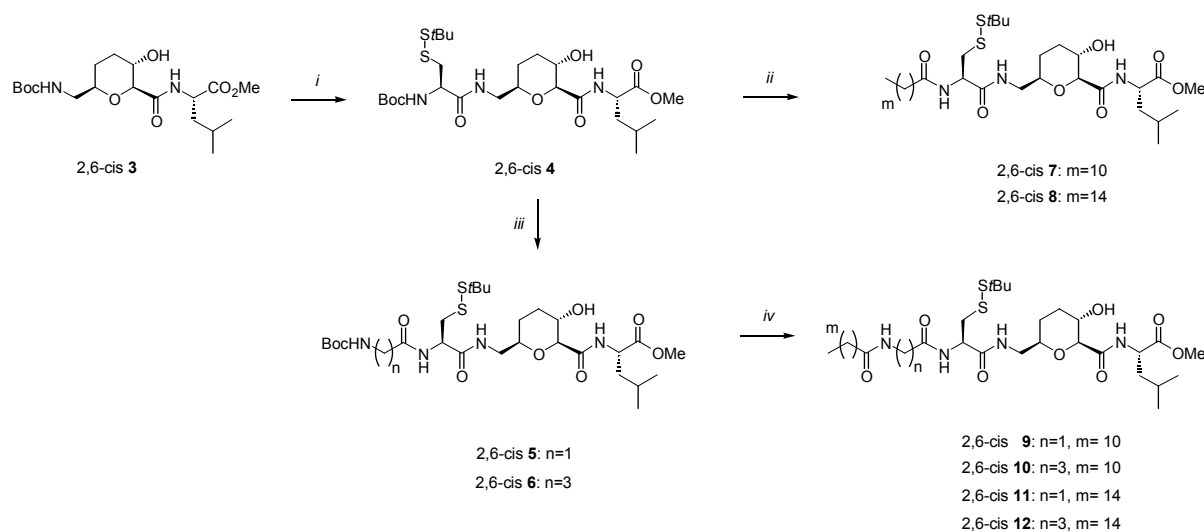
*ᵃ*Reagents and conditions. (*i*) (a) TFA/DCM, *i*Pr₃SiH (b) Boc-Cys(S*t*Bu)-OH, BOP, DIPEA, HOBt, DMF/DCM (72%, over 2 steps); (*ii*) (a) TFA/DCM, *i*Pr₃SiH (b) for 2,6-trans **7** and 2,6-cis **7**: CH₃(CH₂)₁₀CO₂H, for 2,6-trans **8** and 2,6-cis **8**: CH₃(CH₂)₁₄CO₂H, BOP, DIPEA, HOBt, DMF/DCM (2,6-trans **7**: 100%, 2,6-trans **8**: 87%, 2,6-cis **7**: 91%, 2,6-cis **8**: 84%, 2 steps); (*iii*) (a) TFA/DCM, *i*Pr₃SiH (b) for 2,6-trans **5** and 2,6-cis **5**: Boc-Gly-OH, for 2,6-trans **6** and 2,6-cis **6**: Boc-4-aminobutyric acid, BOP, DIPEA, HOBt, DMF/DCM (2,6-trans **5**: 75%, 2,6-trans **6**: 75%, 2,6-cis **5**: 72%, 2,6-cis **6**: 70%, 2 steps); (*iv*) (a) TFA/DCM, *i*Pr₃SiH (b) for 2,6-trans **9**, 2,6-trans **10**, 2,6-cis **9** and 2,6-cis **10**: CH₃(CH₂)₁₀CO₂H, for 2,6-trans **11**, 2,6-trans **12**, 2,6-cis **11** and 2,6-cis **12**: CH₃(CH₂)₁₄CO₂H, BOP, DIPEA, HOBt, DMF/DCM (2,6-trans **9**: 95%, 2,6-trans **10**: 64%, 2,6-trans **11**: 90%, 2,6-trans **12**: 85%, 2,6-cis **9**: 100%, 2,6-cis **10**: 92%, 2,6-cis **11**: 98%, 2,6-cis **12**: 78%, 2 steps).

Scheme 4.3 Deprotection 2,6-trans **7**-**12** and 2,6-cis **7**-**12**.[a]
Biological evaluation of target compounds 2,6-trans **19**-**24** and 2,6-cis **19**-**24**.[b]

	Activity (%)[b]				Activity (%)[b]	
	10 μM	100 μM			10 μM	100 μM
2,6-trans **7**: R₁= CH₃ R₂= StBu 2,6-trans **13**: R₁= H; R₂= StBu 2,6-trans **19**: R₁= R₂= H	96	32		2,6-cis **7**: R₁= CH₃ R₂= StBu 2,6-cis **13**: R₁= H; R₂= StBu 2,6-cis **19**: R₁= R₂= H	>100	44
2,6-trans **8**: R₁= CH₃ R₂= StBu 2,6-trans **14**: R₁= H; R₂= StBu 2,6-trans **20**: R₁= R₂= H	82	21		2,6-cis **8**: R₁= CH₃ R₂= StBu 2,6-cis **14**: R₁= H; R₂= StBu 2,6-cis **20**: R₁= R₂= H	57	31
2,6-trans **9**: R₁= CH₃ R₂= StBu 2,6-trans **15**: R₁= H; R₂= StBu 2,6-trans **21**: R₁= R₂= H	71	19		2,6-cis **9**: R₁= CH₃ R₂= StBu 2,6-cis **15**: R₁= H; R₂= StBu 2,6-cis **21**: R₁= R₂= H	80	57
2,6-trans **10**: R₁= CH₃ R₂= StBu 2,6-trans **16**: R₁= H; R₂= StBu 2,6-trans **22**: R₁= R₂= H	>100	79		2,6-cis **10**: R₁= CH₃ R₂= StBu 2,6-cis **16**: R₁= H; R₂= StBu 2,6-cis **22**: R₁= R₂= H	78	27
2,6-trans **11**: R₁= CH₃ R₂= StBu 2,6-trans **17**: R₁= H; R₂= StBu 2,6-trans **23**: R₁= R₂= H	57	15		2,6-cis **11**: R₁= CH₃ R₂= StBu 2,6-cis **17**: R₁= H; R₂= StBu 2,6-cis **23**: R₁= R₂= H	89	43
2,6-trans **12**: R₁= CH₃ R₂= StBu 2,6-trans **18**: R₁= H; R₂= StBu 2,6-trans **24**: R₁= R₂= H	48	10		2,6-cis **12**: R₁= CH₃ R₂= StBu 2,6-cis **18**: R₁= H; R₂= StBu 2,6-cis **24**: R₁= R₂= H	87	42
2,6-trans **1**	>100	42		2,6-cis **1**	>100	80

[a](*i*) 1M LiOH, H₂O/1,4-dioxane, 0°C→rt; (*ii*) (a) DTT, Tris buffer (pH 7.4), MeOH or EtOH (b) RP-HPLC purification. [b]Activity of PGGT-1 at 10 or 100 μM of compound: expressed as % of control activity (*i.e.* without test compound).

4.2.2 Biological Evaluation – All compounds (2,6-trans **19** – **24** and 2,6-cis **19** – **24**) were evaluated for their inhibitory potency against PGGT-1 (Scheme 4.3) by determining the residual enzyme activity *in vitro* at 2 different concentrations (10 and 100 μM) according to a procedure previously described.[4d] The results of the biological evaluation are presented in Scheme 4.3. Similar to the monosubstrate analogs (2,6-trans **1** and 2,6-cis **1**, Figure 4.1 and Scheme 4.3), the most potent compounds 2,6-trans **23** and 2,6-trans **24** feature a 2,6-trans substitution pattern on the SAA moiety. Determination of the IC_{50}-values revealed that 2,6-trans **23** (IC_{50}= 12.7±1.3 μM) and 2,6-trans **24** (IC_{50}= 12.3±1.0 μM), differing in the nature of the linker, inhibit PGGT-1 with equal efficacy, representing a ≈6-fold improvement in potency compared to the corresponding monosubstrate 2,6-trans **1** (IC_{50}= 68±16 μM).

Whereas monosubstrate 2,6-cis **1** showed little activity below 1000 μM, compounds 2,6-cis **19** – **24**, featuring an isoprenyl analog, all appeared to exhibit an enhanced activity. The most potent member of the 2,6-cis series was found to be 2,6-cis **20**, in which the C_{16} palmitic acid is directly connected to the cysteine. In contrast to the 2,6-trans series, where the introduction of a longer alkyl chain and linker gradually increases the inhibitory potency, introduction of a linker or increasing the length of the alkyl chain in the 2,6-cis series seems to have no additional effect on the potency. Although inhibitors based on 2,6-cis **1** yielded (slightly) less potent inhibitors, the gain of inhibition potency is more pronounced in comparison with the series based on 2,6-trans **1**.

4.3 Conclusions

Summarizing, it has been shown that the attachment of simple lipids such as lauric or palmitic acid (with or without a linker) to the previously presented Ca_1a_2L analogues 2,6-trans and 2,6-cis **1**, is a promising approach to increase their inhibition potency against PGGT-1. The most potent compounds (2,6-trans **23** and 2,6-trans **24**) were found to inhibit PGGT-1 in the low micromolar range (≈6 fold improvement over the corresponding monosubstrate analogs). At the moment there is no experimental prove that the inhibitors presented in this chapter actually act by occupying the peptide and isoprenyl pyrophosphate pocket of the enzyme. Due to the presence of different hydrophobic pockets in the active site of PGGT-1, in which the introduced acyl residues

could bind, alternative binding modes are feasible. Furthermore, as the lipophilic Ca_1a_2L analogs are provided with the zinc-binding thiol function, it is unlikely that they adopt a product-like conformation in the active site of the enzyme.[2bf]

4.4 Experimental Section

4.4.1 General – ^1H NMR and ^{13}C NMR spectra were recorded with a Bruker AC-200 (200, 50.1 MHz), a Bruker DPX-300 (300, 75 MHz), a Bruker Avance-400 (400, 100 MHz) or a Bruker DMX-600 (600, 150 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Mass spectra were recorded with a Perkin Elmer/SCIEX API 165 mass instrument and HR-Mass spectra were recorded with an API QSTAR^{tm} Pulsar (Applied Biosystems). LC-MS analysis was performed on a Jasco HPLC system (detection simultaneously at 214 and 254 nm) coupled to the Perkin Elmer/SCIEX API 165 mass instrument. Column chromatography was performed on silica gel 60 (0.04–0.063 mm, Fluka). DMF (Biosolve p.a.), 1,4-dioxane (Biosolve p.a.), DCM (Biosolve, p.a.) and toluene (Biosolve, p.a.) were stored over molecular sieves (4Å). EtOAc and PE (40-60) were of technical grade and distilled before use. Leu-OMe·HCl (Nova Biochem), BOP (Nova Chemicals), DIPEA (Biosolve), iPrSi$_3$H (Aldrich), TFA (Biosolve), Boc-Cys(StBu)-OH (NovaBiochem), Boc-Gly-OH (Bissendorf Biochemicals), Boc-4-aminobutyric acid (NeoSystems), palmitic acid (Janssen Chimica), lauric acid (Acros), Boc$_2$O (Fluka) were used as received. Reactions were followed by TLC analysis on silicagel (Schleicher & Schuell, F 1500 LS 254) or HPTLC aluminium sheets (Merck, silicagel 60, F254), with detection by UV-absorption (254 nm) where applicable and charring at 150°C with 20% H_2SO_4 in EtOH (25 g L^{-1}), ninhydrin (3 g L^{-1}) in EtOH/AcOH (100/3 v/v), $NH_4(Mo)_7O_{24}·4H_2O$ (25 g L^{-1}) and $NH_4Ce(SO_4)_4·2H_2O$ (10 g L^{-1}) in 10% aq. H_2SO_4 or KMnO$_4$ (2%) in 1% aq. K_2CO_3. Column chromatography was performed with silica gel (Fluka; 0.063-0.200 mm).

4.4.2 General procedures – *General procedure 1a (GP 1a); removal of the Boc protective group:* to a ~0.05M soln. of the dimer in DCM were added 1.3 equiv. iPr$_3$SiH or Et$_3$SiH and TFA (→TFA/DCM 1/1 v/v). After TLC analysis (PE/EtOAc 1/1 v/v) showed consumption of the starting material, the TFA was coevaporated with toluene (5×10 mL). The free amine can be visualised with TLC analysis by employing Et$_2$O/EtOH/25% aq. ammonia (6/3/1 v/v/v) and spraying with ninhydrin.

General procedure 1b (GP 1b); condensation with RCO$_2$H: to a ~0.1M soln. of the amine in DMF were added 1.2 equiv. of the appropriate acid, 1.2 equiv. BOP and 4 equiv. DIPEA. After TLC analysis (DCM/MeOH 9/1 v/v KMnO$_4$) showed consumption of the starting material, DMF was removed *in vacuo*. The residue was dissolved in EtOAc and washed with water (2×), sat. aq. NaHCO$_3$ (2×), 5% KHSO$_4$ (2×) and brine. The organic phase was dried (MgSO$_4$) and concentrated *in vacuo*.

General procedure 2 (GP 2); saponification methyl ester: to a stirred solution of the methyl ester in 1,4-dioxane/H$_2$O (1/1 v/v) at 0°C was added aq. LiOH (1.0 M, 1.0 equiv.) and the temperature was allowed to rise to room temperature. After TLC analysis (DCM/MeOH 9/1 v/v) showed consumption of the starting material (30-45 min) the reaction mixture was neutralised (pH ~7) by addition of Amberlite-H$^+$. The Amberlite-H$^+$ was filtered off and the solvents were removed *in vacuo* by co-evaporation with toluene. Dissolving the crude acid in DCM allowed precipitation of the product in cold petroleum ether. For LC-MS analysis eluent A: 50% aq. MeOH, B: CH$_3$CN and C: 0.1% methanolic TFA were employed.

General procedure 3 (GP 3); DTT mediated removal of StBu group: to a solution of the disulfide in MeOH or EtOH (c 0.025–0.05 M) is added Tris-HCl buffer (pH 7.4, 1 mL) after which the solution is

degassed with argon. Subsequently DTT (25-50 equiv.) is added and the reaction mixture is stirred under argon for 24 h. Next, the reaction mixture is diluted with *t*BuOH/CH$_3$CN/H$_2$O (1/1/1 v/v/v, *c* = 5 – 10 mg mL^{-1}) and the crude thiol is purified by RP-HPLC employing a linear gradient of CH$_3$CN (= eluent B) in 50% aq. MeOH (= eluent A) and 0.1% methanolic TFA (= eluent C).

N-(6-[(*N*-tert-butyloxycarbonyl)-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono

pyranosyl)-L-leucine methyl ester (2,6-trans 4). Following *GP 1a* and *1b* employing 2,6-trans **3** (1.8 g, 4.6 mmol) and Boc-Cys(S*t*Bu)-OH (1.7 g, 5.5 mmol) gave after purification by silica gel chromatography the titl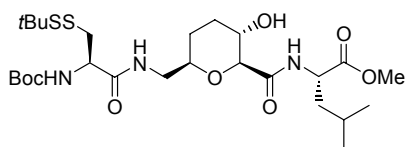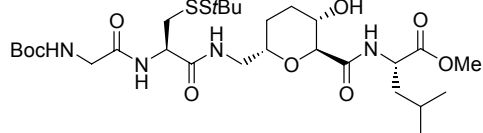e compound (R$_f$ = 0.5, EtOAc) in 72% yield as a colorless oil. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.57, 7.40 (2m, 2H, NHC$_\alpha^{Leu}$, NHC$_7$), 5.89 (bs, 1H, NHBoc), 4.60 (bs, 1H, H$_\alpha^{Leu}$), 4.39 (bs, 1H, H$_\alpha^{Cys}$), 4.06 (d, 1H, H$_6$, *J* = 7.2 Hz), 3.97 (bs, 1H, H$_6$), 3.87 (bs, 1H, H$_3$), 3.76 (s, 3H, OCH$_3$), 3.43-3.38 (m, 2H, H$_7$), 3.10 (m, 2H, H$_\beta^{Cys}$), 1.90, 1.73-1.68 (m, 7H, H$_{4\&5}$, H$_{\beta\gamma}^{Leu}$), 1.46, 1.34 (2×s, 18H, 2×*t*Bu), 0.96 (m, 6H, 2×CH$_3^{Leu}$); ^{13}C-NMR (75 MHz, CDCl$_3$) δ 173.2-171.0 (C=O$^{ester\&amide}$), 154.7 (C=O^{Boc}), 79.1 (C$_q$ *t*Bu^{Boc}), 73.5, 71.6, 66.2 (C$_2$, C$_3$, C$_6$), 54.1, 51.7, 49.6 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$, OCH$_3$), 47.2 (C$_7$), 41.8, 39.5 (C$_q$ S*t*Bu , C$_\beta^{Leu}$, C$_\beta^{Cys}$), 29.0, 27.4 (2×*t*Bu), 26.1, 23.2 (C$_4$, C$_5$), 24.1 (C$_\gamma^{Leu}$), 22.1, 20.8 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 594.5 (M+H)$^+$, 616.4 (M+Na)$^+$. HR-MS: calc. for [C$_{26}$H$_{47}$N$_3$O$_8$S$_2$+H]$^+$ 594.28773, found 594.28674. [α]$_D^{20}$ = +2.0 (CHCl$_3$, *c* = 0.5).
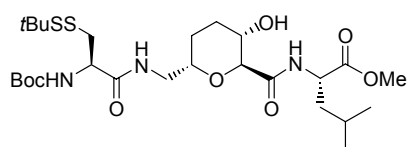
N-(6-[(*N*-tert-butyloxycarbonyl)-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono
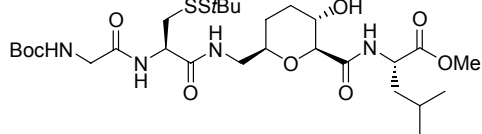

pyranosyl)-L-leucine methyl ester (2,6-cis 4). Following *GP 1a* and *1b* using 2,6-cis **3** (0.8 g, 2.1 mmol) and Boc-Cys(S*t*Bu)-OH (0.8 g, 2.5 mmol) gave after purification by silica gel chromatography the title compound (R$_f$ = 0.5, EtOAc) in 72% yield as a colorless oil. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.13 (d, 1H, NH, *J* = 7.2 Hz), 6.80 (t, 1H, NH, *J* = 4.8 and 5.6 Hz), 5.53 (bs, 1H, NHBoc), 4.51 (m, 1H, H$_\alpha^{Leu}$), 4.29 (dd, 1H, H$_\alpha^{Cys}$, *J* = 6.6 and 6.9 Hz), 3.95 (d, 1H, H$_2$, *J* = 8.0 Hz), 3.88 (m, 1H, H$_6$), 3.71 (m, 1H, H$_3$), 3.67 (s, 3H, CH$_3$O), 3.64 (m, 1H, H$_{7a}$), 3.17 (m, 1H, H$_{7b}$), 3.05 (m, 2H, H$_\beta^{Cys}$), 1.84 (m, 1H, H$_{4a}$), 1.69 (m, 1H, H$_{5a}$), 1.57 (m, 5H, H$_{4b}$, H$_{5b}$, H$_{\beta\gamma}^{Leu}$), 1.37, 1.25 (2×s, 18H, 2×*t*Bu), 0.86 (dd, 6H, 2×CH$_3^{Leu}$ *J* = 6.2 and 6.5 Hz); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 172.8-170.9 (C=O$^{ester\&amide}$), 155.4 (C=O^{Boc}), 79.7 (C$_q^{tBu Boc}$), 77.8, 76.6, 67.9 (C$_2$, C$_3$, C$_6$), 53.8, 52.0, 49.7 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$, OCH$_3$), 47.7 (C$_7$), 43.2, 41.7, 40.7 (C$_q^{StBu}$, C$_\beta^{Leu}$, C$_\beta^{Cys}$), 30.4, 26.8 (C$_4$, C$_5$), 29.4, 27.9 (2×*t*Bu), 24.5 (C$_\gamma^{Leu}$), 22.4, 21.5 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 594.4 (M+H)$^+$, 616.4 (M+Na)$^+$. HR-MS: calc. for [C$_{26}$H$_{47}$N$_3$O$_8$S$_2$+H]$^+$ 594.28773, found 594.28687. [α]$_D^{20}$ = −42 (CHCl$_3$, *c* = 0.5).

N-(6-[(*N*-(*N*-tert-Butyloxycarbonyl-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-

glucuronopyranosyl)-L-leucine methyl ester (2,6-trans 5). Following *GP 1a* and *1b* using 2,6-trans **4** (94 mg, 0.16 mmol) and Boc-Gly-OH (33.3 mg, 0.19 mmol) gave the title compound (R$_f$ = 0.5, EtOAc/acetone 1/1 v/v) in 75% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.40 (d, 1H, NH, *J* = 7.4 Hz), 7.30 (m, 1H, NH), 5.40 (bs, 1H, NHBoc), 4.78 (dt, 1H, H$_\alpha^{Cys}$, *J* = 5.5, 5.6 and 5.7 Hz), 4.65 (m, 1H, H$_\alpha^{Leu}$), 4.13 (m, 1H, H$_6$), 4.02 (d, 1H, H$_2$, *J* = 8.5 Hz), 3.85 (dd, 1H, H$_\alpha^{Gly}$, *J* = 5.7 Hz), 3.76 (m, 5H, H$_3$, H$_\alpha^{Gly}$, OCH$_3$), 3.57, 3.45 (2×m, 2H, H$_{7ab}$), 3.34 (m, 1H, H$_\beta^{Cys}$), 3.06 (dd, 1H, H$_\beta^{Cys}$ *J* = 5.1 Hz), 1.95 (m, 1H, H$_{4a}$), 1.85 (m, 1H, H$_{5a}$), 1.67 (m, 5H, H$_{4b5b}$, H$_{\beta\gamma}^{Leu}$), 1.45, 1.33 (2×s, 18H, 2×*t*Bu), 0.86 (m, 6H, 2×CH$_3^{Leu}$); ^{13}C-NMR (100 MHz, acetone-*d6*) δ 173.5-170.6 (C=O$^{ester\&amide}$), 157.2 (C=O^{Boc}), 79.7 (C$_q^{tBu Boc}$), 76.2 (C$_2$), 72.5 (C$_6$), 66.7 (C$_3$), 54.0, 52.4 (OCH$_3$, C$_\alpha^{Cys}$), 50.8 (C$_\alpha^{Leu}$), 48.3 (C$_q^{tBu}$), 44.8 (C$_7$), 42.8 (C$_\alpha^{Gly}$), 41.6 (C$_\beta^{Cys}$), 40.9 (C$_\beta^{Leu}$), 30.5, 29.2 (2×*t*Bu), 27.4 (C$_4$), 25.4 (C$_\gamma^{Leu}$), 24.0 (C$_5$), 23.3, 21.6 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 651.4 (M+H)$^+$, 673.5 (M+Na)$^+$. HR-MS: calc. for [C$_{28}$H$_{50}$N$_4$O$_9$S$_2$+H]$^+$ 651.30920, found 651.30963. [α]$_D^{20}$ = −25.6 (CHCl$_3$, *c* = 0.25).

N-(6-[(N-(N-tertButyloxycarbonyl-glycine))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-cis 5). Followi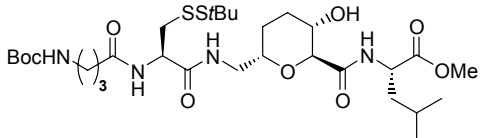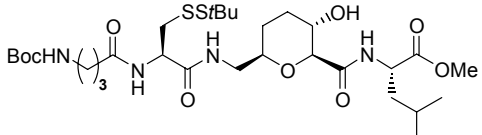ng *GP 1a* and *1b* using 2,6-cis **4** (70 mg, 0.12 mmol) and Boc-Gly-OH (25 mg, 0.14 mmol) gave 2,6-cis **5** (R_f 0.56, EtOAc/acetone 1/1 v/v) in 72% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.40 (d, 1H, NH, J= 7.3 Hz), 7.17 (m, 1H, NH), 5.41 (bs, 1H, NHBoc), 4.78 (dd, 1H, H$_\alpha^{Cys}$, J= 6.6 and 6.7 Hz), 4.66 (m, 1H, H$_\alpha^{Leu}$), 3.76 (m, 5H, H$_\alpha^{Gly}$, CH$_3$O), 3.63 (d, H$_2$, 1H, J= 9.3 Hz), 3.54 (m, H$_3$, H$_6$), 3.46 (m, 1H, H$_{7a}$), 3.28, 3.25 (2×dd, 1H, H$_{7b}$, J= 2.2, 2.5 and 2.6 Hz), 3.20 (dd, 1H, H$_\beta^{Cys}$, J= 6.0 and 6.4 Hz), 3.06 (dd, 1H, H$_\beta^{Cys}$, J= 6.4 and 6.5 Hz), 2.17 (m, 1H, H$_{4a}$), 1.67 (m, 4H, H$_{4b}$, H$_{\beta\gamma}^{Leu}$), 1.55–1.40 (m, 2H, H$_{4b\&5b}$), 1.45, 1.33 (2×s, 18H, 2×*t*Bu), 0.86 (dd, 6H, 2×CH$_3^{Leu}$, J= 6.1 Hz); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 173.7, 172.2, 170.3 (C=O$^{ester\&amide}$), 156.1 (C=O^{Boc}), 80.1 (C$_q^{tBu\ Boc}$), 77.7 (C$_2$), 76.7 (C$_6$), 68.2 (C$_3$), 53.0, 52.4 (C$_\alpha^{Leu}$, OCH$_3$), 49.7 (C$_\alpha^{Cys}$), 48.1 (C$_q^{tBu}$), 44.1 (C$_\alpha^{Gly}$), 43.6 (C$_7$), 41.1, 40.8 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 30.5 (C$_4$), 29.6, 28.2 (2×*t*Bu), 27.1 (C$_5$), 24.7 (C$_\gamma^{Leu}$), 22.6, 21.6 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 650.3 (M+H)$^+$, 673.5 (M+Na)$^+$. HR-MS: calc. for [C$_{28}$H$_{50}$N$_4$O$_9$S$_2$+H]$^+$ 651.30920, found 651.31061. [α]$_D^{20}$= −67 (CHCl$_3$, *c*= 1).

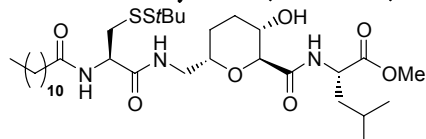**N-(6-[(N-(4-N-tertButyloxycarbonyl-aminobutyric acid))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-trans 6).** Following *GP 1a* and *1b* using 2,6-trans **4** (254 mg, 0.43 mmol) and Boc-4-aminobutyric acid (104 mg, 0.51 mmol) gave the title compound (R_f 0.7, EtOAc/acetone 1/1 v/v) in 75% yield. ^{13}C-NMR (50 MHz, acetone-*d6*) δ 173.4, 172.2, 170.7 (C=O$^{ester\&amide}$), 157.0 (C=O^{Boc}), 78.5 (C$_q^{tBu\ Boc}$), 76.1 (C$_2$), 72.4 (C$_6$), 66.9 (C$_3$), 54.2, 52.2 (C$_\alpha^{Leu}$, OCH$_3$), 50.7 (C$_\alpha^{Cys}$), 48.1 (C$_q$, S*t*Bu), 42.8, 41.4. 40.7, 39.8 (C$_7$, C$_\beta^{Leu}$, C$_\beta^{Cys}$, CH$_2$), 33.0, 27.2, 26.6, 24.0 (C$_4$, C$_5$, 2×CH$_2$), 29.9, 28.5 (2×*t*Bu), 25.3 (C$_\gamma^{Leu}$), 23.2, 21.4 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 679.5 (M+H)$^+$, 701.4 (M+Na)$^+$. HR-MS: calc. for [C$_{30}$H$_{54}$N$_4$O$_9$S$_2$+H]$^+$ 679.34050, found 679.34375. [α]$_D^{20}$= −16 (CHCl$_3$, *c*= 1).

N-(6-[(N-(4-N-tertButyloxycarbonyl-aminobutyric acid))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-cis 6). Following *GP 1a* and *1b* using 2,6-cis **4** (0.25 g, 0.42 mmol) and Boc-4-aminobutyric acid (0.10 g, 0.51 mmol) gave 2,6-cis **6** (R_f 0.65, EtOAc/acetone 1/1 v/v) in 70% yield. δ 7.21 (m, 1H, NH, J= 7.3 Hz), 7.12 (m, 1H, NH), 4.80 (bs, 1H, NHBoc), 4.63 (m, 2H, H$_\alpha^{Cys}$, H$_\alpha^{Leu}$), 3.71 (s, 3H, CH$_3$O), 3.49–3.42 (m, 3H, H$_2$, H$_3$, H$_6$), 3.09–3.01 (m, 4H, H$_7$, H$_\beta^{Cys}$), 2.20 (2×CH$_2$), 2.10 (m, 1H, H$_{4a}$), 1.74 (m, 2H, CH$_2$), 1.63 (m, 4H, H$_{4b}$, H$_{\beta\gamma}^{Leu}$), 1.49–1.27 (m, 2H, H$_{4b}$, H$_{5b}$), 1.38, 1.27 (2×s, 18H, 2×*t*Bu), 0.86 (dd, 6H, 2×CH$_3^{Leu}$, J= 6.1 Hz); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 174.3, 173.6, 172.4, 171.3 (C=O$^{ester\&amide}$), 156.4 (C=O^{Boc}), 79.5 (C$_q^{tBu\ Boc}$), 77.8 (C$_6$), 76.9 (C$_2$), 68.2 (C$_3$), 52.9, 52.3 (C$_\alpha^{Leu}$, OCH$_3$), 49.7 (C$_\alpha^{Cys}$), 47.8 (C$_q$, S*t*Bu), 43.4, 41.0. 40.7, 39.3 (C$_7$, C$_\beta^{Leu}$, C$_\beta^{Cys}$, CH$_2$), 29.6, 28.2 (2×*t*Bu), 32.9, 30.4, 27.0, 26.1 (C$_4$, C$_5$, 2×CH$_2$), 24.7 (C$_\gamma^{Leu}$), 22.6, 21.5 (2×CH$_3^{Leu}$). MS (ESI): *m/z* 677.7 (M+H)$^+$, 701.4 (M+Na)$^+$. HR-MS: calc. for [C$_{30}$H$_{54}$N$_4$O$_9$S$_2$+H]$^+$ 679.34050, found 679.34039. [α]$_D^{20}$= −69 (CHCl$_3$, *c*= 1).

N-(6-[(N-(N-lauric acid))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-trans 7). Following *GP 1a* and *1b* using 2,6-trans **4** (44 mg, 0.07 mmol) and lauric acid (18 mg, 0.09 mmol) gave 2,6-trans **7** (R_f 0.5, DCM/MeOH 9/1 v/v) in 100% yield. ^{1}H-NMR (600 MHz, CDCl$_3$) δ 7.30 (d, 1H, NH, J= 8.4 Hz), 6.97 (m, 1H, NH), 6.77 (d, 1H, NH, J= 7.5 Hz), 4.77 (dd, 1H, H$_\alpha^{Cys}$, J= 7.0 Hz), 4.65 (m, 1H, H$_\alpha^{Leu}$), 4.04 (m, 1H, H$_6$), 3.99 (d, 1H, H$_2$, J= 7.9 Hz), 3.84 (m, 1H, H$_3$), 3.76 (s, 3H, OCH$_3$), 3.76 (m, 1H, H$_{7a}$), 3.31 (m, 1H, H$_{7b}$), 3.20, 3.08 (m, 2H, H$_\beta^{Cys}$), 2.35 (t, 2H, CH$_2^{lipid}$, J= 7.5 and 7.6 Hz), 2.27 (t, 2H, CH$_2^{lipid}$, J= 7.5 and 7.7 Hz), 1.95 (m, 1H, H$_{4a}$),

1.75–1.58 (m, 6H, H$_{\beta\gamma}$^{Leu}, H$_{5a}$, CH$_2$^{lipid}), 1.42–1.27 (m, *t*Bu, H$_{4b\&5b}$, CH$_2$^{lipid}), 0.95 (t, 6H, 2×CH$_3$^{Leu}, *J*= 6.2 and 6.3 Hz), 0.88 (t, 3H, CH$_3$^{lipid}, *J*= 6.8 and 7.1 Hz); ^{13}C-NMR (150 MHz, CDCl$_3$) δ 174.0, 173.4, 171.9, 170.5 (C=O$^{ester\&amide}$), 73.4 (C$_6$), 71.7 (C$_2$), 67.4 (C$_3$), 52.9, 52.5 (C$_\alpha$^{Leu}, OCH$_3$), 50.1 (C$_\alpha$^{Cys}), 48.5 (C$_q$^{tBu}), 42.0 (C$_\beta$^{Cys}), 41.0 (C$_\beta$^{Leu}), 39.6 (C$_7$), 36.5, 33.8, 31.9 (3×CH$_2$^{lipid}), 29.6 (*t*Bu), 29.6 (CH$_2$^{lipid}), 26.5, 25.6, 24.0, 24.8, 22.7 (C$_4$, C$_5$, 3×CH$_2$^{lipid}), 24.8 (C$_\gamma$^{Leu}), 22.8, 21.7 (2×CH$_3$^{Leu}), 14.0 (CH$_3$^{lipid}). MS (ESI): *m/z* 676.5 (M+H)$^+$. HR-MS: calc. for [C$_{33}$H$_{61}$N$_3$O$_7$S$_2$+H]$^+$ 676.40237, found 676.40063. [α]$_D$^{20}= −2.4 (CHCl$_3$, *c*= 0.25).

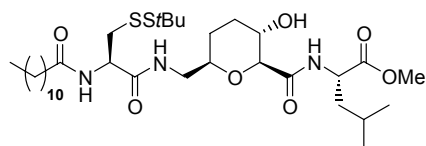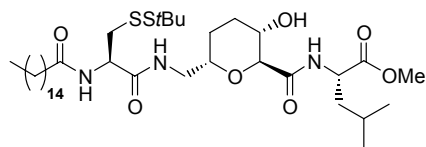### *N*-(6-[(*N*-(*N*-lauric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-cis 7).

Following *GP 1a* and *1b* using 2,6-cis **4** (53 mg, 0.09 mmol) and lauric acid (21.5 mg, 0.11 mmol) gave 2,6-cis **7** (R$_f$= 0.4, DCM/MeOH 9/1 v/v) in 91% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.35 (d, 1H, NH, *J*= 8.9 Hz), 7.10 (bt, 1H, NH), 4.73 (dd, 1H, H$_\alpha$^{Cys}, *J*= 7.2 and 7.4 Hz), 4.69 (dd, 1H, H$_\alpha$^{Cys}, *J*= 7.3 and 8.3 Hz), 3.77 (s, 3H, CH$_3$O), 3.62–3.42 (m, 4H, H$_2$, H$_3$, H$_6$, H$_{7a}$), 3.12 (m, 1H, H$_{7b}$), 3.02 (dd, 2H, H$_\beta$^{Cys}, *J*= 6.9, 7.2 and 7.3 Hz), 2.32–2.17 (m, 4H, 2×CH$_2$^{lipid}), 1.70–1.32 (m, H$_{\beta\gamma}$^{Leu}, H$_4$, H$_5$, CH$_2$^{lipid}), 1.34–1.20 (m, *t*Bu, CH$_2$^{lipid}), 0.95 (dd, 6H, 2×CH$_3$^{Leu}, *J*= 5.9 Hz), 0.89 (t, 3H, CH$_3$^{lipid}, *J*= 6.8 and 7.1 Hz); ^{13}C-NMR (150 MHz, CDCl$_3$) δ 175.3, 175.2, 173.1, 172.4 (C=O$^{ester\&amide}$), 78.8 (C$_6$), 78.2 (C$_2$), 69.2 (C$_3$), 53.4, (C$_\alpha$^{Leu}, OCH$_3$), 50.6 (C$_\alpha$^{Cys}), 49.0 (C$_q$^{tBu}), 44.4, (C$_7$), 42.0. 41.5 (C$_\beta$^{Leu}, C$_\beta$^{Cys}), 37.1, 32.8, 31.5, 31.0– 30.0 (CH$_2$^{lipid}), 30.2 (*t*Bu), 28.0, 26.6 (C$_4$, C$_5$), 23.5 (CH$_2$^{lipid}), 25.8 (C$_\gamma$^{Leu}), 23.7, 22.6 (2×CH$_3$^{Leu}), 15.0 (CH$_3$^{lipid}). MS (ESI): *m/z* 676.3 (M+H)$^+$, 698.5 (M+Na)$^+$. HR-MS: calc. for [C$_{33}$H$_{61}$N$_3$O$_7$S$_2$+H]$^+$ 676.40237, found 676.40149. [α]$_D$^{20}= −115.2 (CHCl$_3$, *c*= 0.25).

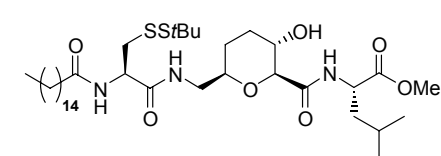### *N*-(6-[(*N*-(*N*-palmitic acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine methyl ester (2,6-trans 8).

Following *GP 1a* and *1b* using 2,6-trans **4** (91 mg, 0.15 mmol) and palmitic acid (47.2 mg, 0.18 mmol) gave the title compound (R$_f$= 0.67, EtOAc/acetone 1/1 v/v) in 87% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.30 (m, 2H, 2×NH), 7.00 (d, 1H, NH, *J*= 7.8 Hz), 4.77 (m, 1H, H$_\alpha$^{Cys}), 4.62 (m, 1H, H$_\alpha$^{Leu}), 4.06 (m, 2H, H$_6$, H$_2$), 3.84 (m, 1H, H$_3$), 3.76 (s, 3H, CH$_3$O), 3.60, 3.30 (2×m, 2H, H$_7$), 3.14 (m, 2H, H$_\beta$^{Cys}), 2.27 (m, 2H, CH$_2$^{lipid}), 1.95 (m, 1H, H$_{4a}$), 1.75, 1.55 (m, 6H, H$_\beta$^{Leu}, H$_\gamma$^{Leu}, H$_{5a}$, CH$_2$^{lipid}), 1.42–1.20 (m, *t*Bu, H$_{4b5b}$, CH$_2$^{lipid}), 0.95 (t, 6H, 2×CH$_3$^{Leu}, *J*= 5.8 and 5.9 Hz), 0.88 (t, 3H, CH$_3$^{lipid}, *J*= 6.6 and 7.0 Hz); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 174.1, 173.4, 171.9, 170.7 (C=O$^{ester\&amide}$), 74.2 (C$_6$), 71.3 (C$_2$), 66.6 (C$_3$), 53.3, 52.6 (C$_\alpha$^{Leu}, OCH$_3$), 50.7 (C$_\alpha$^{Cys}), 48.2 (C$_q$^{tBu}), 42.0 (C$_\beta$^{Cys}), 40.8 (C$_\beta$^{Leu}), 39.7 (C$_7$), 36.3, 31.8 (2×CH$_2$^{lipid}), 29.6 (*t*Bu), 29.6 (CH$_2$^{lipid}), 26.5, 25.6, 24.0. 22.6 (C$_4$, C$_5$, 2×CH$_2$^{lipid}), 25.1 (C$_\gamma$^{Leu}), 22.6, 21.3 (2×CH$_3$^{Leu}), 14.0 (CH$_3$^{lipid}). MS (ESI): *m/z* 733.9 (M+H)$^+$, 754.6 (M+Na)$^+$. HR-MS: calc. for [C$_{37}$H$_{69}$N$_3$O$_7$S$_2$+H]$^+$ 732.46497, found 732.46338. [α]$_D$^{20}= −2.0 (CHCl$_3$, *c*= 0.5).

N-(6-[(*N*-*N*-(palmitic acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine methyl ester (2,6-cis 8).

Following *GP 1a* and *1b* using 2,6-cis **4** (76 mg, 0.13 mmol) and palmitic acid (39.4 mg, 0.15 mmol) gave the title compound (R$_f$= 0.8, EtOAc/acetone 1/1 v/v) in 84% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.37 (1H, NH), 7.20 (1H, NH), 7.12 (1H, NH), 4.70 (m, 2H, H$_\alpha$^{Cys}, H$_\alpha$^{Leu}), 3.76 (s, 3H, CH$_3$O), 3.53 (m, 4H, H$_2$, H$_3$, H$_6$, H$_{7a}$), 3.20 (m, 1H, H$_{7b}$), 3.00 (m, 2H, H$_\beta$^{Cys}), 2.40–2.17 (m, 3H, CH$_2$^{lipid}, H$_{4a}$), 1.50–1.40 (m, 6H, H$_\beta$^{Leu}, H$_\gamma$^{Leu}, H$_{5a}$, CH$_2$^{lipid}), 1.40–1.20 (m, *t*Bu, H$_{4b5b}$, CH$_2$^{lipid}), 0.95 (dd, 6H, 2×CH$_3$^{Leu}, *J*= 6.1 and 6.0 Hz), 0.88 (t, 3H, CH$_3$^{lipid}, *J*= 6.6 and 7.0 Hz); ^{13}C-NMR (100 MHz methanol-*d4*) δ 174.4, 172.2, 171.4 (C=O$^{ester\&amide}$), 77.9 (C$_6$), 77.4 (C$_2$), 68.3 (C$_3$), 53.6, 52.4 (C$_\alpha$^{Leu}, OCH$_3$), 50.7 (C$_\alpha$^{Cys}), 48.2 (C$_q$, S*t*Bu), 43.9, (C$_7$), 42.7. 41.0 (C$_\beta$^{Leu}, C$_\beta$^{Cys}), 36.5, 32.5, 31.5, 30.0–29.0 (CH$_2$^{lipid}), 30.2 (*t*Bu), 27.1, 25.7, 24.8, 22.7 (C$_4$, C$_5$, CH$_2$), 24.8 (C$_\gamma$^{Leu}), 22.7, 21.7 (2×CH$_3$^{Leu}), 14.1 (CH$_3$^{lipid}). MS (ESI): *m/z* 732.5 (M+H)$^+$, 754.8 (M+Na)$^+$. HR-MS: calc. for [C$_{37}$H$_{69}$N$_3$O$_7$S$_2$+H]$^+$ 732.46497, found 732.46295. [α]$_D$^{20}= −43.6 (CHCl$_3$, *c*= 0.5).

N-(6-[(*N*-(*N*-(lauric acid)-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine methyl ester (2,6-trans 9).

Following *GP 1a* and *1b* using 2,6-trans **5** (34 mg, 0.05 mmol) and lauric acid (12.5 mg, 0.06 mmol) gave 2,6-trans **9** (R_f 0.5, DCM/MeOH 9/1 v/v) in 52% yield. ^{13}C-NMR (50 MHz, CDCl$_3$) δ 174.4, 173.5, 171.9, 169.7 (C=O$^{ester\&amide}$), 73.0, 70.9 (C$_2$, C$_6$), 67.1 (C$_3$), 53.0, 52.3 (C$_\alpha$, OCH$_3$), 49.6 (C$_\alpha$), 48.2 (C$_q$^{tBu}), 43.3, 42.0, 41.7, 39.5 (C$_\beta$^{Cys}, C$_\beta$^{Leu}, CH$_2$^{Gly}, C$_7$), 35.9, 31.6 (2×CH$_2$^{lipid}), 29.5 (*t*Bu), 29.6 (CH$_2$^{lipid}), 26.3, 25.3, 23.7, 22.4 (C$_4$, C$_5$, CH$_2$^{lipid}), 24.6 (C$_\gamma$^{Leu}), 22.6, 21.4 (2×CH$_3$^{Leu}), 13.8 (CH$_3$^{lipid}). MS (ESI): *m/z* 733.5 (M+H)$^+$, 755.5 (M+Na)$^+$. HR-MS: calc. for [C$_{35}$H$_{64}$N$_4$O$_8$S$_2$+H]$^+$ 733.42383, found 733.42206.

N-(6-[(*N*-(*N*-(lauric acid)-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine methyl ester (2,6-cis 9).
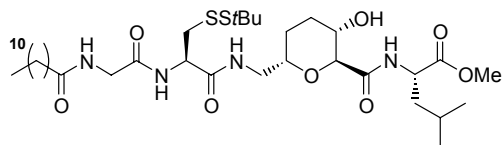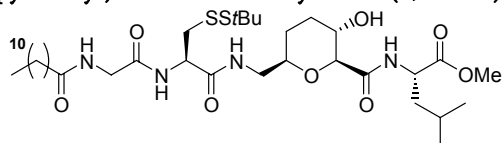
Following *GP 1a* and *1b* using 2,6-cis **5** (64 mg, 0.10 mmol) and lauric acid (23.6 mg, 0.12 mmol) gave 2,6-cis **9** (R_f 0.54, DCM/MeOH 9/1 v/v) in 100% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.42 (dd, 2H, 2×NH, *J*= 5.7 and 6.5 Hz), 7.18 (t, 1H, NH, *J*= 5.8 Hz), 6.54 (t, 1H, NH, *J*= 5.1 Hz), 4.70 (m, 2H, H$_\alpha$^{Cys}, H$_\alpha$^{Leu}), 3.92 (dd, 2H, CH$_2$^{Gly}, *J*= 2.9 and 3.0 Hz), 3.77 (s, 3H, OCH$_3$), 3.66 (m, 1H, H$_6$), 3.55 (m, 2H, H$_2$, H$_3$), 3.43, 3.26 (2×m, 2H, H$_7$), 3.14, 3.00 (2×m, 2H, H$_\beta$^{Cys}), 2.64–2.14 (m, 4H, 2×CH$_2$^{lipid}), 1.72–1.32 (m, H$_{\beta\gamma}$^{Leu}, H$_4$, H$_5$, CH$_2$^{lipid}), 1.32 (s, 9H, *t*Bu), 1.32–1.17 (m, CH$_2$^{lipid}), 0.95 (dd, 6H, 2×CH$_3$^{Leu}, *J*= 6.0 Hz), 0.88 (t, 3H, CH$_3$^{lipid}, *J*= 6.6 and 7.0 Hz); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 174.3, 174.1, 172.3, 170.2, 169.7 (C=O$^{ester\&amide}$), 77.6, 76.5 (C$_2$, C$_6$), 68.3 (C$_3$), 53.2, 52.6 (C$_\alpha$^{Leu}, OCH$_3$), 49.6 (C$_\alpha$^{Cys}), 48.4 (C$_q$^{tBu}), 43.8, 43.4, 41.3, 41.0 (C$_\beta$^{Cys}, C$_\beta$^{Leu}, CH$_2$^{Gly}, C$_7$), 36.2, 31.9, 30.7 (3×CH$_2$^{lipid}), 29.6 (*t*Bu), 29.3 (CH$_2$^{lipid}), 27.3, 25.6, 22.6 (C$_4$, C$_5$, CH$_2$^{lipid}), 24.9 (C$_\gamma$^{Leu}), 22.8, 21.8 (2×CH$_3$^{Leu}), 14.1 (CH$_3$^{lipid}). MS (ESI): *m/z* 733.5 (M+H)$^+$, 755.5 (M+Na)$^+$. HR-MS: calc. for [C$_{35}$H$_{64}$N$_4$O$_8$S$_2$+H]$^+$ 733.42383, found 733.42236. [α]$_D$^{20}= −55.6 (CHCl$_3$, *c*= 0.5).

N-(6-[(*N*-(*N*-4-(*N*-lauric acid)-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine methyl ester. (2,6-trans 10).

Following *GP 1a* and *1b* using 2,6-trans **6** (73 mg, 0.11 mmol) and lauric acid (26.0 mg, 0.13 mmol) gave the title compound (R_f 0.5, DCM/MeOH 9/1 v/v) in 64% yield. ^{1}H-NMR (600 MHz, CDCl$_3$) δ 7.66 (m, 1H, NH), 7.27 (m, 1H, NH), 7.17 (d, 1H, NH, *J*= 8.1 Hz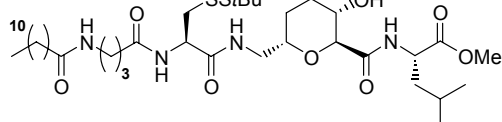), 5.88 (m, 1H, NH), 4.96 (m, 1H, H$_\alpha$^{Cys}), 4.85–4.60 (m, 3H, H$_\alpha$^{Leu}, CH$_2$), 4.17 (m, 1H, H$_6$), 4.10 (d, 1H, H$_2$, *J*= 8.2 Hz), 4.06 (m, CH$_2$), 3.80 (m, 1H, H$_3$), 3.75 (s, 3H, CH$_3$O), 3.71–3.25 (m, H$_7$, CH$_2$), 3.32 (dd, H$_\beta$^{Cys}, *J*= 6.5 Hz), 3.12 (m, H$_\beta$^{Cys}), 2.31 (m, CH$_2$), 2.13 (m, CH$_2$), 1.91 (m, H$_{4a}$, CH$_2$), 1.77 (m, 1H, H$_{5a}$), 1.97–1.55 (m, H$_{\beta\gamma}$^{Leu}, H$_4$, H$_4$, CH$_2$), 1.40 (CH$_2$), 1.33 (s, 9H, *t*Bu), 1.25 (m, CH$_2$), 0.95 (m, 6H, 2×CH$_3$^{Leu}), 0.88 (t, 3H, CH$_3$^{lipid}, *J*= 6.8 and 7.1 Hz); ^{13}C-NMR (150 MHz, CDCl$_3$) δ 174.2, 173.6, 173.0, 172.4, 170.5 (C=O$^{ester\&amide}$), 72.9, 71.9 (C$_2$, C$_6$), 70.7 (C$_3$), 53.0, 52.3 (C$_\alpha$^{Leu}, OCH$_3$), 50.1 (C$_\alpha$^{Cys}), 48.3 (C$_q$^{tBu}), 42.8, 42.8, 40.9, 39.6, 38.0, 36.9 (C$_\beta$^{Cys}, C$_\beta$^{Leu}, CH$_2$, C$_7$), 32.5, 31.9 (CH$_2$), 29.6 (*t*Bu), 29.6 (CH$_2$), 26.7, 26.6, 26.0, 25.9 (C$_4$, C$_5$, CH$_2$), 24.8 (C$_\gamma$^{Leu}), 22.9, 21.6 (2×CH$_3$^{Leu}), 14.0 (CH$_3$^{lipid}). MS (ESI): *m/z* 761.7 (M+H)$^+$, 783.5 (M+Na)$^+$. HR-MS: calc. for [C$_{37}$H$_{68}$N$_4$O$_8$S$_2$+H]$^+$ 761.45513, found 761.45355. [α]$_D$^{20}= −8 (CHCl$_3$, *c*= 0.25).

N-(6-[(*N*-(*N*-4-(*N*-lauric acid)-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-cis 10).
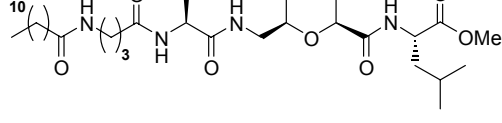
Following *GP 1a* and *1b* using 2,6-cis **6** (55 mg, 0.08 mmol) and lauric acid (19.5 mg, 0.10 mmol) gave 2,6-cis **10** (R_f 0.50, DCM/MeOH 9/1 v/v) in 92% yield. ^{1}H-NMR (600 MHz, CDCl$_3$) δ 7.36 (d, 1H, NH, *J*= 8.7 Hz), 7.29 (d, 1H, NH, *J*= 8.1 Hz), 7.22 (m, 1H, NH), 5.92 (m, 1H,

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NH), 4.72–4.62 (m, 2H, H_α^{Cys}, H_α^{Leu}), 3.76 (s, 3H, OMe), 3.61–3.46 (m, 5H, H_2, H_3, H_6, H_{7a}), 3.25 (m, 2H, CH_2), 3.17 (m, 1H, H_{7b}), 3.06 (m, 2H, H_β^{Cys}), 2.27 (m, 2H, CH_2), 2.17 (t, 2H, CH_2, J= 7.7 and 7.2 Hz), 1.90 (m, 2H, CH_2), 1.82–1.39 (m, $H_{\beta\gamma}^{Leu}$, CH_2^{lipid}, H_{45}), 1.33 (s, 9H, *t*Bu), 1.26 (m, CH_2^{lipid}), 0.88 (dd, 6H, $2\times CH_3^{Leu}$, J= 5.8 and 5.6 Hz), 0.87 (t, 3H, CH_3^{lipid}, J= 6.7 and 7.2 Hz); ^{13}C-NMR (50 MHz, CDCl$_3$) δ 173.8, 173.6, 173.3, 172.1, 170.9 (C=O$^{ester\&amide}$), 77.7 (C$_6$), 76.7 (C$_2$), 68.1 (C$_3$), 52.8, 52.3 (OCH$_3$, C$_\alpha^{Cys}$), 49.7 (C$_\alpha^{Leu}$), 47.9 (C$_q^{tBu}$), 43.4 (C$_7$), 40.7, 40.6 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 38.2, 36.5, 33.0, 31.6, 30.3 (CH$_2$), 29.6 (*t*Bu), 29.3, 29.1 (CH$_2^{lipid}$), 27.2, 25.8, 22.7 (C$_{45}$, CH$_2$), 24.8 (C$_\gamma^{Leu}$), 24.1, 21.7 ($2\times$CH$_3^{Leu}$), 14.1 (CH$_3^{lipid}$). MS (ESI): *m/z* 761.8 (M+H)$^+$, 783.6 (M+Na)$^+$. HR-MS: calc. for [C$_{37}$H$_{68}$N$_4$O$_8$S$_2$+H]$^+$ 761.45513, found 761.45288. [α]$_D^{20}$= −59.2 (CHCl$_3$, *c*= 0.25).

N-(6-[(N-(N-(palmitic acid)-glycine))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine methyl ester (2,6-trans 11). Following *GP 1a* and *1b* using 2,6-trans **5** (50 mg, 0.08 mmol) and palmitic acid (23.6 mg, 0.09 mmol) gave 2,6-trans **11** (R$_f$= 0.7, EtOAc/acetone 1/1 v/v) in 90% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.40 (d, 1H, NH, J= 8.2 Hz), 7.30 (m, 2H, $2\times$NH), 6.56 (t, 1H, NH, J= 5.0 Hz), 4.77 (dd, 1H, H$_\alpha^{Cys}$, J= 5.8 and 6.0 Hz), 4.66 (m, 1H, H$_\alpha^{Leu}$), 4.10 (m, 1H, H$_6$), 4.06 (d, 1H, H$_2$, J= 8.2 Hz), 3.92 (ddd, 2H, CH$_2^{Gly}$, J= 5.0 and 5.4 Hz), 3.77 (m, 4H, H$_3$, OCH$_3$), 3.54 (m, 1H, H$_{7a}$), 3.38 (m, 2H, H$_{7b}$, H$_\beta^{Cys}$), 3.09 (dd, 1H, H$_\beta^{Cys}$, J= 5.1 and 5.2 Hz), 2.36–2.22 (m, 2H, CH$_2^{lipid}$), 1.94 (m, 1H, H$_{4a}$), 1.80 (m, 1H, H$_{5a}$), 1.70–1.55 (m, 9H, H$_{\beta\gamma}^{Leu}$, H$_{4b}$, H$_{5b}$, $2\times$CH$_2^{lipid}$), 1.33 (s, 9H, *t*Bu), 1.39–1.15 (m, CH$_2^{lipid}$), 0.95 (t, 6H, $2\times$CH$_3^{Leu}$, J= 3.9), 0.88 (t, 3H, CH$_3^{lipid}$, J= 6.6 and 7.0 Hz); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 174.6, 173.8, 172.2, 169.9 (C=O$^{ester\&amide}$), 73.0, 71.0 (C$_2$, C$_6$), 67.5 (C$_3$), 53.2, 52.6 (C$_\alpha^{Leu}$, OCH$_3$), 49.9 (C$_\alpha^{Cys}$), 48.5 (C$_q^{tBu}$), 43.7, 42.3, 41.0, 39.6 (C$_\beta^{Cys}$, C$_\beta^{Leu}$, CH$_2^{Gly}$, C$_7$), 36.1, 31.9 ($2\times$CH$_2^{lipid}$), 29.6 (*t*Bu), 29.6 (CH$_2^{lipid}$), 26.6, 25.5, 24.0, 22.6 (C$_4$, C$_5$, $2\times$CH$_2^{lipid}$), 24.1 (C$_\gamma^{Leu}$), 22.6, 21.6 ($2\times$CH$_3^{Leu}$), 14.1 (CH$_3^{lipid}$). MS (ESI): *m/z* 789.5 (M+H)$^+$, 811.6 (M+Na)$^+$. HR-MS: calc. for [C$_{37}$H$_{72}$N$_4$O$_8$S$_2$+H]$^+$ 789.48643, found 789.48407. [α]$_D^{20}$= −17.6 (CHCl$_3$, *c*= 0.5).
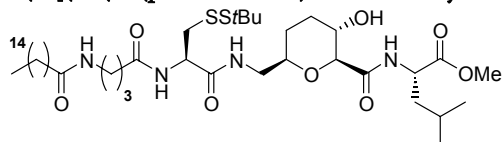
N-(6-[(N-(N-(palmitic acid)-glycine))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine methyl ester (2,6-cis 11). Following *GP 1a* and *1b* using 2,6-cis **5** (63 mg, 0.10 mmol) and palmitic acid (29.8 mg, 0.12 mmol) gave 2,6-cis **11** (R$_f$= 0.8, EtOAc/acetone 1/1 v/v) in 98% yield. ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.77 (m, 1H, NH), 7.61 (d, 1H, NH, J= 7.8 Hz), 7.52 (d, 1H, NH, J= 8.7 Hz), 7.00 (m, 1H, NH), 4.66 (m, 2H, H$_\alpha^{Cys}$, H$_\alpha^{Leu}$), 3.90 (dd, 2H, CH$_2^{Gly}$), 3.76 (s, 3H, CH$_3$O), 3.76–3.27 (m, 4H, H$_2$, H$_3$, H$_6$, H$_7$, H$_\beta^{Cys}$), 3.09 (dd, 1H, H$_\beta^{Cys}$, J= 1.6, 1.9 and 6.4, 6.7 Hz), 2.27 (m, 2H, CH$_2^{lipid}$), 2.15 (m, 1H, H$_{4a}$), 1.74–1.38 (m, 10H, H$_\beta^{Leu}$, H$_\gamma^{Leu}$, H$_{4b}$, H$_5$, $2\times$CH$_2^{lipid}$), 1.32 (s, 9H, *t*Bu), 1.32–1.15 (m, CH$_2^{lipid}$), 0.95 (m, 6H, $2\times$CH$_3^{Leu}$), 0.88 (t, 3H, CH$_3^{lipid}$, J= 6.6 and 7.0 Hz); ^{13}C-NMR (100 MHz, CDCl$_3$) δ 174.7, 173.9, 172.4, 170.3, 169.7 (C=O$^{ester\&amide}$), 78.0, 76.7 (C$_2$, C$_6$), 68.3 (C$_3$), 53.1, 52.5 (C$_\alpha^{Leu}$, OCH$_3$), 49.9 (C$_\alpha^{Cys}$), 48.2 (C$_q^{tBu}$), 43.7, 42.0, 41.6, 40.8 (C$_\beta^{Cys}$, C$_\beta^{Leu}$, CH$_2^{Gly}$, C$_7$), 36.1, 31.8 ($2\times$CH$_2^{lipid}$), 29.6 (*t*Bu), 29.6 (CH$_2^{lipid}$), 27.1, 25.5, 23.8, 21.6 (C$_4$, C$_5$, $2\times$CH$_2^{lipid}$), 24.8 (C$_\gamma^{Leu}$), 22.7, 21.6 ($2\times$CH$_3^{Leu}$), 14.0 (CH$_3^{lipid}$). MS (ESI): *m/z* 789.5 (M+H)$^+$, 811.6 (M+Na)$^+$. HR-MS: calc. for [C$_{37}$H$_{72}$N$_4$O$_8$S$_2$+H]$^+$ 789.48643, found 789.48444. [α]$_D^{20}$= −55 (*c*= 0.5, CHCl$_3$).

N-(6-[(N-(N-(palmitic acid)-4-aminobutyric acid))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-trans 12). Following *GP 1a* and *1b* using 2,6-trans **6** (57 mg, 0.08 mmol) and palmitic acid (26 mg, 0.1 mmol) gave 2,6 trans **12** (R$_f$= 0.5, EtOAc/acetone 1/1 v/v) in 85% yield. ^{1}H-NMR (600 MHz, CDCl$_3$) δ 7.66 (m, 1H, NH), 7.29 (m, 1H, NH), 7.17 (d, 1H, NH, J= 7.9 Hz), 5.97 (m, 1H, NH), 4.82 (m, 1H, H$_\alpha^{Cys}$), 4.67 (m, 1H, H$_\alpha^{Leu}$), 4.16 (m, 1H, H$_6$), 4.10 (d, 1H, H$_2$, J= 8.0 Hz), 4.05 (m, CH$_2$), 3.81 (m, 1H, H$_3$), 3.75 (s, 3H, CH$_3$O), 3.59 (m, 1H, H$_{7a}$), 3.48, 3.42 (m, CH$_2$), 3.32 (m, H$_{7b}$, H$_\beta^{Cys}$, CH$_2$), 3.11 (m, H$_\beta^{Cys}$,

CH$_2$), 2.35 (m, CH$_2$), 2.15 (m, CH$_2$), 1.91 (m, H$_{4a}$, CH$_2$), 1.77 (m, 1H, H$_{5a}$), 1.68–1.54 (m, H$_{\beta\gamma}^{Leu}$, H$_{4b}$, H$_{5b}$, CH$_2$), 1.33 (s, 9H, *t*Bu), 1.39–1.15 (m, CH$_2$), 0.95 (t, 6H, 2×CH$_3^{Leu}$, *J*= 5.8 and 5.4 Hz), 0.88 (t, 3H, CH$_3^{lipid}$, *J*= 6.9 and 7.1 Hz); ^{13}C-NMR (150 MHz, CDCl$_3$) δ 176.9, 174.2, 173.1, 172.3, 170.5 (C=O$^{ester\&amide}$), 73.0, 70.7 (C$_2$, C$_6$), 67.4 (C$_3$), 53.0, 52.4 (C$_\alpha^{Leu}$, OCH$_3$), 49.8 (C$_\alpha^{Cys}$), 48.3 (C$_q^{tBu}$), 42.7, 40.7, 39.6, 37.8 (C$_\beta^{Cys}$, C$_\beta^{Leu}$, CH$_2$, C$_7$), 33.9, 32.5, 31.8 (CH$_2$), 29.6 (*t*Bu), 29.6 (CH$_2$), 26.5, 26.0, 25.8, 22.6, 21.6 (C$_4$, C$_5$, CH$_2$), 24.8 (C$_\gamma^{Leu}$), 22.8, 21.5 (2×CH$_3^{Leu}$), 14.0 (CH$_3^{lipid}$). MS (ESI): *m/z* 817.8 (M+H)$^+$, 839.6 (M+Na)$^+$. HR-MS: calc. for [C$_{41}$H$_{76}$N$_4$O$_8$S$_2$+H]$^+$ 817.51773, found 817.51459. [α]$_D^{20}$= −7.6 (CHCl$_3$, *c*= 0.5).

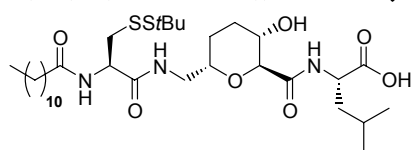### *N*-(6-[(*N*-(*N*-(palmitic acid)-4-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine methyl ester (2,6-cis 12).

Following *GP 1a* and *1b* using 2,6-cis **6** (44 mg, 0.07 mmol) and palmitic acid (20 mg, 0.08 mmol) gave the title compound (R$_f$ 0.54, EtOAc/acetone 1/1 v/v) in 78% yield. ^{1}H-NMR (600 MHz, CDCl$_3$) δ 7.34–7.39 (m, 3H, NH), 6.11 (m, 1H, NH), 4.70 (m, 1H, H$_\alpha^{Cys}$), 4.64 (m, 1H, H$_\alpha^{Leu}$), 4.00 (m, CH$_2$), 3.76 (s, 3H, CH$_3$O), 3.50–3.57 (m, H$_2$, H$_3$, H$_6$, CH$_2$), 3.36–3.18 (m, H$_7$, CH$_2$), 3.06 (m, H$_\beta^{Cys}$, CH$_2$), 2.33–2.22 (m, CH$_2$), 2.16 (m, CH$_2$), 1.80 (m, CH$_2$), 1.69 (m, H$_{\beta\gamma}^{Leu}$, H$_{4a}$, H$_{5a}$, CH$_2$), 1.33 (s, 9H, *t*Bu), 1.50–1.32 (m, H$_{4b}$, H$_{5b}$, CH$_2$), 1.25 (m, CH$_2$), 0.95 (t, 6H, 2×CH$_3^{Leu}$, *J*= 5.4 and 5.5 Hz), 0.88 (t, 3H, CH$_3^{lipid}$, *J*= 6.8 and 7.1 Hz); ^{13}C-NMR (150 MHz, CDCl$_3$) δ 175.0, 174.6, 174.5, 173.2, 171.9 (C=O$^{ester\&amide}$), 79.0, 77.7 (C$_2$, C$_6$), 69.2 (C$_3$), 54.7, 53.3 (C$_\alpha^{Leu}$, OCH$_3$), 49.0 (C$_\alpha^{Cys}$), 48.3 (C$_q^{tBu}$), 44.5, 41.9, 41.8, 39.4, 37.6, 34.1, 32.8, 31.5 (C$_\beta^{Cys}$, C$_\beta^{Leu}$, CH$_2$, C$_7$), 29.6 (*t*Bu), 30.0 (CH$_2$), 28.1, 26.7, 26.6, 23.5 (C$_4$, C$_5$, CH$_2$), 24.8 (C$_\gamma^{Leu}$), 22.8, 21.5 (2×CH$_3^{Leu}$), 14.0 (CH$_3^{lipid}$). MS (ESI): *m/z* 817.8 (M+H)$^+$, 839.6 (M+Na)$^+$. HR-MS: calc. for [C$_{41}$H$_{76}$N$_4$O$_8$S$_2$+H]$^+$ 817.51773, found 817.51849. [α]$_D^{20}$= −50.8 (CHCl$_3$, *c*= 0.5).

N-(6-[(*N*-(*N*-lauric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine (2,6-trans 13).

From 2,6-trans **7** (31 mg, 0.05 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.54 (dd, 1H, *J*= 5.6 Hz, H$_\alpha^{Cys}$), 4.36 (m, 1H, H$_\alpha^{Leu}$), 4.10 (d, 1H, *J*= 3.6 Hz, H$_2$), 4.02 (m, 1H, H$_3$), 3.69 (m, 1H, H$_6$), 3.26 (m, 2H, H$_7$), 3.06 (1H, H$_\beta^{Cys}$), 2.88 (dd, 1H, *J*= 6.0 Hz, H$_\beta^{Cys}$), 2.16 (m, 2H, CH$_2$), 1.69-1.42 (m, CH$_2^{lipid}$, H$_{\beta\gamma}^{Leu}$, H$_{4\&5}$), 1.30-1.10 (m, *t*Bu, CH$_2^{lipid}$), 0.85 (t, 6H, *J*= 6.4 Hz, 2×CH$_3^{Leu}$), 0.79 (t, 3H, *J*= 6.8 Hz, CH$_3^{lipid}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 175.2-170.5 (C=O$^{amide\&carboxyl}$), 73.8 (C$_6$), 72.0 (C$_2$), 66.7 (C$_3$), 52.7, 50.2 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$), 48.1 (C$_7$), 41.7 (C$_q^{tBu}$), 40.2, 36.2 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 29.5 (*t*Bu), 29.4 (CH$_2^{lipid}$), 25.4 (C$_4$), 24.7 (C$_\gamma^{Leu}$), 22.6 (CH$_3^{Leu}$), 22.4 (C$_5$), 21.3 (CH$_3^{Leu}$), 13.8 (CH$_3^{lipid}$). MS (ESI): *m/z* 684.6 (M+Na)$^+$. HR-MS: calc. for [C$_{32}$H$_{59}$N$_3$O$_7$S$_2$+H]$^+$ 662.3867, found 662.3922.

N-(6-[(*N*-(*N*-lauric acid))-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine (2,6-trans 19).

From 2,6-trans **13** (7 mg, 11 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 16.3 min, *m/z* 574.5 (M+H)$^+$. Linear gradient of B (30→90%) in 26 min.

N-(6-[(*N*-(*N*-lauric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine (2,6-cis 13).

From 2,6-cis **7** (55 mg, 0.08 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.60 (t, 1H, *J*= 6.8 and 7.2 Hz, H$_\alpha^{Cys}$), 4.24 (m, 1H, H$_\alpha^{Leu}$), 3.52 (m, 3H, H$_2$, H$_3$, H$_{7a}$), 3.35 (m, 1H, H$_6$), 3.10 (m, 1H, H$_{7b}$), 3.00 (dd, 1H, *J*= 6.0 Hz, H$_\beta^{Cys}$), 2.84 (dd, 1H, *J*= 6.0 Hz, H$_\beta^{Cys}$), 2.14 (m, 2H, CH$_2$), 2.00 (m, 1H, H$_{4a}$), 1.69-1.42 (m, CH$_2^{lipid}$, H$_{\beta\gamma}^{Leu}$, H$_{4b}$, H$_5$), 1.30-1.10 (m, *t*Bu, CH$_2^{lipid}$), 0.83 (bs, 6H, 2×CH$_3^{Leu}$), 0.76 (t, 3H, *J*= 6.4 and 6.8 Hz, CH$_3^{lipid}$). ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176.0-172.5 (C=O$^{amide\&carboxyl}$), 80.8 (C$_6$), 77.6 (C$_2$), 69.2 (C$_3$), 54.0, 51.3 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$), 44.1 (C$_7$), 42.7 (C$_q^{tBu}$), 41.5, 36.6 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 30.5 (CH$_2^{lipid}$), 30.0 (*t*Bu), 28.1 (C$_4$), 26.7 (C$_5$), 25.9 (C$_\gamma^{Leu}$), 23.1,

21.7 ($2 \times CH_3^{Leu}$), 14.2 (CH_3^{lipid}). MS (ESI): *m/z* 662.3 (M+H)$^+$, 684.6 (M+Na)$^+$. HR-MS: calc. for [$C_{32}H_{59}N_3O_7S_2$+H]$^+$ 662.3867, found 662.3909.

N-(6-[(N-(N-lauric acid))- L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine (2,6-cis 19). From 2,6-cis **13** (12 mg, 18 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 4.8 min, *m/z* 574.5 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min.

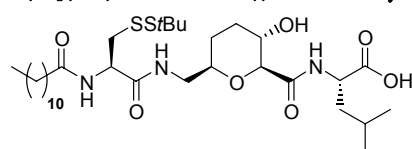**N-(6-[(N-(N-palmitic acid))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine (2,6-trans 14).** From 2,6-trans **8** (98 mg, 0.13 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.50 (dd, 1H, *J*= 5.6 Hz, H$_α^{Cys}$), 4.40 (m, 1H, H$_α^{Leu}$), 4.06 (d, 1H, *J*= 3.6 Hz, H$_2$), 4.01 (m, 1H, H$_3$), 3.68 (m, 1H, H$_6$), 3.25 (m, 2H, H$_7$), 3.14 (dd, 1H, H$_β^{Cys}$, *J*= 5.6 Hz), 2.85 (m, 1H, H$_β^{Cys}$), 2.15 (m, 2H, CH$_2$), 1.68-1.29 (m, CH$_2^{lipid}$, H$_{βγ}^{Leu}$, H$_{4\&5}$), 1.22-1.17 (m, *t*Bu, CH$_2^{lipid}$), 0.86-0.77 (m, 9H, $2 \times CH_3^{Leu}$), 0.88 (m, 3H, CH$_3^{lipid}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176.0-172.3 (C=O$^{amide\&carboxyl}$), 78.1 (C$_6$), 72.8 (C$_2$), 65.8 (C$_3$), 54.0, 51.5 (C$_α^{Leu}$, C$_α^{Cys}$), 43.5 (C$_7$), 43.3 (C$_q^{tBu}$), 41.4, 36.9 (C$_β^{Leu}$, C$_β^{Cys}$), 30.6 (CH$_2^{lipid}$), 30.2 (*t*Bu), 26.7 (C$_4$), 26.1 (C$_γ^{Leu}$), 23.7 (C$_5$), 23.5, 21.8 ($2 \times CH_3^{Leu}$), 14.4 (CH$_3^{lipid}$). LC-MS analysis: R$_t$ 24.4 min, *m/z* 718.6 (M+H)$^+$. Linear gradient of B (10→95%) in 26 min. HR-MS: calc. for [$C_{36}H_{67}N_3O_7S_2$+H]$^+$ 718.4493, found 718.4442.

N-(6-[(N-(N-palmitic acid))-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine (2,6-trans 20). From 2,6-trans **14** (25 mg, 35 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 7.0 min, *m/z* 630.6 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min.

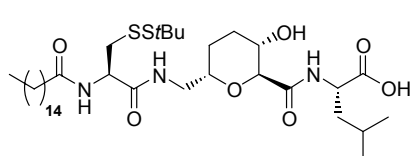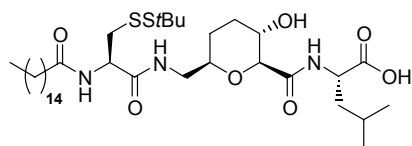**N-(6-[(N-(N-palmitic acid))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine (2,6-cis 14).** From 2,6-cis **8** (79 mg, 0.11 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.53 (dd, 1H, *J*= 6.0 and 6.4 Hz, H$_α^{Cys}$), 4.44 (t, 1H, *J*= 6.4 and 7.6 Hz, H$_α^{Leu}$), 3.51 (d, 1H, *J*= 9.6 Hz, H$_2$), 3.43-3.36 (m, 2H, H$_3$, H$_{7a}$), 3.27 (dd, 1H, *J*= 3.0 and 3.6 Hz, H$_6$), 3.17 (m, 1H, H$_{7b}$), 3.14 (dd, 1H, *J*= 6.0 Hz, H$_β^{Cys}$), 2.85 (dd, 1H, *J*= 8.0 and 9.2 Hz, H$_β^{Cys}$), 2.15 (m, 2H, CH$_2$), 2.00 (m, 1H, H$_{4a}$), 1.64–1.60 (m, 4H, H$_{βγ}^{Leu}$, H$_{5a}$), 1.50–1.27 (m, CH$_2^{lipid}$, H$_{4b\&5b}$), 1.27–1.17 (m, *t*Bu, CH$_2^{lipid}$), 0.85–0.77 (dd, 6H, *J*= 6.0 Hz, $2 \times CH_3^{Leu}$), 0.79 (t, 3H, *J*= 6.4 Hz, CH$_3^{lipid}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176–170 (C=O$^{amide\&carboxyl}$), 80.8 (C$_6$), 77.6 (C$_2$), 69.2 (C$_3$), 54.0, 51.4 (C$_α^{Leu}$, C$_α^{Cys}$), 44.1 (C$_7$), 42.8 (C$_q^{tBu}$), 41.5, 36.7 (C$_β^{Leu}$, C$_β^{Cys}$), 30.5 (CH$_2^{lipid}$), 30.0 (*t*Bu), 28.1 (C$_4$), 26.7 (C$_5$), 25.9 (C$_γ^{Leu}$), 23.1, 21.7 ($2 \times CH_3^{Leu}$), 14 (CH$_3^{lipid}$). LC-MS analysis: R$_t$ 23.6 min, *m/z* 718.6 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min. HR-MS: calc. for [$C_{36}H_{67}N_3O_7S_2$+H]$^+$ 718.4493, found 718.4420.

N-(6-[(N-(N-palmitic acid))-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine (2,6-cis 20). From 2,6-cis **14** (8 mg, 11 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 7.0 min, *m/z* 630.7 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min.

N-(6-[(N-(N-(lauric acid)-glycine))-S-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine (2,6-trans 15). From 2,6-trans **9** (20 mg, 0.03 mmol) following *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.51 (dd, 1H, *J*= 5.2 and 5.6 Hz, H$_α^{Cys}$), 4.40 (dd, 1H, *J*= 4.0 and 4.4 Hz, H$_α^{Leu}$), 4.10 (d, 1H, *J*= 3.6 Hz, H$_2$), 4.03 (m, 1H, H$_3$), 3.77 (s, 2H, H$_α^{Gly}$), 3.71 (m, 1H, H$_6$), 3.25 (bt, 2H, *J*= 5.2 and 5.6 Hz, H$_7$), 3.11 (dd, 1H, *J*= 5.2 Hz, H$_β^{Cys}$), 2.94 (dd, 1H, *J*= 8.4 Hz, H$_β^{Cys}$), 2.17 (t, 2H, CH$_2$, *J*= 7.2 and 8.0 Hz), 1.68-1.29 (m, CH$_2^{lipid}$, H$_{βγ}^{Leu}$, H$_{4\&5}$), 1.28-1.15 (m, *t*Bu, CH$_2^{lipid}$), 0.85 (dd, 6H, $2 \times CH_3^{Leu}$, *J*= 5.6 and 6.0 Hz), 0.80 (t, 3H, *J*= 7.2 and 7.6 Hz, CH$_3^{lipid}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 172.4-171.4 (C=O$^{amide\&carboxyl}$), 78.2 (C$_6$), 72.7 (C$_2$), 65.7 (C$_3$), 54.3, 51.4 (C$_α^{Leu}$, C$_α^{Cys}$), 43.6 (C$_7$), 42.8 (C$_q^{tBu}$), 42.4, 37.2 (C$_β^{Leu}$, C$_β^{Cys}$), 36.6 (C$_α^{Gly}$), 30.3 (CH$_2^{lipid}$), 30.0 (*t*Bu), 26.6 (C$_4$), 22.8 (C$_γ^{Leu}$), 21.3 (C$_5$), 19.6, 18.9

($2\times$CH$_3^{\text{Leu}}$), 14.4 (CH$_3^{\text{lipid}}$). LC-MS analysis: R$_t$ 19.9 min, *m/z* 719.5 (M+H)$^+$. Linear gradient of B (05→95%) in 26 min. HR-MS: calc. for [C$_{34}$H$_{62}$N$_4$O$_8$S$_2$+H]$^+$ 719.4081, found 719.4051.

***N*-(6-[(*N*-(*N*-(lauric acid)-glycine))-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine (2,6-trans 21).** From 2,6-trans **15** (20 mg, 28 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 4.3 min, *m/z* 631.7 (M+H)$^+$. Linear gradient of B (00→90%) in 26 min.

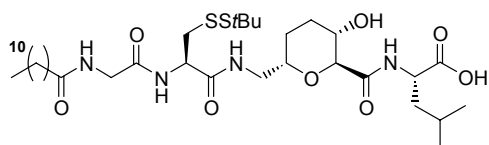***N*-(6-[(*N*-(*N*-(lauric acid)-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine (2,6-cis 15).** From 2,6-cis **9** (44 mg, 0.07 mmol) following *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.59 (t, 1H, *J*= 6.4 and 7.2 Hz, H$_\alpha^{\text{Cys}}$), 4.27 (dd, 1H, *J*= 4.0 Hz, H$_\alpha^{\text{Leu}}$), 3.77 (dd, 2H, *J*= 16.4 Hz, H$_\alpha^{\text{Gly}}$), 3.52 (d, 1H, *J*= 9.6 Hz, H$_2$), 3.47 (m, 2H, H$_6$, H$_{7a}$), 3.37 (m, 1H, H$_4$), 3.07 (m, 2H, H$_{7b}$, H$_\beta^{\text{Cys}}$), 2.94 (dd, 1H, *J*= 7.6 and 8.0 Hz, H$_\beta^{\text{Cys}}$), 2.18 (t, 2H, *J*= 7.6 Hz, CH$_2^{\text{lipid}}$), 2.02 (m, 1H, H$_{4a}$), 1.61-1.40 (m, 8H, H$_{\beta\gamma}^{\text{Leu}}$, H$_{5ab}$, CH$_2^{\text{lipid}}$, H$_{4b}$), 1.25-1.19 (m, *t*Bu, CH$_2^{\text{lipid}}$), 0.87 (d, 6H, *J*= 6.0 Hz, $2\times$CH$_3^{\text{Leu}}$), 0.80 (t, 3H, *J*= 6.8 and 7.2 Hz, CH$_3^{\text{lipid}}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 175.3-170.1 (C=O), 78.0 (C$_6$), 76.7 (C$_2$), 68.3 (C$_3$), 53.4, 50.1 (C$_\alpha^{\text{Leu}}$, C$_\alpha^{\text{Cys}}$), 48.4 (C$_7$), 43.9 (C$_q^{t\text{Bu}}$), 43.3, 41.5 (C$_\beta^{\text{Leu}}$, C$_\beta^{\text{Cys}}$), 36.2 (C$_\alpha^{\text{Gly}}$), 30.9 (*t*Bu), 29.8 (CH$_2^{\text{lipid}}$), 25.6 (C$_4$), 24.9 (C$_\gamma^{\text{Leu}}$), 22.8 (CH$_3^{\text{lipid}}$), 22.6 (C$_5$), 21.8 (CH$_3^{\text{Leu}}$), 14.1 (CH$_3^{\text{lipid}}$). MS (ESI): *m/z* 719.4 (M+H)$^+$. HR-MS: calc. for [C$_{34}$H$_{62}$N$_4$O$_8$S$_2$+H]$^+$ 719.4081, found 719.4105.

***N*-(6-[(*N*-(*N*-(lauric acid)-glycine))-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine (2,6-cis 21).** From 2,6-cis **15** (12 mg, 17 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 8.7 min, *m/z* 631.7 (M+H)$^+$. Linear gradient of B (60→90%) in 26 min.

***N*-(6-[(*N*-(*N*-(lauric acid)-4-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucuronopyranosyl)-L-leucine (2,6-trans 16).** From 2,6-trans **10** (29 mg, 0.04 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.82 (dd, 1H, *J*= 7.6 Hz, H$_\alpha^{\text{Cys}}$), 4.28 (m, 1H, H$_\alpha^{\text{Leu}}$), 4.14 (m, 2H, H$_{2\&3}$), 3.73 (m, 1H, H$_6$), 3.44 (m, 1H, H$_{7a}$), 3.29-3.13 (m, 4H, CH$_2^{\text{lipid}}$, H$_{7a}$, H$_\beta^{\text{Cys}}$), 3.00 (dd, 1H, *J*= 7.6 and 8.0 Hz, H$_\beta^{\text{Cys}}$), 2.30, 2.16 ($2\times$m, 4H, $2\times$CH$_2$), 1.82-1.50 (m, CH$_2^{\text{lipid}}$, H$_{\beta\gamma}^{\text{Leu}}$, H$_4$, H$_5$), 1.35-1.20 (m, *t*Bu, CH$_2^{\text{lipid}}$), 0.85 (m, 9H, $2\times$CH$_3^{\text{Leu}}$, CH$_3^{\text{lipid}}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176.2-171.8 (C=O$^{\text{amide\&carboxyl}}$), 78.6 (C$_6$), 73.7 (C$_2$), 65.5 (C$_3$), 53.9, 53.6 (C$_\alpha^{\text{Leu}}$, C$_\alpha^{\text{Cys}}$), 44.5 (C$_7$), 43.8 (C$_q^{t\text{Bu}}$), 43.5, 39.4 (C$_\beta^{\text{Leu}}$, C$_\beta^{\text{Cys}}$), 37.0, 33.3, 32.8 (CH$_2$), 30.5 (*t*Bu), 30.2 (CH$_2^{\text{lipid}}$), 27.6 (C$_4$), 26.8 (C$_5$), 26.1 (C$_\gamma^{\text{Leu}}$), 23.4, 22.2 ($2\times$CH$_3^{\text{Leu}}$), 14 (CH$_3^{\text{lipid}}$). LC-MS analysis: R$_t$ 24.6 min, *m/z* 747.5 (M+H)$^+$. Linear gradient of B (00→95%) in 26 min. HR-MS: calc. for [C$_{36}$H$_{66}$N$_4$O$_8$S$_2$+H]$^+$ 747.4394, found 747.4446.

***N*-(6-[(*N*-(*N*-(lauric acid)-4-aminobutyric acid))-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine (2,6-trans 22).** From 2,6-trans **16** (8 mg, 11 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 4.6 min, *m/z* 659.6 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min.

***N*-(6-[(*N*-(*N*-(lauric acid)-4-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucuronopyranosyl)-L-leucine (2,6-cis 16).** From 2,6-cis **10** (40 mg, 0.06 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.61 (t, 1H, *J*= 6.8 and 7.2 Hz, H$_\alpha^{\text{Cys}}$), 4.43 (dd, 1H, *J*= 3.6 Hz, H$_\alpha^{\text{Leu}}$), 3.52 (d, 1H, *J*= 9.2 Hz, H$_2$), 3.46 (m, 2H, H$_3$, H$_{7a}$), 3.36 (m, 1H, H$_6$), 3.20-3.02 (m, 4H, CH$_2^{\text{lipid}}$, H$_{7b}$, H$_\beta^{\text{Cys}}$), 2.90 (dd, 1H, *J*= 4.8 and 5.2 Hz, H$_\beta^{\text{Cys}}$), 2.20 (t, 2H, *J*= 7.2 CH$_2$), 2.08 (t, 2H, *J*= 7.6 Hz, CH$_2$), 2.02 (m, 1H, H$_{4a}$), 1.70 (m, 2H, CH$_2^{\text{lipid}}$), 1.64-1.60 (m, H$_{\beta\gamma}^{\text{Leu}}$, H$_{5a}$), 1.52-1.30 (m, CH$_2^{\text{lipid}}$, H$_{4b\&5b}$), 1.30-1.10 (m, *t*Bu, CH$_2^{\text{lipid}}$), 0.87 (m, 6H, $2\times$CH$_3^{\text{Leu}}$), 0.80 (t, 3H, *J*= 7.2 Hz, CH$_3^{\text{lipid}}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 174.9-170.6 (C=O$^{\text{amide\&carboxyl}}$), 77.7 (C$_6$), 76.9 (C$_2$), 68.1 (C$_3$), 53.1, 49.7 (C$_\alpha^{\text{Leu}}$, C$_\alpha^{\text{Cys}}$), 49.4 (C$_7$), 48.0 (C$_q^{t\text{Bu}}$), 43.6, 41.2 (C$_\beta^{\text{Leu}}$, C$_\beta^{\text{Cys}}$), 40.6,

38.8, 36.4 (3×CH$_2$), 29.3 (*t*Bu), 29.1 (CH$_2$^{lipid}), 25.5 (C$_4$), 24.7 (C$_\gamma$^{Leu}), 22.6 (CH$_3$^{Leu}), 22.4 (C$_5$), 21.6 (CH$_3$^{Leu}), 13.8 (CH$_3$^{lipid}). LC-MS analysis: R$_t$ 25.0 min, *m/z* 747.5 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min. HR-MS: calc. for [C$_{36}$H$_{66}$N$_4$O$_8$S$_2$+H]$^+$ 747.4394 (M+H)$^+$, found 747.4411.

***N*-(6-[(*N*-(*N*-(lauric acid)-4-aminobutyric acid))-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine (2,6-cis 22).** From 2,6-cis **16** (20 mg, 27 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 4.7 min, *m/z* 659.6 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min.

***N*-(6-[(*N*-(*N*-(palmitic acid)-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine (2,6-trans 17).** From 2,6-trans **11** (74 mg, 0.09 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.50 (dd, 1H, *J*= 6.4 Hz, H$_\alpha$^{Cys}), 4.40 (dd, 1H, *J*= 4.0 and 4.4 Hz, H$_\alpha$^{Leu}), 4.10 (d, 1H, *J*= 3.6 Hz, H$_2$), 4.01 (m, 1H, H$_3$), 3.75 (s, 2H, H$_\alpha$^{Gly}), 3.70 (m, 1H, H$_6$), 3.25 (m, 2H, H$_7$), 3.10 (dd, 1H, *J*= 5.2 Hz, H$_\beta$^{Cys}), 2.94 (dd, 1H, H$_\beta$^{Cys}, *J*= 1.2 Hz), 2.16 (t, 2H, CH$_2$, *J*= 7.2 and 8.0 Hz), 1.68-1.29 (m, CH$_2$^{lipid} H$_{\beta\gamma}$^{Leu}, H$_{4\&5}$), 1.26-1.10 (m, *t*Bu, CH$_2$^{lipid}), 0.85 (dd, 6H, 2×CH$_3$^{Leu}, *J*= 5.6 and 6.0 Hz), 0.79 (t, 3H, CH$_3$^{lipid}, *J*= 6.8 Hz); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176.8-171.4 (C=O$^{amide\&carboxyl}$), 78.3 (C$_6$), 72.7 (C$_2$), 65.7 (C$_3$), 54.3, 51.4 (C$_\alpha$^{Leu}, C$_\alpha$^{Cys}), 43.6 (C$_7$), 42.7 (C$_q$^{tBu}), 41.2, 36.6 (C$_\beta$^{Leu}, C$_\beta$^{Cys}), 32.8 (C$_\alpha$^{Gly}), 30.5 (CH$_2$^{lipid}), 30.0 (*t*Bu), 26.6 (C$_4$), 25.9 (C$_\gamma$^{Leu}), 23.6 (C$_5$), 23.3, 21.5 (2×CH$_3$^{Leu}), 14.2 (CH$_3$^{lipid}). MS (ESI): *m/z* 775.5 (M+H)$^+$, 797.4 (M+Na)$^+$. HR-MS: calc. for [C$_{38}$H$_{70}$N$_4$O$_8$S$_2$+H]$^+$ 775.4707, found 775.4756.

***N*-(6-[(*N*-(*N*-(palmitic acid)-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono yranosyl)-L-leucine (2,6-trans 23).** From 2,6-trans **17** (19 mg, 28 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 8.6 min, *m/z* 687.6 (M+H)$^+$. Linear gradient of B (80→90%) in 26 min.

***N*-(6-[(*N*-(*N*-(palmitic acid)-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine (2,6-cis 17).** From 2,6-cis **11** (48 mg, 0.06 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.55 (dd, 1H, *J*= 6.0 Hz, H$_\alpha$^{Cys}), 4.47 (dd, 1H, *J*= 6.8 and 7.6 Hz, H$_\alpha$^{Leu}), 3.76 (s, 2H, H$_\alpha$^{Gly}), 3.53 (d, 1H, *J*= 9.2 Hz, H$_2$), 3.40 (m, 2H, H$_3$, H$_{7a}$), 3.29 (dd, 1H, *J*= 3.2 Hz, H$_6$), 3.18 (1H, H$_{7b}$), 3.09 (dd, 1H, *J*= 5.6 and 6.0 Hz, H$_\beta$^{Cys}), 2.92 (dd, 1H, *J*= 7.6 and 8.0 Hz, H$_\beta$^{Cys}), 2.15 (t, 2H, *J*= 7.2 and 7.6 Hz, CH$_2$), 2.01 (m, 1H, H$_{4a}$), 1.64-1.60 (m, 4H, H$_{\beta\gamma}$^{Leu}, H$_{5a}$), 1.50-1.27 (m, CH$_2$^{lipid}, H$_{4b\&5b}$), 1.25-1.19 (m, *t*Bu, CH$_2$^{lipid}), 0.87 (dd, 6H, *J*= 6.0 Hz, 2×CH$_3$^{Leu}), 0.80 (t, 3H, *J*= 7.2 Hz, CH$_3$^{lipid}); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176-170 (C=O$^{amide\&carboxyl}$), 80.6 (C$_6$), 77.6 (C$_2$), 69.3 (C$_3$), 54.2, 51.2 (C$_\alpha$^{Leu}, C$_\alpha$^{Cys}), 44.3 (C$_7$), 43.5 (C$_q$^{tBu}), 42.6, 41.4 (C$_\beta$^{Leu}, C$_\beta$^{Cys}), 36.6 (C$_\alpha$^{Gly}), 30.5 (CH$_2$^{lipid}), 30.0 (*t*Bu), 26.5 (C$_4$), 25.8 (C$_\gamma$^{Leu}), 23.5 (C$_5$), 23.2, 21.7 (2×CH$_3$^{Leu}), 15.2 (CH$_3$^{lipid}). MS (ESI): *m/z* 775.6 (M+H)$^+$, 797.4 (M+Na)$^+$. HR-MS: calc. for [C$_{38}$H$_{70}$N$_4$O$_8$S$_2$+H]$^+$ 775.4707, found 775.4690.

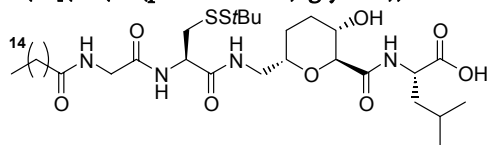***N*-(6-[(*N*-(*N*-(palmitic acid)-glycine))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy-β-D-glucurono pyranosyl)-L-leucine (2,6-cis 23).** From 2,6-cis **17** (10 mg, 15 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 6.8 min, *m/z* 687.6 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min.

***N*-(6-[(*N*-(*N*-4-(palmitic acid)-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy -α-D-glucuronopyranosyl)-L-leucine (2,6-trans 18).** From 2,6-trans **12** (58 mg, 0.07 mmol) following *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.50 (dd, 1H, *J*= 5.2 Hz, H$_\alpha$^{Cys}), 4.40 (m, 1H, H$_\alpha$^{Leu}), 4.11 (d, 1H, *J*= 3.6 Hz, H$_2$), 4.03 (m, 1H, H$_3$), 3.72 (m, 1H, H$_6$), 3.26 (m, 2H, H$_7$), 3.12-3.06 (m, 3H, CH$_2$^{lipid}, H$_\beta$^{Cys}), 2.92 (dd, 1H, *J*= 6.0 Hz, H$_\beta$^{Cys}), 2.16, 2.06 (2×t, 4H, *J*= 7.2 and 7.6 Hz, 2×CH$_2$), 1.73-1.42 (m, CH$_2$^{lipid}, H$_{\beta\gamma}$^{Leu}, H$_4$, H$_5$), 1.30-1.10 (m, *t*Bu, CH$_2$^{lipid}), 0.85 (dd, 6H, *J*= 5.6 and 6.0 Hz, 2×CH$_3$^{Leu}), 0.79 (t, 3H, *J*= 6.8 Hz, CH$_3$^{lipid}); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176.1-172.4 (C=O$^{amide\&carboxyl}$), 78.3 (C$_6$),

72.7 (C$_2$), 65.7 (C$_3$), 54.2, 51.4 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$), 43.5 (C$_7$), 43.0 (C$_q^{tBu}$), 41.1, 39.3 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 37.0, 33.7, 32.8 (CH$_2$), 30.5 (CH$_2^{lipid}$), 30.0 (*t*Bu), 26.4 (C$_4$), 26.0 (C$_\gamma^{Leu}$), 23.6 (C$_5$), 23.3, 21.5 (2×CH$_3^{Leu}$), 14.2 (CH$_3^{lipid}$). MS (ESI): *m/z* 803.5 (M+H)$^+$. HR-MS: calc. for [C$_{40}$H$_{74}$N$_4$O$_8$S$_2$+H]$^+$ 803.5020 (M+H)$^+$, found 803.4991.

***N*-(6-[(*N*-(*N*-4-(palmitic acid)-aminobutyric acid))-L-cysteinyl]-aminomethyl-4,5-dideoxy-α-D-glucurono pyranosyl)-L-leucine (2,6-trans 24).** From 2,6-trans **18** (17 mg, 21 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 6.6 min, *m/z* 715.5 (M+H)$^+$. Linear gradient of B (10→95%) in 26 min.

***N*-(6-[(*N*-(*N*-4-(palmitic acid)-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy -β-D-glucuronopyra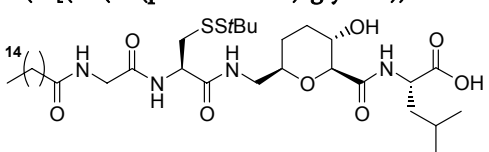nosyl)-L-leucine (2,6-cis 18).** From 2,6-cis **12** (44 mg, 0.05 mmol) according to *GP 2*. Crude yield: 100%. ^{1}H-NMR (400 MHz, methanol-*d4*) δ 4.54 (dd, 1H, *J*= 6.0 Hz, H$_\alpha^{Cys}$), 4.43 (t, 1H, *J*= 6.8 and 7.2 Hz, H$_\alpha^{Leu}$), 3.50 (d, 1H, *J*= 9.2 Hz, H$_2$), 3.42 (m, 2H, H$_3$, H$_{7a}$), 3.29 (dd, 1H, *J*= 3.2 and 3.6 Hz, H$_6$), 3.18 (1H, H$_{7b}$), 3.12-3.02 (m, 2H, CH$_2^{lipid}$), 3.09 (dd, 1H, J= 8.0 Hz, H$_\beta^{Cys}$), 2.92 (dd, 1H, *J*= 7.6 and 8.0 Hz, H$_\beta^{Cys}$), 2.18 (t, 2H, *J*= 7.2 and 7.6 Hz, CH$_2$), 2.08 (t, 2H, *J*= 7.2 and 8.0 Hz, CH$_2$), 2.01 (m, 1H, H$_{4a}$), 1.70 (m, 2H, CH$_2^{lipid}$), 1.64-1.60 (m, H$_{\beta\gamma}^{Leu}$, H$_{5a}$), 1.52-1.30 (m, CH$_2^{lipid}$, H$_{4b\&5b}$), 1.25-1.19 (m, *t*Bu, CH$_2^{lipid}$), 0.87 (dd, 6H, *J*= 5.2 and 5.6 Hz, 2×CH$_3^{Leu}$), 0.80 (t, 3H, *J*= 6.4 and 7.2 Hz, CH$_3^{lipid}$); ^{13}C-NMR (50 MHz, methanol-*d4*) δ 176.0-172.5 (C=O$^{amide\&carboxyl}$), 80.7 (C$_6$), 77.6 (C$_2$), 69.3 (C$_3$), 54.2, 51.6 (C$_\alpha^{Leu}$, C$_\alpha^{Cys}$), 44.2 (C$_7$), 42.8 (C$_q^{tBu}$), 42.3, 41.6 (C$_\beta^{Leu}$, C$_\beta^{Cys}$), 39.4, 36.9, 33.8 (3×CH$_2$), 30.5 (CH$_2^{lipid}$), 30.0 (*t*Bu), 26.4 (C$_4$), 25.9 (C$_\gamma^{Leu}$), 23.5 (C$_5$), 23.2, 21.2 (2×CH$_3^{Leu}$), 14.4 (CH$_3^{lipid}$). LC-MS analysis: R$_t$ 23.1 min, *m/z* 803.5 (M+H)$^+$. Linear gradient of B (10→90%) in 26 min. HR-MS: calc. for [C$_{40}$H$_{74}$N$_4$O$_8$S$_2$+H]$^+$ 803.5020, found 803.5039 (M+H)$^+$.

***N*-(6-[(*N*-(*N*-4-(palmitic acid)-aminobutyric acid))-*S*-tert-butylthio-L-cysteinyl]-aminomethyl-4,5-dideoxy -β-D-glucuronopyrano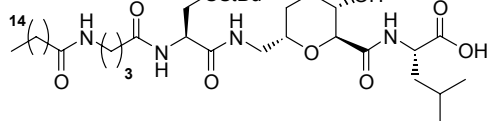syl)-L-leucine (2,6-cis 24).** From 2,6-cis **18** (11 mg, 14 μmol) according to *GP 3*. LC-MS analysis: R$_t$ 7.0 min, *m/z* 715.6 (M+H)$^+$. Linear gradient of B (70→90%) in 26 min.

4.5 References and Notes

(1)   For some examples of bisubstrate design in drug development, see: (a) C. Lerner, B. Masjost, A. Ruf, V. Gramlich, R. Jakob-Roetne, G. Zürchner, E. Borroni, F. Diederich *Org. Biomol. Chem.* **2003**, *1*, 42. (b) W. T. Miller *Nat. Struct. Biol.* **2001**, *8*, 16. (c) K. Parang, J. H. Till, A. J. Ablooglu, R. A. Kohanski, S. R. Hubbard, P. A. Cole *Nat. Struct. Biol.* **2001**, *8*, 37. (d) E. Wolf, J. De Angelis, E. M. Khalil, P. A. Cole, S. K. Burley *J. Mol. Biol.* **2002**, *317*, 215. (e) K. L. Levert, G. L. Waldrop *Biochem. Biophys. Res. Commun.* **2002**, *291*, 1213.

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Chapter 5

Tetrazole as Carboxyl Bioisostere in the Development of Ca_1a_2X Box Based Prenyl Transferase Inhibitors

Abstract. A suitably protected tetrazole analog of methionine (**10**) was synthesised and subsequently used to evaluate whether the tetrazole moiety can be used as a carboxyl isostere in the Ca_1a_2X based approach toward PFT inhibitors as presented in Chapter 3. Biological evaluation of the tetrazole analogs of reference compound CVIM (**15**) and the previously presented PFT inhibitor **1** (**19**) shows that both selectivity and significant inhibitory potency against PFT are preserved, indicating that the tetrazole is a suitable carboxylic acid isostere for SAA modified Ca_1a_2X analogs.

5.1 Introduction

A well established approach (see Chapter 1) toward the development of inhibitors of the prenylating enzymes PFT and PGGT-1 entails the development of analogs of the tetrapeptide sequence Ca_1a_2X (Figure 5.1, C= cysteine, a_1= any (aliphatic) amino acid, a_2= any aliphatic amino acid (*e.g.* Val, Ile), X= Leu for PGGT-1 inhibitors and X= Met (or Ser) for PFT inhibitors. A new type of inhibitors based on the replacement of the central two amino acids by a sugar amino acid (SAA) is described in Chapters 2 and 3. Compound **1** (figure 5.1), having a 2,6-cis arrangement of the SAA part and a benzyl ether substituent on C_3, was found to be a highly selective (PGGT-1: IC_{50} > 1000 µM) and potent inhibitor of PFT (IC_{50}= 250 ± 20 nM). The leucine derivative of **1** is compound **2** and this Ca_1a_2X mimic was found to inhibit PFT with an IC_{50} value of 57 ± 18 µM and PGGT-1 with an IC_{50} value of 14 ± 6 µM.
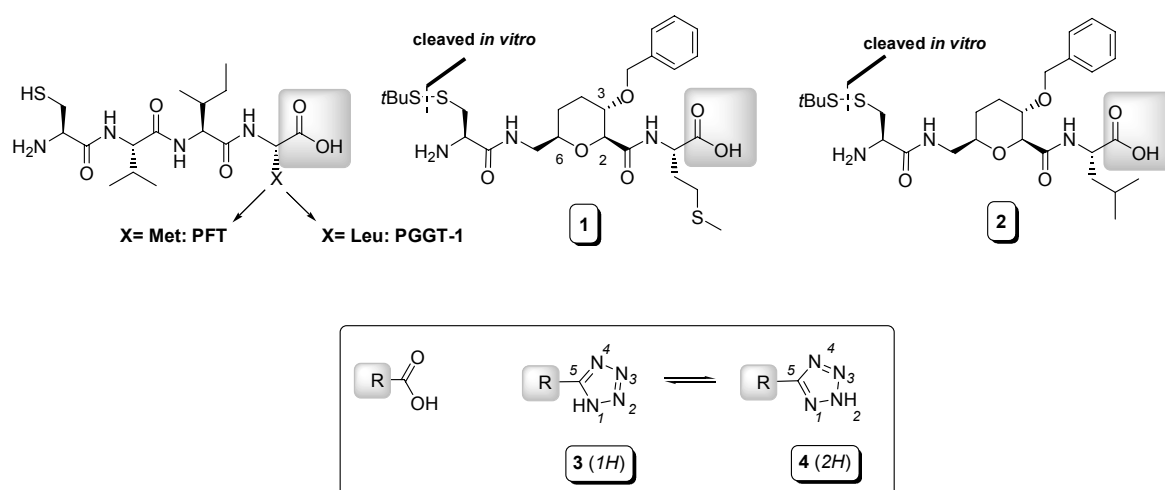
Figure 5.1 Isosteric replacement of the carboxyl group by a tetrazole (**3**↔**4**) in Ca_1a_2X mimics **1** and **2**.

Besides the substitution of the a_1a_2 part in the Ca_1a_2X sequence, several research groups have investigated whether the C or X residues can be modified. As the side chain of the X-residue is an important structural recognition element attaining both inhibitory action and selectivity between PFT and PGGT-1, the application of a suitable carboxylic acid isostere was considered. Based on this, the resemblance of the tetrazole group (**3**↔**4**, Figure 5.1) with the carboxylic acid functionality in terms of acidity, planarity, biostability and lipophilicity make the tetrazole moiety a suitable candidate.[1] Ohkanda *et*

al.[2] have demonstrated that the use of a tetrazole as a carboxyl isostere has a beneficial effect on the potency of their type of PFT inhibitors. In this chapter the synthesis and biological evaluation of Ca_1a_2X analogs **15** and **19** (Scheme 5.3 and 5.4, respectively) is described.

5.2 Results and Discussion

5.2.1 Synthesis – First, the synthesis of the appropriate tetrazole analogs of methionine and leucine (Scheme 5.1) was investigated. For this purpose, the method of Duncia *et al.*[3] was employed, as the thus synthesised tetrazoles are protected at the *N1* position (Figure 5.1) with the base labile propionitrile group.

Boc-Leu-OH (**5**) and Boc-Met-OH (**6**) were converted into the corresponding cyanoethyl amides (**6** and **9**, respectively) in good yield. Treatment of **9** with PPh$_3$, TMSN$_3$ and DIAD and ensuing removal of the Boc group afforded tetrazole compound **10** (TFA salt) in >99% from **9**. Unfortunately, conversion of **6** into **7** failed under several conditions (elevated temperature, various amounts of reagents). The sensitivity of the tetrazole formation to steric hindrance in combination with the bulky leucine side chain[4] probably prevents formation of the tetrazole ring (Scheme 5.2).[5]

Scheme 5.1 Synthesis amino tetrazole **10** and attempted synthesis of **7**.[a]
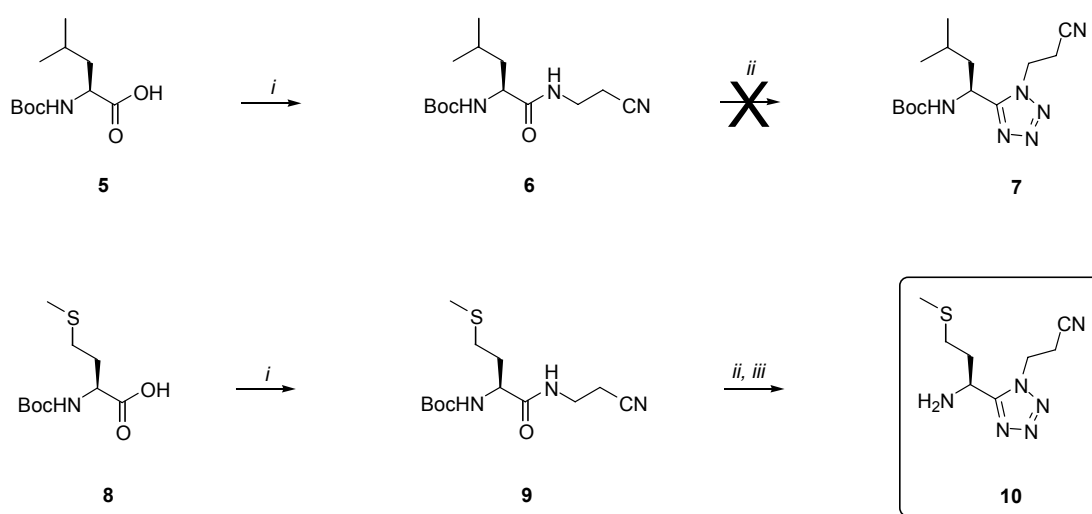
[a]Reagents and conditions. (*i*) fumarate•H$_2$NCH$_2$CH$_2$C≡N, EDC, HOBt, DMF/DCM (**5**: 73%; **9**: 67%); (*ii*) CH$_3$CN, 0 – 4°C, PPh$_3$, DIAD, TMSN$_3$; (*iii*) TFA, DCM, *i*Pr$_3$SiH (**10**: >95%, from **9**).
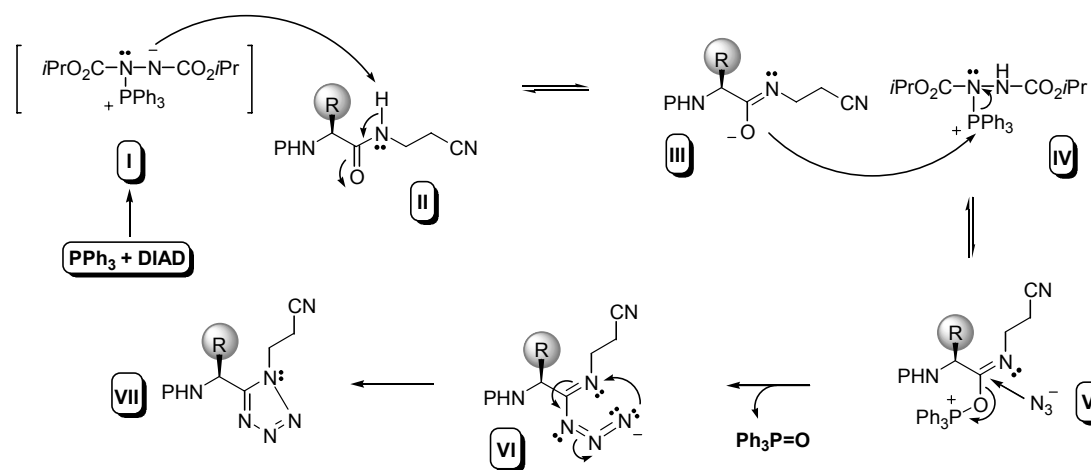
Scheme 5.2 Proposed mechanism of tetrazole formation from an amide with N_3^- and species **V**.

CVIM-*1H*-tetrazole (**15**, Scheme 5.3) was synthesised by Boc chemistry as follows. Thus, amino tetrazole **10** was condensed with Boc-Val-OH (BOP, HOBt) affording dimer **11** in 61% yield. Treatment of **11** with TFA and subsequent coupling with Boc-Ile-OH furnished trimer **12** in 54% over 2 steps. Following the same procedure, Boc-Cys(Tr)-OH was incorporated leading to the fully protected tetramer **13** (70% from **12**). Treatment of **13** with excess DBU (7 equiv.) led to removal of the propionitrile group (yield 95%) and finally deprotection of the cysteine residue (TFA, DCM and R_3SiH) afforded CVIM analog **15** (Scheme 5.3) which after purification by RP-HPLC (purity ≥95%) was isolated in 28% overall yield starting from **13**.

Although both the propionitrile group and Fmoc group can be removed by base mediated β-elimination, the Fmoc group is much more labile.[6] This offered the opportunity to explore the use of Fmoc protected SAA **16b** in the synthesis of target molecule **19** (Scheme 5.4). SAA **16b** was readily obtained from the corresponding Boc derivative **16a** of which the synthesis is presented in Chapter 3. Coupling of **10** and **16b** gave dimer **17** in 71% yield. Selective removal of the Fmoc group in **17** with 1.0 equiv. DBU in DCM was complete after 30 min (TLC analysis). Subsequent addition of HOBt (2.0 equiv.) and coupling of the released amine with Fmoc-Cys(S*t*Bu)-OH gave fully protected **18** in a two-step one-pot procedure. After purification by silica gel chromatography compound **18** was isolated in 83% yield from **17**. Treatment of **18** with excess DBU caused deprotection of the tetrazole and cysteine residue to furnish **19**, which after purification by RP-HPLC (purity ≥ 95%) was isolated in ~20% overall yield from **18**.

Scheme 5.3 Synthesis of CVIM analog **15**.[a]
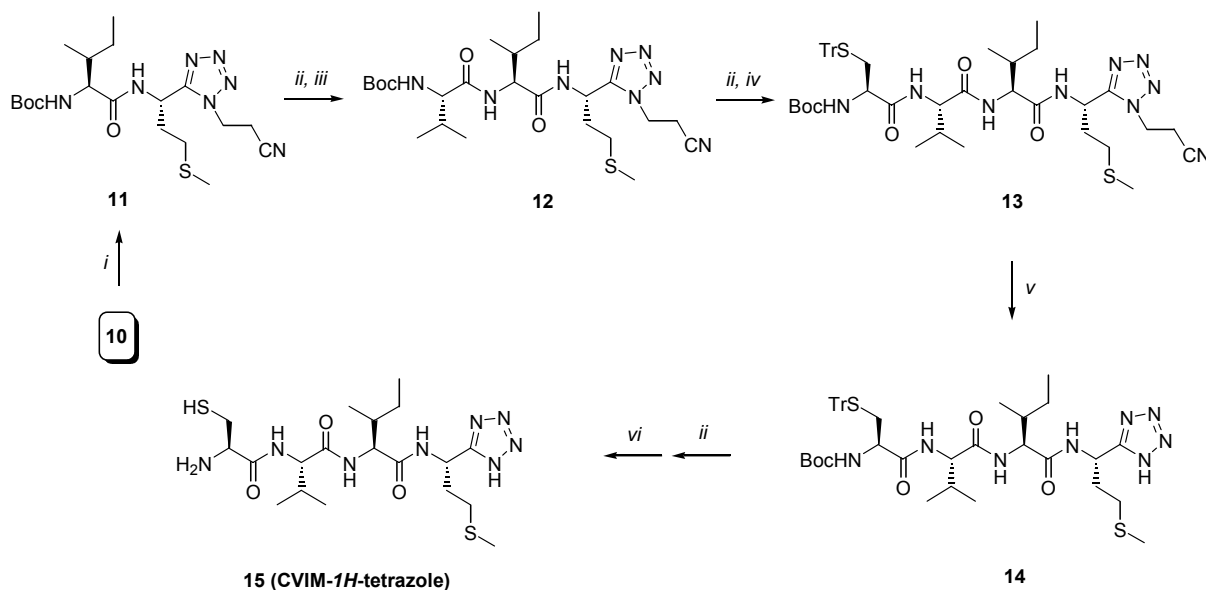
[a]Reagents and conditions. (*i*) Boc-Ile-OH, BOP, HOBt, DIPEA, DMF (**11**: 61%); (*ii*) TFA, DCM, *i*Pr$_3$SiH or Et$_3$SiH; (*iii*) Boc-Val-OH, BOP, HOBt, DIPEA, DMF (**12**: 54%, 2 steps); (*iv*) Boc-Cys(Tr)-OH, BOP, HOBt, DIPEA, DMF (**13**: 70%, 2 steps); (*v*) 7 equiv. DBU, DCM; (*vi*) RP-HPLC (**15**: 28% from **13**).

Scheme 5.4 Synthesis of compound **19**.[a]
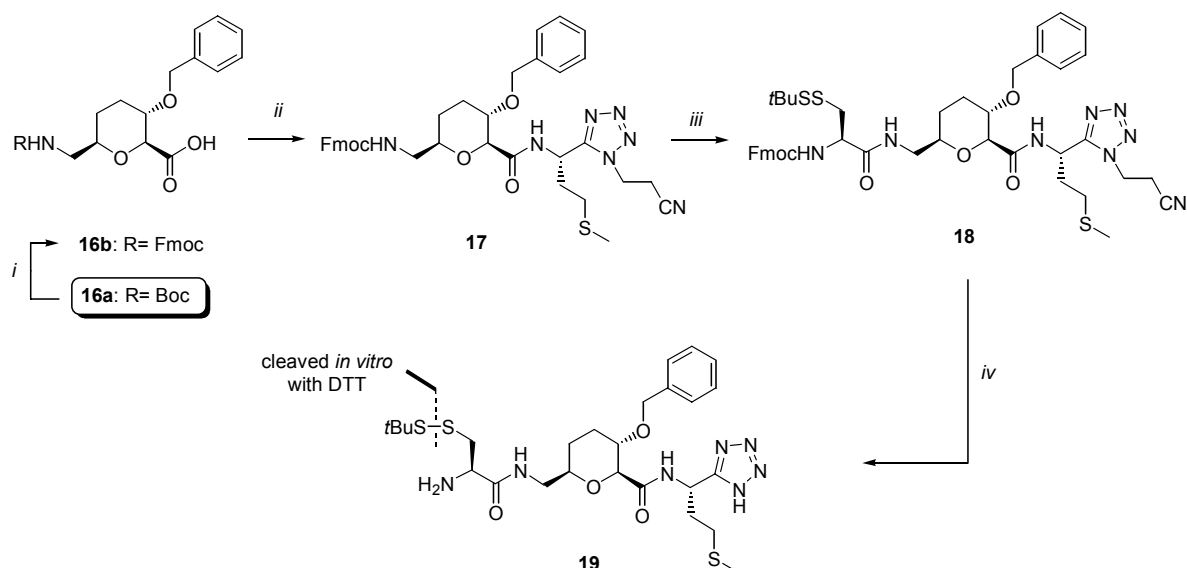
[a]Reagents and conditions. (*i*) (a) TFA, DCM, *i*Pr$_3$SiH (b) FmocOSu, NaHCO$_3$, H$_2$O (**16b**: 92%, 2 steps); (*ii*) **10**, HCTU, DIPEA, DMF (**17**: 71%); (*iii*) (a) DBU (1.0 equiv.), DCM (b) HOBt (c) Fmoc-Cys(S*t*Bu)-OH, HCTU, DIPEA, DMF (**18**: 83%, "one pot procedure"); (*iv*) (a) 8 equiv. DBU, DCM (b) RP-HPLC (**19**: 20% from **18**).

5.2.2 Biological Evaluation – Compounds **15** and **19** were evaluated for their *in vitro* inhibitory activity against pure PFT and PGGT-1.[7] The tetrapeptide CVIM[8] was evaluated as a reference. The S*t*Bu protective group is cleaved under the conditions of the assay (DTT, pH 7.4). In Figure 5.2 the results of the biological evaluation against PFT are depicted.
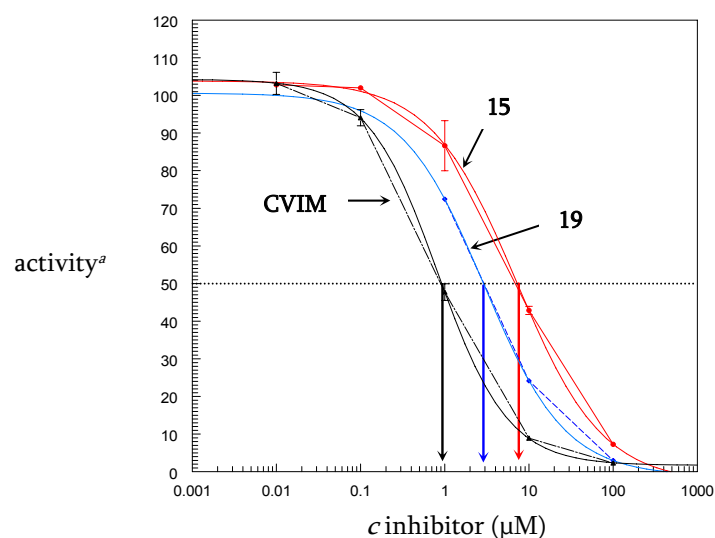
Figure 5.2 Results biological evaluation of **15**, **19** and **CVIM** against PFT. [a]Activity of PFT at a certain concentration of compound: expressed as % of control activity (*i.e.* without test compound).

In line with their carboxylic analogs (Chapter 3), compounds **15** and **19** did not show any inhibitory potency against PGGT-1. The inhibitory action of **15** and **19** toward PFT was slightly reduced in comparison with their carboxylic counterparts.

5.3 Conclusions

This chapter describes the concise synthesis of Ca_1a_2X analogs **15** and **19**. Their biological evaluation shows that tetrazole can be incorporated as a suitable carboxylic acid isostere in the SAA based development of PFT inhibitors. Unfortunately, as the targeted tetrazole analog of leucine (**7**) could not be synthesised according to the method employed in this chapter, it is uncertain whether Ca_1a_2X analogs targeted at inhibiting PGGT-1 can be provided with this carboxylic acid isostere (see Chapter 8).

5.4 Experimental Section

5.4.1 General - ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC-200 (200, 50.1 MHz), a Bruker DPX-300 (^{1}H: 300, ^{13}C: 75 MHz), a Bruker Avance-400 (^{1}H: 400, ^{13}C: 100 MHz) or a Bruker DMX-600 (^{1}H: 600, ^{13}C: 150 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Mass spectra were recorded with a Perkin Elmer/SCIEX API 165 mass instrument and HR-MS spectra were recorded with an API QSTAR^{tm} Pulsar (Applied Biosystems). Eluents were of technical grade and distilled before use. DMF (Biosolve p.a.), DCM (Biosolve p.a.) and toluene (Biosolve p.a.) were stored over molecular sieves (4Å). EtOAc and PE (40-60) were of technical grade and distilled before use. MeOH (Biosolve), HCl (Acros), DIC (Fluka), iPr$_3$SiH (Aldrich), EDC (Aldrich), TFA (Biosolve), Boc-Leucine (Novabiochem), Boc-Methionine (Novabiochem), 3-aminopropionitrile fumarate (Aldrich), PPh$_3$ (Acros), DIAD (Acros), TMSN$_3$ (Fluka), THF (Biosolve), PMe$_3$ (Aldrich), Et$_3$N (Acros) were used as received. Reactions were followed by TLC analysis on silica gel (Schleicher & Schuell, F 1500 LS 254) or HPTLC aluminium sheets (Merck, silica gel 60, F254), with detection by UV-absorption (254 nm) where applicable and charring at 150°C with 20% H$_2$SO$_4$ in EtOH (25 g L^{-1}), ninhydrin (3 g L^{-1}) in EtOH/AcOH (100/3 v/v), NH$_4$(Mo)$_7$O$_{24}$·4H$_2$O (25 g L^{-1}) and NH$_4$Ce(SO$_4$)$_4$·2H$_2$O (10 g L^{-1}) in 10% aq. H$_2$SO$_4$ or 2% KMnO$_4$ in 1% aq. K$_2$CO$_3$. Column chromatography was performed with silica gel (Fluka; 0.063-0.200 mm). RP-HPLC analysis and purification were performed on a Jasco HPLC system equipped with a Merck Lichrosphere C18 100Å column (4×250 mm). The applied solvents were H$_2$O (eluent A), CH$_3$CN (eluent B) and 1% aq. TFA (effective 0.1%, eluent C).

5.4.2 General procedures - *General procedure 1 (GP 1):* a $\approx$0.01M solution of the amino acid in DCM/DMF (1/1, v/v) was cooled to 0°C and 1.1 equiv. EDC and 1.1 equiv. HOBt were added. After 30 min of stirring at 0°C, 3-aminopropionitrile fumarate (1.2 equiv.) was added and the mixture is stirred overnight during which the temperature is allowed to rise to room temperature. After TLC analysis (DCM/MeOH: 9/1 v/v, ninhydrine) showed consumption of starting material, the DCM was evaporated under reduced pressure and the mixture was taken up in EtOAc, washed with saturated aq. KHSO$_4$, aq. NaHCO$_3$ and brine. The organic phase was dried (MgSO$_4$), concentrated and subjected to silica gel column chromatography.

General procedure 2 (GP 2): to a stirred solution of the coupled amino acid in CH$_3$CN, PPh$_3$ (2.5 equiv.) was added at 0°C, DIAD (2.5 equiv.) was added slowly, followed by TMSN$_3$ (10 equiv.), which was added over 20 min The mixture was stirred overnight at 0–4°C, after which it was cooled to 0°C and a solution of NaNO$_2$ (1 equiv.) in H$_2$O was added. After 30 min a solution of ceric-ammonium nitrate (1 equiv.) in H$_2$O was added over 10 min. After 20 min ice-cold water was added and the aqueous solution was extracted with EtOAc (3×). The combined organic phases were washed with brine, dried (MgSO$_4$) and concentrated.

General procedure 3a (GP 3a): to a ~0.05M solution of the dimer in DCM were added iPr$_3$SiH (1.3 equiv.) or Et$_3$SiH (1.3 equiv.) and TFA ($\rightarrow$50% TFA in DCM). After TLC analysis (PE/EtOAc 1/1 v/v) showed total consumption of starting material, the reaction mixture was coevaporated 5× with anh. toluene.

General procedure 3b (GP 3b): to a ~0.1M solution of the amine in DMF was added the appropriate amino acid (1.1 equiv.), BOP (1.1 equiv.), HOBt (1.1 equiv.) and DIPEA (4 equiv.). After TLC analysis (PE/EtOAc 1/1 v/v) showed consumption of the starting material, DMF was removed *in vacuo*. The residue was dissolved in EtOAc and washed with water (2×), sat. aq. NaHCO$_3$ (2×), water (2×), 5% aq. KHSO$_4$ (2×) and brine. The organic phase was dried (MgSO$_4$) and evaporated *in vacuo*.

Boc-Leu-aminopropionitrile (6). Following *GP 1* using Boc-Leu-OH (1.01 g, 4.36 mmol) gave **6** (73% yield, white solid). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.26, 5.53 (2×bs, 2×NH), 4.16 (bs, 1H, H$_\alpha^{Leu}$), 4.57-4.43 (m, 2H, CH$_2$), 3.16 (t, 2H, NCH$_2$, *J*= 6.4 Hz), 1.69-1.48 (2×m, 3H, H$_{\beta\gamma}^{Leu}$), 1.45 (s, 9H, *t*Bu), 0.94 (t, 6H, 2×CH$_3$, *J*= 6.6 and 6.7 Hz). ^{13}C-NMR (100 MHz, CDCl$_3$) δ 173.6 (C=O^{amide}), 155.8 (C=O^{Boc}), 117.9 (C≡N), 80.1 (C$_q^{Boc}$), 53.0 (C$_\alpha^{Met}$), 41.2 (CH$_2$), 35.5 (C$_\beta^{Leu}$), 28.3 (*t*Bu), 23.4 (C$_\gamma^{Leu}$), 22.8, 21.8 (2×CH$_3^{Leu}$), 18.1 (CH$_2$), MS (ESI): *m/z* 284.0 (M+H)$^+$, 306.2 (M+Na)$^+$. HR-MS: calc. for [C$_{14}$H$_{25}$N$_3$O$_3$+H]$^+$ 284.19687, found 284.19678. [α]$_D^{25}$= −40 (*c*= 1, CHCl$_3$).
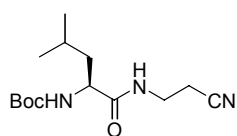
Boc-Met-aminopropionitrile (9). Following *GP 1* and using Boc-Met-OH (**8**, 750 mg, 3.00 mmol) gave **9** (67% yield, white solid). ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.0 (C=O^{amide}), 154.8 (C=O^{Boc}), 117.36 (C≡N), 78.4 (C$_q^{Boc}$), 52.8 (C$_\alpha^{Met}$), 34.6 (CH$_2$), 31.3, 29.2 (C$_{\beta\gamma}^{Met}$), 27.4 (*t*Bu), 17.0 (CH$_2$), 14.2 (SCH$_3$). MS (ESI): *m/z* 302.1 (M+H)$^+$, 324.0 (M+Na)$^+$. HR-MS: calc. for [C$_{13}$H$_{23}$N$_3$O$_3$S+H]$^+$ 302.15329, found 302.15283. [α]$_D^{25}$= −18 (*c*= 1, CHCl$_3$).
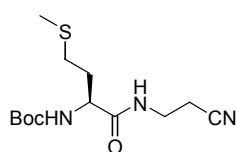
H-Met-*1H*-propionitrile-tetrazole (10). Compound **9** (100 mg, 0.31 mmol) was treated according to *GP 2*. The crude product can be applied to a silica gel column (EtOAc) affording Boc-Met-*1H*-propionitrile-tetrazole as a white solid. ^{1}H-NMR (200 MHz, CDCl$_3$): δ 6.85 (s, 1H, NHBoc), 4.93-5.02 (m, 1H, H$_\alpha^{Met}$), 4.82-4.90 (m, 2H, CH$_2$C≡N), 3.12-3.19 (m, 2H, NCH$_2$), 2.66 (m, 2H, H$_\beta^{Met}$), 2.43 (m, 2H, H$_\gamma^{Met}$), 2.09 (s, 3H, SCH$_3$), 1.41 (s, 9H, *t*Bu). ^{13}C-NMR (50 MHz, CDCl$_3$): δ 164.6 (C=N^{tetrazole}), 155.6 (C=O^{Boc}), 115.9 (C≡N), 80.9 (C$_q^{tBu}$), 42.8 (C$_\alpha^{Met}$), 42.7 (CH$_2$), 32.0, 29.9 (C$_{\beta\gamma}^{Met}$), 28.1 (*t*Bu), 18.4 (CH$_2$), 15.0 (SCH$_3$). MS (ESI): *m/z* 348.9 (M+Na)$^+$. HR-MS: calc. for [C$_{13}$H$_{22}$N$_6$O$_2$S+H]$^+$ 327.15977, found 327.16174. Next, crude Boc-Met-*1H*-propionitrile-tetrazole was deprotected with TFA/DCM/Et$_3$SiH (25/75/2.5 v/v/v) for 30 min. Evaporation and silica gel purification (R$_f$ 0.5, 25% MeOH in EtOAc) gave **10** as a dark-brown oil (yield >99% from **9**). ^{1}H-NMR (200 MHz, MeOD): δ 5.12 (m, 1H, H$_\alpha^{Met}$), 4.85 (m, 2H, CH$_2$C≡N), 3.30 (m, 2H, NCH$_2$), 2.64-2.45 (m, 4H, H$_{\beta\gamma}^{Met}$), 2.06 (s, 3H, SCH$_3$); ^{13}C-NMR (75 MHz, MeOD): δ 154.3 (C=N^{tetrazole}), 118.2 (C≡N), 44.5 (C$_\alpha^{Met}$ and CH$_2$C≡N), 33.0, 29.7 (C$_{\beta\gamma}^{Met}$), 18.8 (CH$_2$), 14.8 (SCH$_3$). IR *ν* (cm^{-1}, film): 3425, 2924, 2361, 1674, 1551, 1435, 1196, 1134. MS (ESI): *m/z* 227.1 (M+H)$^+$, 248.9 (M+Na)$^+$. HR-MS: calc. for [C$_8$H$_{14}$N$_6$S+H]$^+$ 227.10734, found 227.10713.

Boc-Ile-Met-*1H*-propionitrile-tetrazole (11). Compound **10** (0.49 g, 2.15 mmol) was treated according to *GP 3b*. Purification by silica gel chromatography (R$_f$ 0.4, EtOAc/PE 1/1) gave **11** in 61% yield (0.57 g, 1.3 mmol) as a white solid. ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.27 (d, 1H, NHC=O), 5.47 (dd, 1H, H$_\alpha^{Met}$, *J*= 7.2 Hz), 5.11 (d, 1H, NHBoc, *J*= 7.2 Hz), 4.90, 4.75 (2×dt, 2H, CH$_2$C≡N), 3.12 (m, 2H, CH$_2$CH$_2$C≡N), 2.57 and 2.38 (2×m, 4H, H$_{\beta\gamma}^{Met}$), 2.01 (s, 3H, SCH$_3$), 1.70 (bs, 3H, H$_{\beta\gamma}^{Ile}$), 1.32 (s, 9H, *t*Bu), 1.02 and 0.86–0.80 (2×m, 6H, 2×CH$_3^{Ile}$); ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.8 (C=O^{amide}), 155.6, 155.2 (C=N^{tetrazole} and C=O^{Boc}), 116.2 (C≡N), 79.3 (C$_q^{tBu}$), 58.6 (C$_\alpha^{Ile}$), 42.6 (CH$_2$C≡N), 40.9 (C$_\alpha^{Met}$), 36.2 (C$_\beta^{Ile}$), 31.1, 29.3 (C$_{\beta\gamma}^{Met}$), 27.6 (*t*Bu), 24.2 (C$_\gamma^{Ile}$), 17.8 (NCH$_2$), 14.7, 13.4, 10.2 (SCH$_3$ and 2×CH$_3^{Ile}$). MS (ESI): *m/z* 440.3 (M+H)$^+$, 462.2 (M+Na)$^+$, 478.1 (M+K)$^+$. HR-MS: calc. for [C$_{19}$H$_{33}$N$_7$O$_3$S+H]$^+$ 440.24384, found 440.24355; calc. for [C$_{19}$H$_{33}$N$_7$O$_3$S+NH$_4$]$^+$ 457.27038, found 457.27008. [α]$_D^{25}$= −36 (*c*= 0.86, MeOH).

Boc-Val-Ile-Met-*1H*-propionitrile-tetrazole (12). Compound **11** (0.52 g, 1.19 mmol) was treated according to *GP 3a* and *3b*. Silica gel chromatography (EtOAc, R$_f$ 0.4) gave **12** (0.34 g, 0.64 mmol) as a white solid in 54% yield. ^{13}C-NMR (50 MHz, CDCl$_3$): δ 172.6, 172.0 (C=O^{amide}), 155.3, 155.2 (C=N^{tetrazole} and C=O^{Boc}), 116.2 (C≡N), 80.0 (C$_q^{tBu}$), 60.3, 57.6 (C$_\alpha^{Ile}$ and C$_\alpha^{Val}$), 42.9 (CH$_2$C≡N), 41.2 (C$_\alpha^{Met}$), 36.1, 30.1 (2×C$_\beta^{Ile,Val}$), 31.5, 29.7 (C$_{\beta\gamma}^{Met}$), 28.0 (*t*Bu), 24.5 (C$_\gamma^{Ile}$), 18.2 (NCH$_2$), 19.0, 17.8, 15.1, 14.7, 10.6 (SCH$_3$ and CH$_3^{Ile,Val}$). IR *ν* (cm^{-1}, film): 2963, 2361, 1690, 1643, 1528, 1173. HR-MS: calc. for [C$_{24}$H$_{42}$N$_8$O$_4$S+H]$^+$ 539.31225, found 539.31238. [α]$_D^{25}$= −31 (*c*= 1, DCM).
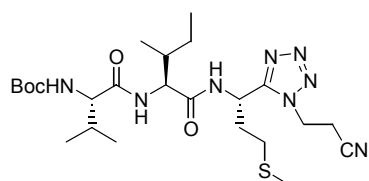
Boc-Cys(Tr)-Val-Ile-Met-*1H*-propionitrile-tetrazole (13). Compound **7** (100 mg, 186 µmol) was treated according to *GP 3a* and *3b*. Silica gel chromatography (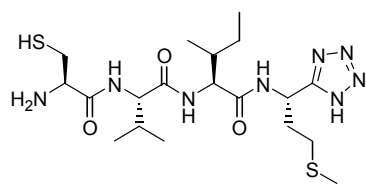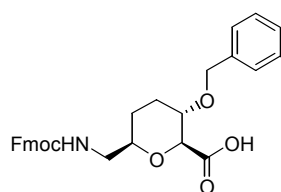EtOAc/PE 5/2, R_f 0.5) gave the title compound as a white solid (115 mg, 130 µmol) in 70% yield. ^{1}H-NMR (400 MHz, CDCl$_3$): δ 7.45–7.18 (m, NHC=O and 15×H^{Tr}), 6.70 (d, 1H, NHC=O, *J* = 8.2 Hz), 6.30 (d, 1H, NHC=O, *J* = 4.6 Hz), 5.54 (m, 1H, H$_\alpha^{Met}$), 4.74 (t, 2H, CH$_2$C≡N, *J* = 6.7 and 7.2 Hz), 4.62 (d, 1H, NHBoc), 4.36 (dd, 1H, H$_\alpha$, *J* = 5.3 Hz), 3.83 (t, 1H, H$_\alpha$, *J* = 4.2 Hz), 3.49 (m, 1H, H$_\alpha$), 3.12 (t, 2H, NCH$_2$, *J* = 3.5 and 4.1 Hz), 2.72 and 2.60 (dd, *J* = 5.0 Hz and m, 4H, H$_{\beta\gamma}^{Met}$ and H$_\beta^{Cys}$), 2.07 (s, 3H, SCH$_3$), 2.01 (m, 1H, H$_\beta^{Val}$), 1.72 (bs, 3H, H$_{\beta\gamma}^{Ile}$), 1.40 (s, 9H, *t*Bu), 0.94–0.79 (m, 12H, 4×CH$_3^{Ile}$). ^{13}C-NMR (100 MHz, CDCl$_3$/MeOD)*: δ 171.9-171.2 (C=O^{amide}*), 156.0, 155.0 (C=N^{tetrazole} and C=O^{Boc}), 144.4, 144.1 (C$_q^{Tr}$), 129.5-126.8 (C^{Tr}*), 116.3 (C≡N), 80.9 (C$_q^{tBu}$), 59.8, 58.3, 54.1 (3×C$_\alpha^{Ile, Val, Cys}$*), 42.9 (*C*H$_2$C≡N), 41.5 (C$_\alpha^{Met}$*), 37.5, 35.7 (2×C$_\beta^{Ile,Val}$*), 32.5, 31.5, 29.7 (C$_{\beta\gamma}^{Met}$, C$_\beta^{Cys}$), 28.1 (*t*Bu^{Boc}*), 24.6 (C$_\gamma^{Ile}$*), 18.2 (NCH$_2$), 19.1, 17.4, 15.4, 15.0, 10.9 (SCH$_3$ and CH$_3^{Ile,Val}$*). IR ν (cm^{-1}, film): 3865, 3742, 3611, 2970, 2322, 1697, 1643, 1520, 1458. MS (ESI): *m/z* 884.6 (M+H)$^+$, 906.5 (M+Na)$^+$. HR-MS: calc. for [C$_{46}$H$_{61}$N$_9$O$_5$S$_2$+H]$^+$ 884.43098, found 884.43110. [α]$_D^{25}$ = −49 (*c* = 0.46, MeOH).
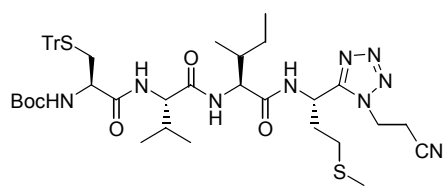
H-Cys-Val-Ile-Met-*1H*-tetrazole (15). Compound **13** (57 mg, 65 µmol) was dissolved in DCM (4 mL) and treated with DBU (7 equiv., 455 µmol, 68 µL) until TLC analysis showed completion (baseline spot, orange color upon spraying with molybdenum solution). Next, the reaction mixture was diluted with EtOAc and washed with 1N HCl and brine. After drying of the organic layer (MgSO$_4$) and concentration *in vacuo*, the crude product (**14**, white solid, crude yield: 49 mg, 59 µmol, 91%) was analyzed by ^{13}C-NMR and mass spectroscopy. No appearance of the propionitrile group was detected by NMR and mass spectroscopy showed the desired mass peaks: MS (ESI) *m/z* 831.5 (M+H)$^+$ and 853.5 (M+Na)$^+$; HR-MS: calc. for [C$_{43}$H$_{58}$N$_8$O$_5$S$_2$+H]$^+$ 831.40443, found 831.40564. Treatment of the crude product according to *GP 3a* afforded the fully deprotected title compound. After evaporation of the volatiles, the reaction mixture was diluted with water and washed with DCM. The aq. layer was concentrated by lyophilisation to afford the crude product as a white powder. Purification by RP-HPLC (linear gradient B, 10→90%) afforded the title compound (8 mg, 16 µmol, 28%) as a white powder. LC-MS analysis: R$_t$ = 9.1 min (linear gradient B 10→90%, 26 min), 489.3 (M+H)$^+$. ^{1}H-NMR (400 MHz, D$_2$O/CD$_3$CN): δ 7.43 (d, 1H, NH, *J* = 8.8 Hz), 5.52 (dd, 1H, H$_\alpha$, *J* = 6.0 and 6.8 Hz), 4.45 (apparent t, 1H, H$_\alpha$, *J* = 7.2 and 7.6 Hz), 4.28 (dt, 1H, H$_\alpha$, *J* = 8.4 and 8.8 Hz), 3.46 (m, 1H, H$_\alpha$), 3.30, 3.00 (2×m, 2H, H$_\beta^{Cys}$), 2.52 (m, 2H, H$_\gamma^{Met}$), 2.27 (m, 2H, H$_\beta^{Met}$), 2.03 (s, 3H, SCH$_3$ and m, 1H, H$_\beta^{Val}$), 1.68, 1.30 (2×m, 3H, H$_{\beta\gamma}^{Ile}$), 0.90 (dd, 6H, 2×CH$_3$), 0.75 (t, 3H, CH$_3$). 0.63 (d, 3H, CH$_3$).

(2S,3S,6R)-3-*O*-Benzyl-1-{*N*-[(9-fluorenyl-methoxycarbonyl)aminomethyl}-tetrahydropyran-1-carboxylic acid (16b). To a solution of SAA **16a** (0.74 g; 2.03 mmol) in DCM (15 mL) were added TFA (15 mL) and 1.1 equiv. Et$_3$SiH. After stirring for 4 h at rt, TLC analysis (EtOAc) indicated complete conversion of the starting. The solvent was removed *in vacuo*, the crude product was coevaporated to dryness with anh. toluene (3×15 mL) and dissolved in dioxane (15 mL). A solution of NaHCO$_3$ (4 equiv.) in H$_2$O (30 mL) was added to the reaction mixture followed by a solution of FmocOSu (1.1 equiv.) in dioxane (15 mL). After stirring overnight at rt, the reaction mixture was washed 2× with 1N HCl and extracted 2× with DCM. The organic layer was washed with brine and dried over MgSO$_4$. After removal of the solvent under reduced pressure, the mixture was purified by silica gel

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* In the case of compound **13** some ^{13}C-NMR resonances appear double - probably due to conformational changes; these resonances are marked with an *.

chromatography (1% AcOH in PE/EtOAc 1/1 v/v) furnishing **16b** (R_f 0.4, EtOAc) as a white foam (0.92 g, 1.88 mmol, 92%). ^{1}H-NMR (CDCl$_3$, 400 MHz): δ 7.73 (d, 2H, 2×H^{Fmoc}, J= 7.6 Hz), 7.57 (d, 2H, 2×H^{Fmoc}, J= 7.6 Hz), 7.37 (t, 2H, 2×H^{Fmoc}, J= 7.2 and 7.6 Hz), 7.30 (m, H$^{Fmoc\&Bn}$), 5.40 (t, 1H, NH), 7.57 (d, 2H, 2×H^{Fmoc}, J= 5.2 and 6.0 Hz), 4.59 (m, 3H, H$^{Fmoc\&Bn}$), 4.37 (d, 1H, H^{Fmoc}, J= 7.2 Hz), 4.19 (bt, 1H, H^{Fmoc}, J= 6.8 Hz), 3.51–3.42 (d, 1H, H$_2$, J= 9.2 Hz), 3.59-3.42 (m, 3H, H$_3$, H$_6$, H$_{7a}$), 3.13-3.1 (m, 1H, H$_{7b}$), 2.31 (m, 1 H, H$_{4a}$), 1.73 (m, 1H, H$_{5a}$), 1.52, 1.37 (2×m, 2H, H$_{4b\&5b}$); ^{13}C-NMR (400 MHz, CDCl$_3$): δ 172.9 (C=O^{acid}), 156.5 (C=O^{Fmoc}), 143.5, 140.9 (C$_q$^{Fmoc}), 137.4 (C$_q$^{Bn}), 128.0, 127.4 126.7, 124.8, 119.6, (CH$^{Fmoc\&Bn}$), 79.0, 76.3, 74.2 (C$_2$, C$_3$, C$_6$), 70.9 (CH$_2$^{Bn}), 66.5 (CH$_2$^{Fmoc}), 46.8 (CH^{Fmoc}), 44.5 (C$_7$), 28.3, 26.5 (C$_4$, C$_5$). MS (ESI): m/z 488.4 [M+H]$^+$. IR ν (cm^{-1}, thin film): 3018, 2930, 2870, 1719, 1522, 1250, 1221, 1099. [α]$_D$^{20}= +3 (c= 1, CHCl$_3$).

N-[(6R)-6-({*N*-[9H-fluoren-9-ylmethoxycarbonyl-amino}methyl-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-methionine-*1H*-propionitrile-tetrazole (17).
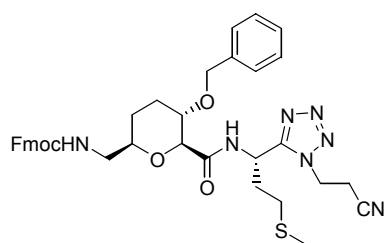
A solution of SAA **16b** (1 equiv., 168 mg, 345 μmol) and **10** (2.2 equiv., 56 mg, 246 μmol) in DMF (5 mL) was treated with HCTU (2.2 equiv., 0.76 mmol, 0.31 g), (2.2 equiv., 0.76 mmol, 0.10 g) and DIPEA (6 equiv., 0.35 mL). After TLC analysis (1% AcOH/EtOAc, R_f 0.9) showed completion, the reaction mixture was concentrated *in vacuo* and washed with sat. NaHCO$_3$, KHSO$_4$ (5%) and brine. The organic phase was dried (MgSO$_4$) and evaporated *in vacuo*. Purification by silica gel chromatography (EtOAc/drop AcOH) furnished **17** as a foam (170 mg, 245 μmol, 71%). ^{1}H-NMR (400 MHz, CDCl$_3$) δ 7.76 (d, 2H, H^{Fmoc}, J= 7.4 Hz), 7.60-7.24 (m, NHC$_\alpha$ and H$^{Fmoc\&Bn}$), 5.43 (m, 2H, NHFmoc, H$_\alpha$^{Met}), 4.78 (m, 2H, CH$_2$C≡N), 4.55–4.30 (m, 4H, PhCH$_2$ and CH$_2$^{Fmoc}), 4.18 (t, 1H, CH^{Fmoc}), 3.72 (d, 1H, H$_2$, J= 9.2 Hz), 3.35 (m, 3H, H$_6$, H$_3$, H$_{7a}$), 3.05 (m, 3H, CH$_2$CH$_2$C≡N and H$_{7b}$), 2.54–2.27 (m, 6H, H$_{\beta\gamma}$^{Met}, H$_{4a}$, H$_{5a}$), 2.01 (s, 3H, SCH$_3$), 1,60–1.40 (m, 2H, H$_{4b\&5b}$). ^{13}C-NMR (50 MHz, CDCl$_3$): δ 170.6 (C=O^{amide}), 156.9, 155.1 (C=O^{Fmoc} and C=N^{tetrazole}), 143.8, 143.7, 141.1 (C$_q$^{Fmoc}), 137.3 (C$_q$^{Bn}), 128.4-126.9, 125.0, 119.8 (CH$^{Fmoc\&Bn}$), 116.3 (C≡N), 78.8 (C$_2$), 77.2 (C$_6$), 74.3 (C$_3$), 70.9 (CH$_2$^{Bn}), 66.6 (CH$_2$^{Fmoc}), 47.0 (CH^{Fmoc}), 44.5 (C$_7$), 42.8 (CH$_2$), 41.2 (C$_\alpha$^{Met}), 31.3 (C$_\beta$^{Met}), 29.8 (C$_\gamma$^{Met}), 28.6 (C$_4$), 28.3 (C$_5$), 18.2 (CH$_2$), 14.9 (SCH$_3$). HR-MS: calc. for [C$_{37}$H$_{41}$N$_7$O$_5$S+H]$^+$ 696.29626, found 696.29617; calc. for [C$_{37}$H$_{41}$N$_7$O$_5$S+Na]$^+$ 718.27821, found 718.27773. [α]$_D$^{25}= −17 (c= 1, CHCl$_3$).

N-[(6R)-6-({*N*-(*S*-[tert-Butyl)sulfanyl]-*N*-{[(9H-fluoren-9-yl)methoxy]carbonyl}-L-cysteinyl)amino]-methyl}-4,5,6-trideoxy-3-benzyloxy-1-oxo-D-arabino-hexo-pyranos-1-yl]-L-methionine-*1*-propionitrile-tetrazole (18).
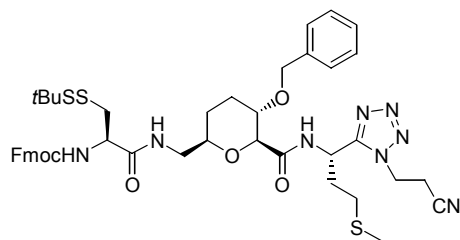
Compound **17** (96 mg, 138 μmol) was dissolved in DCM (2 mL) and treated with 1.0 equiv. DBU (138 μmol, 21 μL). After TLC analysis showed completion, HOBt (37 mg, 2 equiv.) was added followed by a solution of HCTU (69 mg, 166 μmol, 1.2 equiv.), DIPEA (70 μL, 3 equiv.) and Fmoc-Cys(S*t*Bu)-OH (72 mg, 166 μmol, 1.2 equiv.) in DMF (2 mL). After stirring overnight at rt, the reaction mixture was concentrated *in vacuo* and washed with sat. aq. NaHCO$_3$, aq. KHSO$_4$ (5%) and brine. The organic phase was dried (MgSO$_4$) and evaporated *in vacuo*. Purification by silica gel chromatography (R_f 0.7, EtOAc) gave **18** as a white foam (102 mg, 115 μmol, 83%). ^{1}H-NMR (400 MHz, CDCl$_3$/MeOD): δ 7.73-7.62 (m, 4H, CH^{Fmoc}), 7.45 (t, 2H, CH^{Fmoc}, J= 7.2 and 7.6 Hz), 7.30–7.11 (m, CH$^{Fmoc\&Bn}$, NH), 7.14 (bs, 1H, NH), 6.10 (bs, 1H, NHFmoc), 5.34 (m, 1H, H$_\alpha$^{Met}), 4.73 (m, 2H, CH$_2$), 4.49–4.30 (m, 4H, CH$_2$^{Bn} and CH$_2$^{Fmoc}), 4.18 (m, 2H, CH^{Fmoc} and H$_\alpha$^{Cys}), 3.69 (d, 1H, H$_2$, J= 9.2 Hz), 3.49 (m, 1H, H$_1$), 3.37–3.25 (m, 3H, H$_3$ and H$_{7ab}$), 3.12–2.92 (m, 4H, CH$_2$ and H$_\beta$^{Cys}), 2.53–2.30 (m, 4H, H$_{\beta\gamma}$^{Met}), 2.21 (m, 1H, H$_{4a}$), 2.05 (s, 3H, SCH$_3$), 1.60 (m, 1H, H$_{5a}$), 1.40 (m, 2H, H$_{4b\&5b}$), 1.30 (s, 9H, *t*Bu); ^{13}C-NMR (100 MHz, MeOD/CDCl$_3$): δ 170.6, 170.7 (2×C=O^{amide}), 156.5, 155.5 (C=O^{Fmoc} and C=N^{tetrazole}), 143.9, 143.7, 141.3 (4×C$_q$^{Fmoc}), 137.6 (C$_q$^{Bn}), 128.5-127.2, 125.2, 120.0 (CH$^{Bn\&Fmoc}$), 116.1 (C≡N), 78.8 (C$_2$), 76.5 (C$_6$), 74.7 (C$_3$), 71.2 (CH$_2$^{Bn}), 67.5 (CH$_2$^{Fmoc}), 54.7 (C$_\alpha$^{Cys}), 48.4 (C$_q$^{t}^{Bu}), 47.1 (CH^{Fmoc}), 43.4, 42.9, 42.0 (C$_7$, C$_\beta$^{Cys}, CH$_2$),

41.2 (C_α^{Met}), 31.3, 30.1 ($C_{\beta\gamma}^{Met}$), 29.9 (*t*Bu), 28.9, 26.9 (C_4, C_5), 18.5 (CH_2), 15.2 (SCH_3). IR ν (cm^{-1}, film): 3300, 2924, 1670, 1508, 1450, 1234, 1090. HR-MS: calc. for $[C_{44}H_{54}N_8O_6S_3+NH_4]^+$ 904.36667, found 904.36603. $[\alpha]_D^{25} = -21$ (*c* = 0.5, CHCl$_3$).

***N*-[(6R)-6-({*N*-(*S*-[tert-Butyl)sulfanyl]-L-cysteinyl)-amino]-methyl}-4,5,6-trideoxy-4-benzyloxy-1-oxo-D-arabino-hexopyranos-1-yl]-L-methionine-1*H*-tetrazole (19).** A solution of **18** (25 mg, 28 μmol) in DCM (4 mL) was treated dropwise with 8 equiv. DBU (224 μmol, 34 μL) until TLC analysis (EtOAc) showed completion. Next, the reaction mixture was diluted with water and organic impurities were removed by washing with DCM. The aq. layer was lyophilised and purification by RP-HPLC (linear gradient B, 7→54%) gave **19** (3.5 mg, 5.7 μmol, 20%) as a white powder. ^{1}H-NMR (400 MHz, D$_2$O/CD$_3$CN): δ 7.43 (dd, 3H, CH^{Bn}, *J* = 1.2 and 2.4 Hz), 7.27 (2×d, 2H, CH^{Bn}, *J* = 2.8 and 3.6 Hz), 5.55 (dd, 1H, H$_\alpha^{Met}$, *J* = 6.4 Hz), 4.55 and 4.42 (2×d, 2H, CH$_2^{Bn}$, *J* = 11.6 Hz), 4.25 (dd, 1H, H$_\alpha^{Cys}$, *J* = 5.6 Hz), 3.81 (d, 1H, H$_2$, *J* = 9.6 Hz), 3.71–3.61 (m, 2H, H$_6$, H$_{7a}$), 3.56–3.50 (dt, 1H, H$_3$, *J* = 3.6 and 4.4 Hz), 3.28 (dd, 1H, H$_\beta^{Cys}$, *J* = 6.4 Hz), 3.20 (m, 2H, H$_\beta^{Cys}$ and H$_{7b}$), 2.59–2.55 (m, 2H, H$_\gamma^{Met}$), 2.43 (m, 1H, H$_{4a}$), (m, 2H, H$_\beta^{Met}$), 2.12 (s, 3H, SCH$_3$), 1.80 (m, 1H, H$_{5a}$), 1.59–1.49 (m, 2H, H$_{4b\&5b}$), 1.33 (s, 9H, *t*Bu); ^{13}C-NMR (100 MHz, D$_2$O/CD$_3$CN): δ 171.5, 167.8 (2×C=O^{amide}), 156 (C=N^{tetrazole}), ~138 (C$_q^{Bn}$, *due to low concentration difficult to detect*), 128.8, 128.4, 128.3 (CH^{Bn}), 79.9 (C$_2$), 75.8, 75.1 (C$_3$, C$_6$), 71.0 (CH$_2^{Bn}$), 52.8 (C$_\alpha^{Cys}$), 48.7 (C$_q^{tBu}$), 43.5, 40.5 (C$_7$, C$_\beta^{Cys}$), 42.3 (C$_\alpha^{Met}$), 31.2, 29.4 (C$_{\beta\gamma}^{Met}$), 29.1 (*t*Bu), 28.5, 26.8 (C$_4$, C$_5$), 14.4 (SCH$_3$). LC-MS analysis R$_t$ = 11.4 min (linear gradient B 10→90%, 26 min); *m/z* 612.3 (M+H)$^+$.
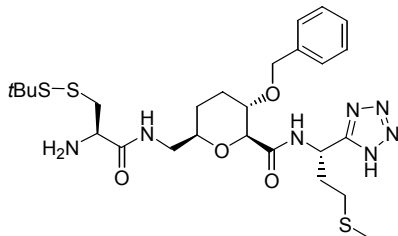
5.5 References and Notes

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Chapter 6

Incorporation of an Azide in Farnesyl Pyrophosphate Enables Bioorthogonal Labeling of Farnesylated Proteins by Bertozzi-Staudinger Ligation

Abstract. A novel labeling strategy for visualising farnesylated proteins from living cells is presented. The strategy uses the FPP analog azidoFPP (**2**) as alternative substrate for PFT. The azidofarnesylated proteins are modified by means of a Bertozzi-Staudinger ligation between phosphine reagent **1** (containing a biotin as identification tool) and the azide group. SDS-PAGE analysis and avidin-horseradish peroxidase chemiluminescence enables visualisation of the isolated proteins. Co-treatment with PFT inhibitors decreased the amount of isolated proteins.

6.1 Introduction

In recent years, several strategies for the chemical tagging of proteins with bioorthogonal functional groups have come to the fore.[1] In general, a chemical probe that has the property to modify distinct protein families is equipped with a small, physiologically inert functionality and applied to a relevant tissue culture. In a later stage, the bioorthogonal group is targeted through a chemoselective reaction, enabling labeling of the modified proteins with biotin or fluorescent groups. Two reactions that are exceptionally suitable for this purpose are the copper-catalysed *Huisgen* cycloaddition of an azide and an acetylene,[2,3] and the *Bertozzi* modification of the *Staudinger* reaction.[4]

Two-step labeling techniques have been applied for the tagging of a number of cysteine, serine and threonine dependent enzymatic activities.[5,6] Further, Bertozzi and co-workers developed the two-step labeling, by metabolic oligosaccharide engineering, of cell surface glycoproteins.[3] In this pioneering report, *N*-azidoacetyl-D-mannosamine proved to be a suitable substrate in the sialic acid biosynthetic pathway, resulting, eventually, in the decoration of cell surface sialic acid residues with azides for subsequent functionalisation by a modified Staudinger reaction. More recently, the same group revealed that a similar strategy but with azidoacetyl glucosamine as a modified metabolite proved to be viable in the tagging of nuclear *O*-GlcNAcylated proteins.[7] These results were an incentive to investigate whether two-step labeling using modified metabolites can be applied to the farnesylation of proteins by protein:farnesyl transferase (PFT).[8]

The following strategy was devised for the two-step labeling of farnesylated proteins (Scheme 6.1).[9,*] After addition of azidofarnesyl pyrophosphate **2**[10] to living cells it is transferred, through the action of PFT, to proteins susceptible to farnesylation. After the culture is lysed and denatured, biotinylated phosphine reagent **1**[11] is added, resulting in selective biotinylation through a Bertozzi-Staudinger reaction. At this stage, the selectivity of the two-step labeling can be ascertained by the addition of PFT specific inhibitors (Figure 6.1).

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* It should be noted that a related strategy employing azidofarnesyl pyrophosphate, but using both different tissue and different inhibitors, appeared in the literature: see reference 9.

It should be noted that the viability of this strategy relies on the following issues. First, the phosphine functionality is a biologically inert species.[12] Although azides are reduced by thiols *in vivo*,[13] this process is too slow to interfere under the timecourse of the employed *in vivo* experiments and thus can be regarded as biologically inert species. Second, the relatively small size and nonpolar character of the azide group make it suited to function as tagging handle and to date it is has not been identified in any known natural biological molecule.
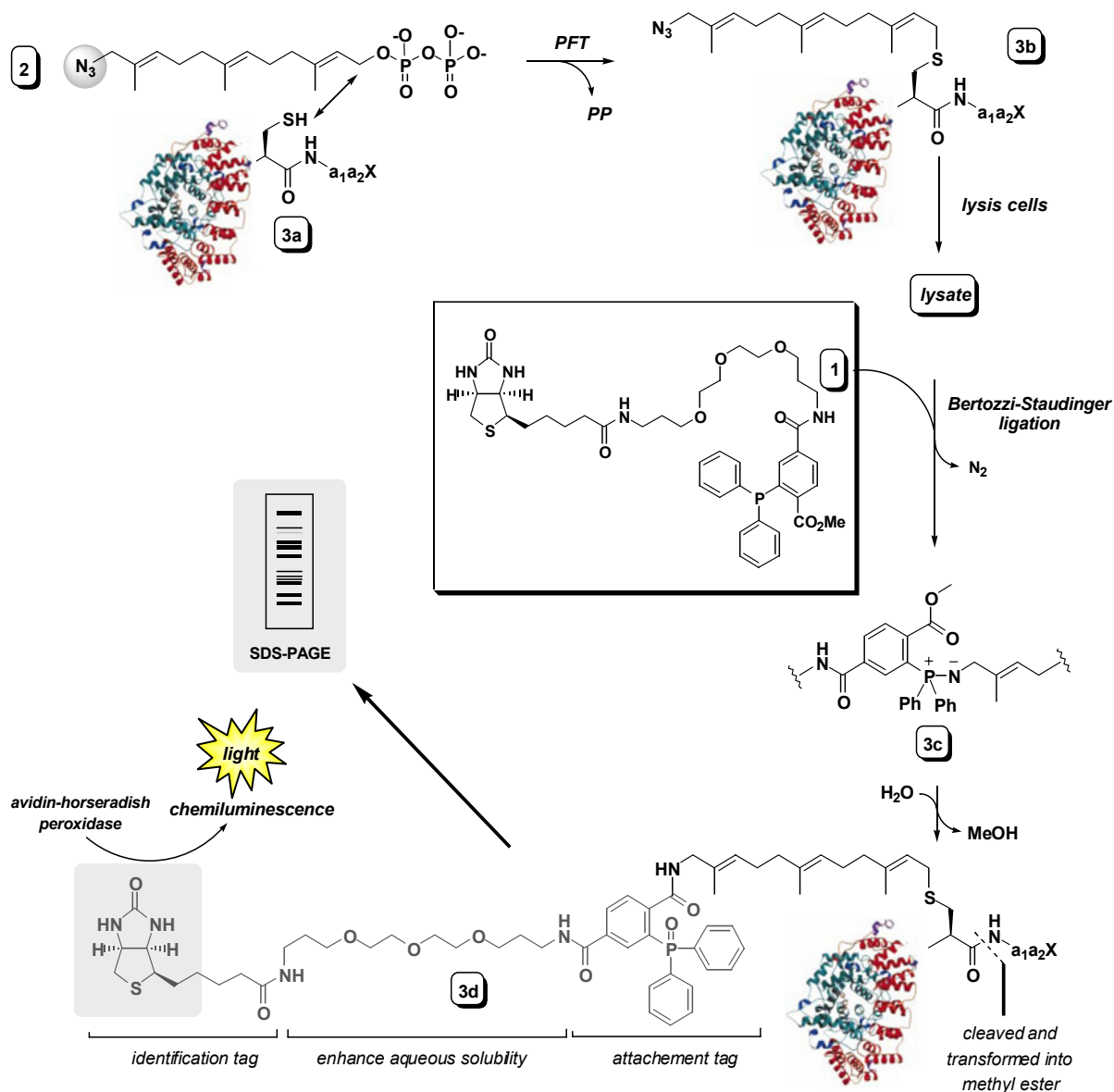
Scheme 6.1 Bertozzi-Staudinger ligation procedure for the identification of isoprenylated proteins.

6.2 Results and Discussion

6.2.1 Synthesis – The synthesis of azidofarnesyl pyrophosphate **2** was accomplished as follows (Scheme 6.2). Known THP-protected farnesol **5**,[14] obtained from farnesol **4** by treatment with dihydropyran and acid, was reacted with selenoxide, *tert*-butyl hydroperoxide and salicylic acid[15] to afford allylic alcohol **6**[16] in 32% yield (**5** recovered in 30% yield). Treatment of **6** with $(PhO)_2P(O)N_3$ and subsequent unmasking of the remaining primary alcohol afforded azidofarnesol **8** in good yield (85% from **6**).[17] The pyrophosphate moiety was now introduced following a well established procedure, by treating **8** with PBr_3 furnishing allylic bromide **9** in 77% yield.[18] Treatment with tetra-*n*-butylammonium pyrophosphate furnished **2** as the tris(tetra-*n*-butylammonium) salt. Consecutive exposure to Dowex-NH_4^+ and lyophilisation afforded the product as the tris(ammonium) salt which could be purified by RP-HPLC, providing homogeneous azidofarnesyl pyrophosphate **2** (50% from **9**).

Scheme 6.2 Synthesis of azide-FPP (**2**).[a]
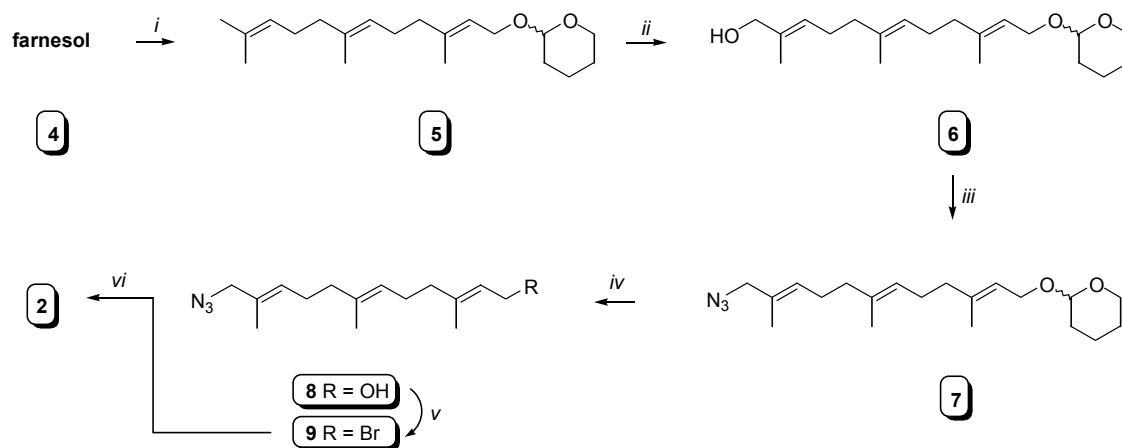
[a]Reagents and conditions: (*i*) DHP, PPTS, DCM (**5**: >99%); (*ii*) $SeO_2·H_2O$ (cat), *t*BuOOH, salicylic acid, DCM (**6**: 32%, recovered **5**: 30%); (*iii*) $(PhO)_2P(O)N_3$, DBU, toluene (**7**: >99%); (*iv*) PPTS, EtOH, 60°C (**8**: 85%); (*v*) PBr_3, pentane, 0–4°C (**9**: 77%); (*vi*) (a) $(nBu_4N)_3HP_2O_7$, CH_3CN, 0°C→rt (b) Dowex-NH_4^+, (c) RP-HPLC (**2**: 50% from **9**).

6.2.2 Labeling Experiment – The efficacy of the Staudinger ligation pair **1**[11] and **2** in the selective labeling of farnesylated proteins was established in the following experiment. Cultured J774 cells, a mouse macrophage cell line, incubated with the HMG-CoA reductase inhibitor simvastatin (**10**) to suppress endogenous farnesyl pyrophosphate, were treated with azidofarnesyl pyrophosphate **2** (100 μM final concentration).

Figure 6.1 Labeling experiment with phosphine **1** and probe **2** in J774 cells.[a]

	1	2	3	4	5	6	7	8
10:	+	+	+	+	+	+	+	+
11:	-	-	+	-	-	-	+	-
12:	-	-	-	+	-	-	-	+
EtOH:	-	-	-	-	+	+	+	+
2:	-	+	+	+	-	-	-	-
8:	-	-	-	-	-	+	+	+
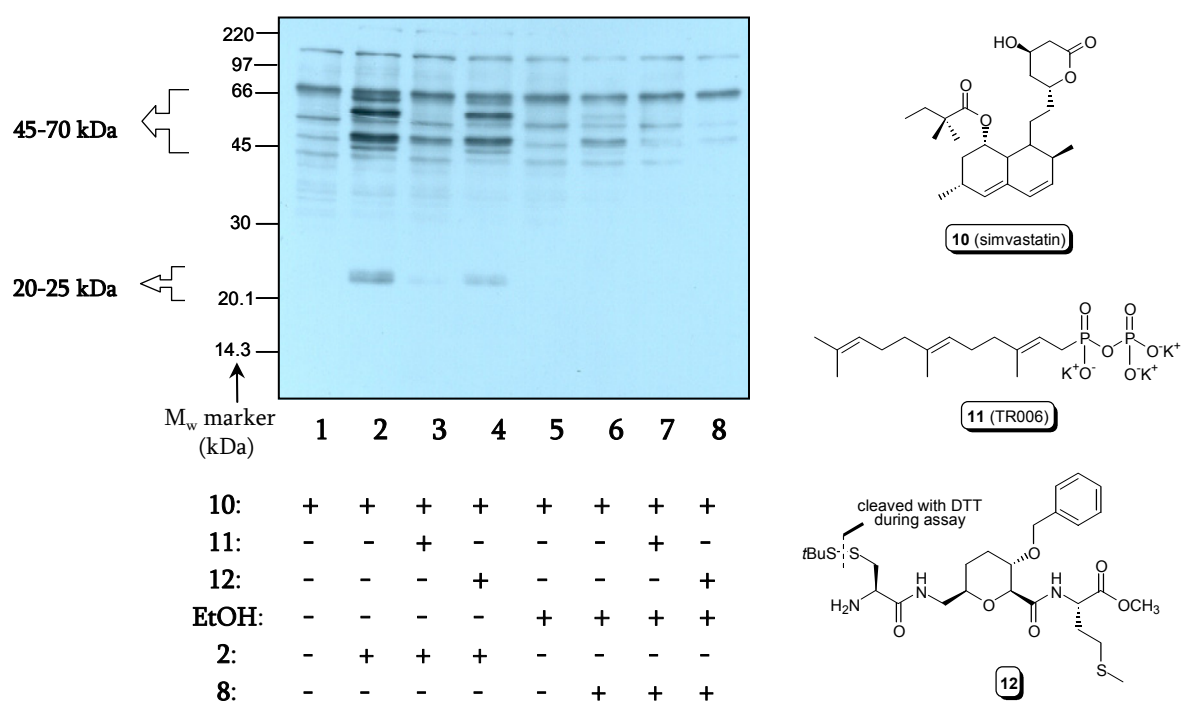
[a]J774 cells were incubated with 5 μM simvastatin **10**, (lanes 1 – 8), 100 μM **2** (lanes 2 – 4), 100 μM **8** (lanes 6 – 8), 100 μM **2** and 100 μM **11** (lanes 3 & 7), 100 μM **2** and 100 μM **12** (lanes 4 & 8). EtOH, the solvent of **8**, was present at the latter 4 incubation conditions (lanes 5 – 8). After incubation for 24 h at 37°C, the cells were lysed, treated with **1** (final concentration 333 μM) prior to SDS-PAGE resolvement of the protein content and avidin-HRP chemiluminescence identification of the modified proteins.

After incubating for 24 hours, the cells were lysed, treated with an excess of reagent **1**, and applied to SDS-PAGE separation. Biotinylated proteins were visualised by an avidin-horseradish peroxidase (HRP) chemiluminescence assay[19] and the results were compared with those of cells which were incubated without azide **2**, but with Staudinger reagent **1** (Figure 6.1, lanes 1 and 2). As can be seen in lane 2 (see arrows), at least five bands appear over the background, four in the region corresponding to 45 – 70 kDa proteins and one band at around 23 kDa. The lamin proteins, which are known to be PFT substrates, reside

in the 67 – 80 kDa region, (nuclear lamin B2 is a 68 kDa protein and probably corresponds to the upper band detected), whereas at 20 – 25 kDa small GTP-binding proteins such as Ras are expected.

Addition of pyrophosphonate **11**, next to azide **2**, to the tissue medium almost completely abolished labeling (lane 3). Compound **11** (**TR006**),[20] developed by van Boom and co-workers is a potent inhibitor of PFT (IC$_{50}$= 0.16 μM); this result clearly demonstrates the selectivity of probe **2** to be processed by PFT. Application of the less potent PFT inhibitor tetrapeptide mimetic **12** (see Chapter 3) also resulted in a decrease of labeling intensity, but with lower efficiency. Lanes 5 – 8 entail a repetition of the experiments, but with azidofarnesol **8** as the chemoselective probe and in the presence of 0.1% of ethanol, the solvent of azidofarnesol. As can be seen, labeling efficiency drops (lane 6), however, longer exposure reveals a similar labeling pattern. This result indicates that azidofarnesol is transformed to the pyrophosphate within the cell and that **11** and **12** prevented the protein farnesylation from azidofarnesol (lanes 7, 8). This is in line with metabolic labeling experiments which showed that the isoprenoid alcohols farnesol and geranylgeraniol can serve as FPP and GGPP precursors *in vivo*.[21]
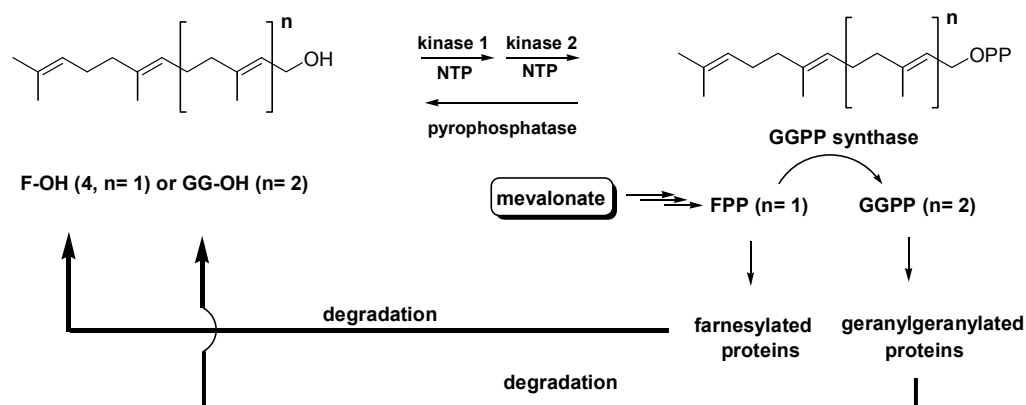
Scheme 6.3 Proposed salvage pathway for the *in vivo* transformation of isoprenoid alcohol in the corresponding pyrophosphate (NTP= nucleoside triphosphate).

The exact salvage pathway by which the isoprenoid alcohols are converted into the corresponding pyrophosphates has not been clarified yet. However, in rat liver microsomes it is has been shown that two subsequent kinases utilizing a nucleoside triphosphate (adenine triphosphate or cytosine triphosphate), transform the hydroxyl

first into a monophosphate and finally into the pyrophosphate (Scheme 6.3).[22] In contrast to FPP and GGPP, which are conventionally synthesised *via* the mevalonate pathway,[*] the isoprenoid alcohols are derived from either dephosphorylation of the corresponding pyrophosphate isoprenoid[23] or degradation of isoprenylated proteins.[21,22]

6.3 Conclusions

In conclusion, this chapter presents a novel labeling strategy for visualising farnesylated proteins from living cells. The strategy does not rely on the use of radiolabels and has the advantage of being rapid, efficient and selective (detection through [³H] labeled mevalonate or farnesylpyrophosphate normally takes up to several weeks). The here presented methodology may find application for the assessment of the characteristics of PFT and PGGT-1 inhibitors within the context of the living cell. Additionally, these techniques will enable the isolation/further characterisation and subcellular localisation of prenylated proteins. Finally, it is obvious that the here presented strategy but with an azidogeranygeranyl pyrophosphate probe instead of N_3FPP should be of great value in terms of obtaining non-radioactively, biotin labeled geranylgeranylated proteins (See Future Prospects, Chapter 8).

6.4 Experimental Section

6.4.1 General – ¹H-NMR and ¹³C-NMR spectra were recorded with a Bruker AC-200 (¹H-NMR 200 Mhz, 50.1 MHz), Bruker DPX-300 (¹H-NMR 300 Mhz, 75 MHz), Bruker Avance-400 (¹H-NMR 400 Mhz, ¹³C-NMR 100 MHz) or Bruker DMX-600 (¹H-NMR 600 Mhz, ¹³C-NMR 150 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Mass spectra were recorded with a Perkin Elmer/SCIEX API 165 mass instrument and HR-MS spectra were recorded with an API QSTAR[tm] Pulsar (Applied Biosystems). Eluents were of technical grade and distilled before use. DMF, DCM, MeOH and toluene (all from Biosolve, p.a.) were stored over MS4Å. EtOAc and PE (40-60) were of technical grade and distilled before use. DBU (Fluka), PPTS (Acros), $SeO_2 \cdot HO_2$ (Aldrich) and farnesol (all trans, Aldrich) were used as received. Commercial tetra-*n*-butylammonium pyrophosphate (Fluka) was purified by recrystallisation.[24] Reactions were followed by TLC analysis on silica gel (Schleicher & Schuell, F 1500 LS 254) or HPTLC aluminium sheets (Merck, silica gel 60, F254), with detection by UV-absorption (254 nm) where applicable and charring at 150°C with 20% H_2SO_4 in EtOH (25 g L^{-1}), ninhydrin (3 g L^{-1}) in EtOH/AcOH (100/3, v/v), $NH_4(Mo)_7O_{24} \cdot 4H_2O$ (25 g L^{-1}) and $NH_4Ce(SO_4)_4 \cdot 2H_2O$ (10 g L^{-1}) in 10% aq. H_2SO_4 or 2% $KMnO_4$ in 1% aq. K_2CO_3. Column chromatography was performed with silica gel (Fluka; 0.063-0.200 mm). RP-HPLC analysis and purification were performed on a Jasco HPLC system equipped with a Merck Lichrosphere C18 100Å column (4×250 mm).

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* See Chapter 1, Scheme 1.2.

(*E,E,E*)-3,7,11-trimethyl-*O*-tetrahydropyranyl-2,6,10-dodecatrien-12-ol (6). The known THP-ether **5** (23 mmol, 7.0 g, PE/Et$_2$O 4/1 v/v, R$_f$ = 0.7) was added dropwise to a cooled (0°C) solution of SeO$_2$·HO$_2$ (1.2 mmol, 128 mg, 0.05 equiv.), salicylic acid (2.3 mmol, 317 mg, 0.1 equiv.) and *t*BuOOH (80% soln, 10 mL, 3.5 equiv.) in DCM (23 mL). The reaction mixture was stirred for 4 days,[25] diluted with toluene and concentrated *in vacuo*. After dilution with ether, the organic layer was washed with sat. aq. NaHCO$_3$ and brine and evaporated. The crude product was dried by coevaporation with anh. toluene, dissolved in MeOH (50 mL), cooled to –15°C and treated in portions with NaBH$_4$ (4.6 mmol, 174 mg, 0.2 equiv.).[26] After 15 min, acetone was added to destroy any excess NaBH$_4$ followed by evaporation of the MeOH. The residue was dissolved in EtOAc and washed with sat. aq. NH$_4$Cl and brine. Silica gel chromatography (EtOAc/PE v/v 1/1) gave **6** (PE/Et$_2$O 4/1 v/v, R$_f$ = 0.2) as an oil (2.35 g, 7.3 mmol, 32%) along with recovered **5** (2.15 g, 7.0 mmol, 30%). MS (ESI): *m/z* 345.0 (M+Na)$^+$, 361.2 (M+K)$^+$.
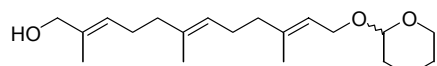
(*E,E,E*)-3,7,11-trimethyl-*O*-tetrahydropyranyl-2,6,10-dodecatrien-12-azide (7).[10] Allylic alcohol **6** (1.7 g, 5.3 mmol) and diphenylphosphoryl azide (2.5 equiv., 13.3 mmol, 2.9 mL) were dissolved in anh. toluene (25 mL) and cooled on ice. DBU (2.5 equiv., 13.3 mmol, 2.0 mL) was added dropwise during which the reaction solidified partially. After ½h the reaction mixture was allowed to warm to rt and stir overnight (reaction mixture turned light pink). After TLC analysis showed completion the reaction was quenched by addition of water and extracted with EtOAc, washed with brine and 1N HCl and dried over MgSO$_4$. The crude product was purified by silica gel chromatography (PE/EtOAc 4/1 v/v, R$_f$ = 0.7, isomeric mixture co-spotted on TLC) to give **7** as an oil (1.8 g, 5. 3 mmol, 100%). ^{1}H-NMR (300 MHz CDCl$_3$) δ 5.38 (m, 2H), 5.11–4.93 (m, 2H), 4.62 (d, 1H, *J* = 2.9 Hz), 4.23 (dd, 1H, *J* = 6.1 and 6.3 Hz), 4.03 (dd, 1H, *J* = 6.9 and 7.6 Hz), 3.89–3.77 (m, 2H), 3.50 (dd, 1H, *J* = 4.1 and 5.3 Hz), 2.27–1.97, 1.82–1.41 (2×m, CH$_2$ and CH$_3$); ^{13}C-NMR (50 MHz CDCl$_3$): δ 149.3–133 (C$_q$), 130–120 (=CH), 113.7 (H$_2$*C*=C), 96.8 (CH^{THP}), 67.2 (H$_2$C-C(CH$_3$)*C*H(N$_3$)), 62.7–61.2 (N$_3$CH$_2$ and CH$_2$OTHP), 38.8, 35.2, 30.0, 25.5, 25.0, 18.8 (CH$_2$), 15–20 (CH$_3$). MS (ESI): *m/z* 370.1 (M+Na)$^+$. HR-MS: calculated for [C$_{20}$H$_{33}$N$_3$O$_2$+NH$_4$]$^+$ 365.29110, found 365.29123.
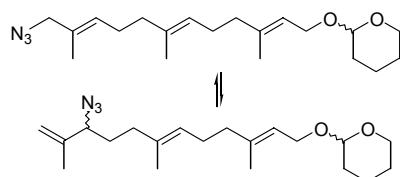
(*E,E,E*)-3,7,11-trimethyl-1-ol-2,6,10-dodecatrien-12-azide (8). To a solution of **7** (998 mg, 2.87 mmol) in EtOH (14 mL) was added PPTS (0.2 equiv., 0.6 mmol, 144 mg). The reaction mixture was stirred at 60°C until TLC analysis showed completion (6–12 hrs) and concentrated *in vacuo*. Silica gel chromatography (PE/Et$_2$O 3/1 v/v, R$_f$ = 0.4, isomeric mixture co-spotted on TLC) gave **8** as a yellow/brown oil (643 mg, 2.44 mmol, 85%). ^{1}H-NMR (300 MHz CDCl$_3$) δ 5.40 (m, 2H, =C*H*CH$_2$), 5.12 (m, 1H, =C*H*CH$_2$), 4.97 (m, 1H, *H*$_2$C=C), 4.13 (d, 2H, CH$_2$OH, *J* = 6.8 Hz), 3.80 (dd, 1H , H$_2$C=C*H*N$_3$, *J* = 7.2 and 6.8 Hz), 3.65 (m, 2H, C*H*$_2$N$_3$), 2.20–1.94, 1.78–1.60 (2×m, CH$_2$ and CH$_3$); ^{13}C-NMR (50 MHz CDCl$_3$): δ 142.1, 139.0, 138.8, 134.3, 133.7 (C$_q$), 129.5, 129.3, 125.1, 124.8, 124.2, 123.5, 123.4 (=*C*H), 114.3 (H$_2$*C*=C), 67.7 (H$_2$C-C(CH$_3$)*C*H(N$_3$)), 59.2, 59.0 (N$_3$CH$_2$ and *C*H$_2$OTHP), 39.2, 39.0, 35.7, 30.3, 26.1 (CH$_2$), 17.4, 16.0, 15.7 (CH$_3$). IR *v* (cm^{-1}, film): 3348, 2924, 2091 (N$_3$), 1443, 1381, 1242. MS (ESI): *m/z* 286.0 (M+Na)$^+$. HR-MS: calculated for [C$_{15}$H$_{25}$N$_3$O+NH$_4$]$^+$ 281.23359, found 281.23342.
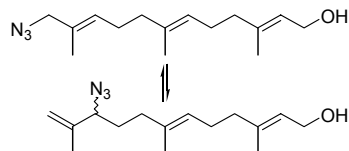
(*E,E,E*)-3,7,11-trimethyl-1-bromide-2,6,10-dodecatrien-12-azide (9). Azide **8** (91 mg, 347 μmol) was dissolved in pentane (1 mL) and the solution was cooled on ice (0–4 °C). Next, PBr$_3$ (1.1 equiv., 380 μmol, 103 μl) was added slowly and after stirring for 1 h, TLC analysis showed completion (EtOAc/DCM 2/8 v/v, R$_f$ = 0.9). MeOH (3.3. equiv., 1.16 mL) was added in order to quench any residual PBr$_3$ and stirring was continued at 0–4 °C for 5 min. Extraction
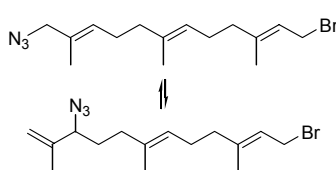
with Et$_2$O (10 mL) and 1× washing with water, 2× with 10% aq. NaHCO$_3$ and 1× with water gave, after drying (MgSO$_4$) and concentration *in vacuo* at 0–4 °C (flask was kept cold in ice-bath) compound **9** as a brown oil (87 mg, 268 μmol, 77%). Bromide **9** can be purified by flash silica gel chromatography (Et$_2$O, R$_f$= 0.9), however, due to their reactive character, allylic bromides are commonly used directly without purification. MS (ESI): *m/z* 326.0 (M+H)$^+$. ^{1}H-NMR (200 MHz, CDCl$_3$) δ 5.40 (m, 1H, =C*H*CH$_2$), 5.12–4.93 (m, =C*H*CH$_2$, *H$_2$C*=C), 4.00 (d, 2H, CH$_2$OH, *J*= 8.0 Hz), 3.81 (m, 1H , H$_2$C=C*H*N$_3$), 3.63 (bs, 2H, C*H$_2$*N$_3$), 2.26–1.90, 1.81–1.50 (2×m, CH$_2$, CH$_3$); ^{13}C-NMR (50 MHz CDCl$_3$): δ 143.3, 142.3, 134.8, 134.3 (C$_q$), 130.2, 129.7, 125.2, 124.4, 123.9, 121.4, 120.6, 120.0 (C=*C*H), 114.5 (H$_2$*C*=C), 68.0 (H$_2$C-C(CH$_3$)*C*H(N$_3$), 59.4 (N$_3$*C*H$_2$), 39.3, 39.1, 35.8, 30.5, 29.6, 29.5, 29.3, 26.3, 26.0 (CH$_2$), 17.6, 15.9, 14.6 (CH$_3$).

(*E,E,E*)-3,7,11-trimethyl-1-yl diphosphate-2,6,10-dodecatrien-12-azide, tris ammonium salt (2). Recrystallised tetra-*n*-butylammonium pyrophosphate[24] (2.0 equiv., 284 μmol, 256 mg) was dissolved in dry CH$_3$CN (1 mL) and cooled in an ice-bath. Next, a solution of bromide **9** (46 mg, 142 μmol) in dry CH$_3$CN (2 mL) was added after which stirring is continued at rt for 2 h. The reaction mixture was subsequently concentrated *in vacuo* and the tetra-*n*-butylammonium salt of N$_3$FPP was dissolved in 2 mL NH$_4$HCO$_3$ chromatography buffer (*i.e.* 2 g NH$_4$HCO$_3$ in 1000 mL *i*PrOH/H$_2$O 1/49 v/v) and loaded onto an ion-exchange column (Dowex$^®$ 50×4, NH$_4^+$ form).[27] Next, eluting the column with buffer (10 mL) and lyophilisation of the crude ammonium salt of compound **2** gave a white solid. RP-HPLC purification (linear gradient B 05→90%, 26 min) in H$_2$O (eluent A) and 0.1M NH$_4$Ac (eluent C) gave compound **2** as a white powder in 50% yield. LC-MS R$_t$ (**2**)= 9.9 min.[28] MS (ESI): *m/z* 441.2 (M+NH$_4$)$^+$. ^{1}H-NMR (600 MHz, DMSO-*d6*) δ 5.40 (m, 1H, C=C*H*CH$_2$), 5.28, 5.11, 4.99 (3×m, 3H, C=C*H*CH$_2$ and *H$_2$*C=C(CH$_3$)), 4.25 (bs, 2H, CH$_2$OH), ~3.55 (H$_2$C=C*H*N$_3$ and C*H$_2$*N$_3$): obscured by DMSO peak, 2.12-1.90, 1.68–1.54 (m, CH$_2$ and CH$_3$). ^{31}P-NMR (133 MHz, 25 mM NH$_4$HCO$_3$ in D$_2$O): δ –5.5 and –9.8.
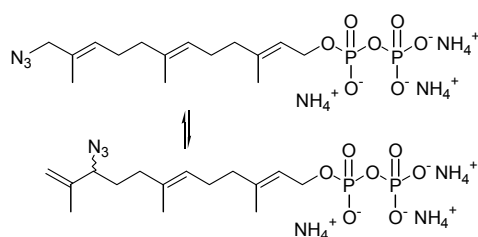
6.5 References and Notes

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(25) Although TLC analysis still showed presence of starting material, stirring for a longer period only decreases the yield as more baseline material is formed. This may be due to overoxidation and degradation.

(26) For the reduction of any formed aldehyde (R_f ~0.6, EtOAc/PE 3/1 v/v) to the desired alcohol.

(27) Prepared from Dowex® 50×4–400 or Dowex® 50×8–400, H⁺ form by ion-exchange with NH_4OH (28–30 wt%). The resin is washed with H_2O and NH_4HCO_3 buffer until the eluent is neutral.

(28) R_t of the monophosphate= 11.2 min (linear gradient of B 05→90%, 26 min).

Chapter 7

A Combinatorial and Optimisation Approach toward Ambiphilic Peptide-based Inhibitors of Protein:geranylgeranyl Transferase-1

Abstract. An *in silico* iterative optimisation procedure is presented that was used in a combinatorial approach (Fmoc based solid phase synthesis) toward a library of ambiphilic peptide-based compounds as potential PGGT-1 inhibitors. Starting with 16 randomly synthesised compounds, their structures were artificially evolved into a new set of compounds which then were synthesised and evaluated for their inhibitory activity against PGGT-1. Each cycle afforded a new generation of compounds and a progressive improvement of the average inhibitory activity was observed until the fifth generation (seven generations were synthesised). The obtained compounds were found to inhibit PGGT-1 in the low micromolar range (IC$_{50}$: 3.8 – 8.1 μM).

7.1 Introduction

   Most isoprenyl pyrophosphate mimic inhibitors contain a hydrophobic tail (isoprenyl mimic) connected to a negatively charged region (pyrophosphate mimic). The allowance of amide bonds in farnesyl pyrophosphate based PFT inhibitors[1] in combination with the promiscuous nature of PGGT-1 indicate that ambiphilic peptide-based compounds, having a negatively charged *C*-terminus and a hydrophobic cap connected *via* spacer molecules (Figure 7.1), can serve as novel lead compounds for the development of PGGT-1 inhibitors. A suitably large set of different building blocks and a parallel solid phase approach offers the opportunity to construct a large library of ambiphilic peptides. However, the combination of large sets of building blocks (important for a broad screening of possible leads) gives rise to such large libraries that synthesis becomes nearly impossible. The substantial effort that is required to construct and evaluate a library of enormous size can be diminished by using an optimisation procedure. The purpose of such a procedure is to guide the synthesis of a manageable library with a greater probability of obtaining good (not necessarily the best) hits for a given (biological) target, in this case PGGT-1.
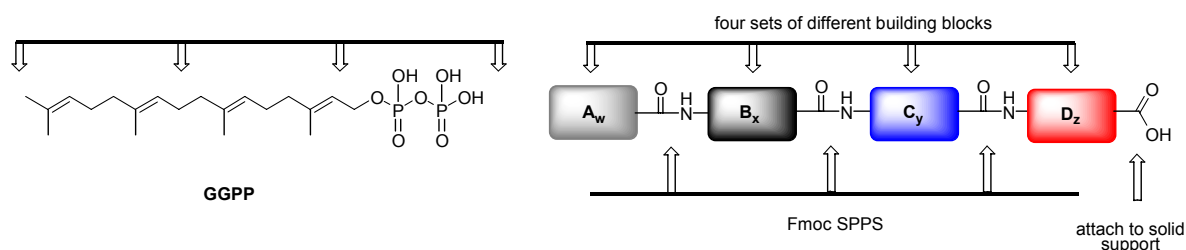
Figure 7.1 Ambiphilic peptides as potential GGPP analogs.

   Several optimisation methods, mainly *in silico*, have been described in the literature.[2] In this chapter the use of an optimisation procedure involving single building block mutations of selected inhibitors was investigated. In Scheme 7.1 a schematic representation of the followed procedure is depicted. The approach comprised the following steps. Initially (step **1**, Scheme 7.1), 30 ambiphilic peptides, designated as generation 0, were constructed as single compounds on Wang solid support using Fmoc

SPPS from a set of building blocks having a variety of structural characteristics.[3] The target peptides were released from the resin with concomitant deprotection by acidolysis, analysed by LC-MS and purified by RP-HPLC. After screening (step **2**, Scheme 7.1) in a PGGT-1 bioassay (see Experimental Section) using a pilot screening method (determination of % inhibition at a predefined concentration), the 16 most potent inhibitors of this initial set of compounds were selected (step **3**), ranked with decreasing inhibitory potency and assigned as generation 0 (Table 7.1a). Next, *in silico* (step **4**: see Scheme 7.2 for a schematic outline), one arbitrarily chosen building block of each of these 16 selected compounds was chosen for replacement by a new randomly chosen building block (see 7.4 and the example in Scheme 7.2). This gives a new set of 16 mutant compounds which are then synthesised, purified and screened following the same procedure (Tables 7.1a and 7.1b). From the combined results of both sets of compounds, that is generation 0 and their mutants, the 16 best inhibitors were selected to make up generation 1. This procedure of single building block mutation *in silico*, synthesis, purification, screening and selection was repeated several times. Thus, overall one first evaluates the molecular diversity of a given library prior to synthesis allowing the biological response (*i.e.* inhibitory potency) to guide the selection of compounds for successive steps.
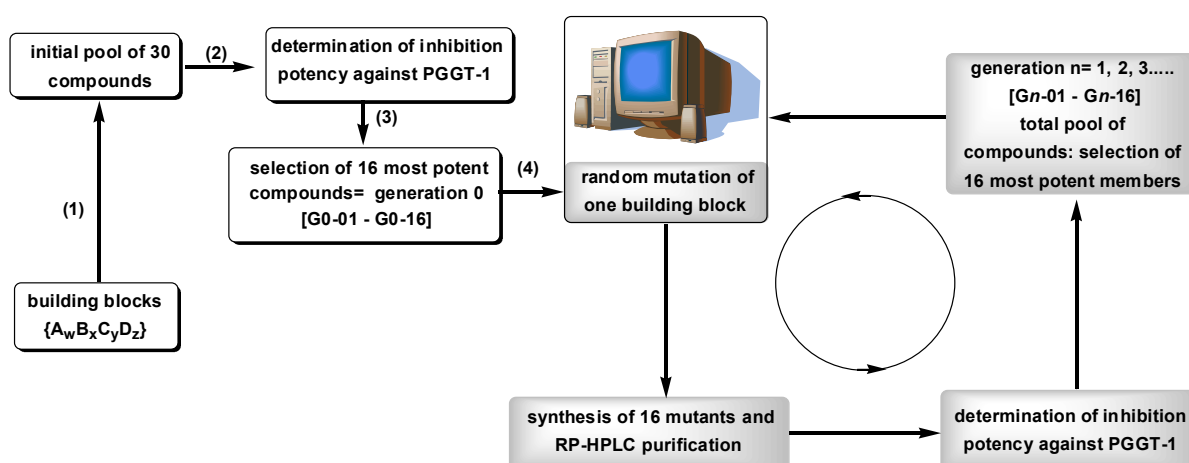
Scheme 7.1 Schematic presentation of the followed optimisation procedure.
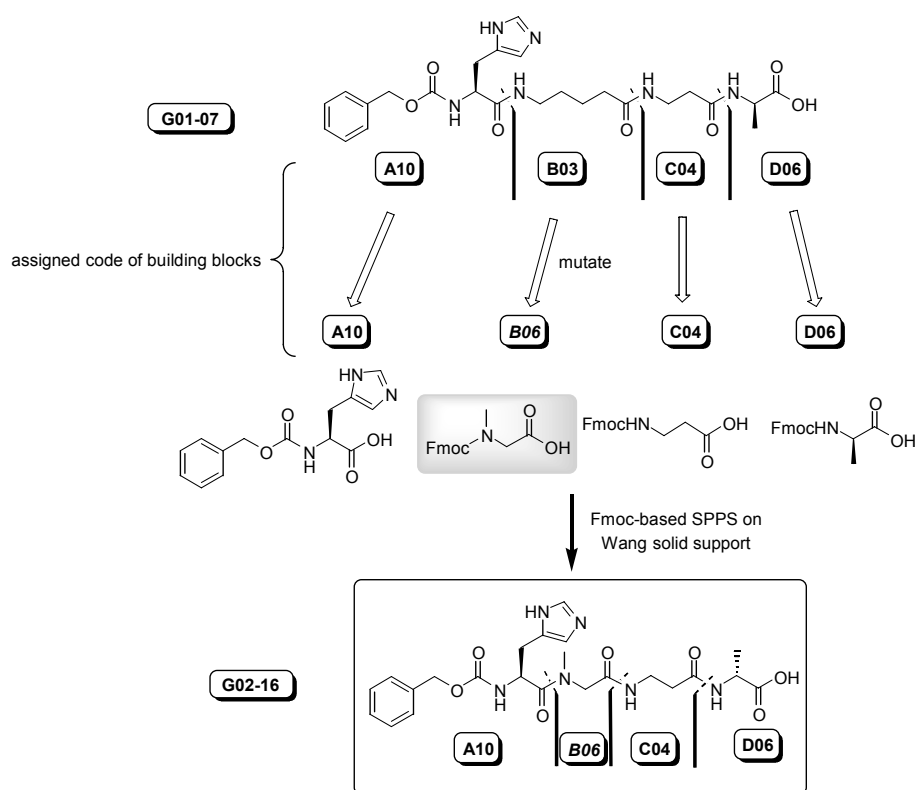
Scheme 7.2 Schematic example of one building mutation procedure performed *in silico*. **G01-07**= compound from generation 1 (G1) ranked #7 according to inhibitory potency.

7.2 Results and Discussion

The assembly of the projected potential inhibitors of PGGT-1 ($A_wB_xC_yD_z$, Figure 7.1) started with the selection of commercially available building blocks for the hydrophobic *N*-terminal and hydrophilic *C*-terminal subunits (A_w and D_z, respectively) as well as two spacer moieties (B_x and C_y). 24 Building blocks make up set A_w (Chart 7.1), 22 building blocks make up set B_x (Chart 7.2), 24 building blocks make up set C_y (Chart 7.3) and 18 building blocks make up set D_z (Chart 7.4). As can be seen, mainly aromatic building blocks were selected for the hydrophobic *N*-terminal part (A_w set). The number of available protected acidic amino acids suitable for incorporation as *C*-terminal building blocks (D_z set) was extended with residues, having uncharged or basic side-chains. Based on the assumption that the length of the hydrophobic tail in the ambiphiles $A_wB_xC_yD_z$ is important with regard to inhibitory potency,[6] spacer molecules that vary in length and conformational restriction were selected with the option to omit (**B01** and **C01**) one or both of these building blocks. Note that after incorporation of a building block *via* amide bond formation, the designated code of the building block is maintained (Scheme 7.2).
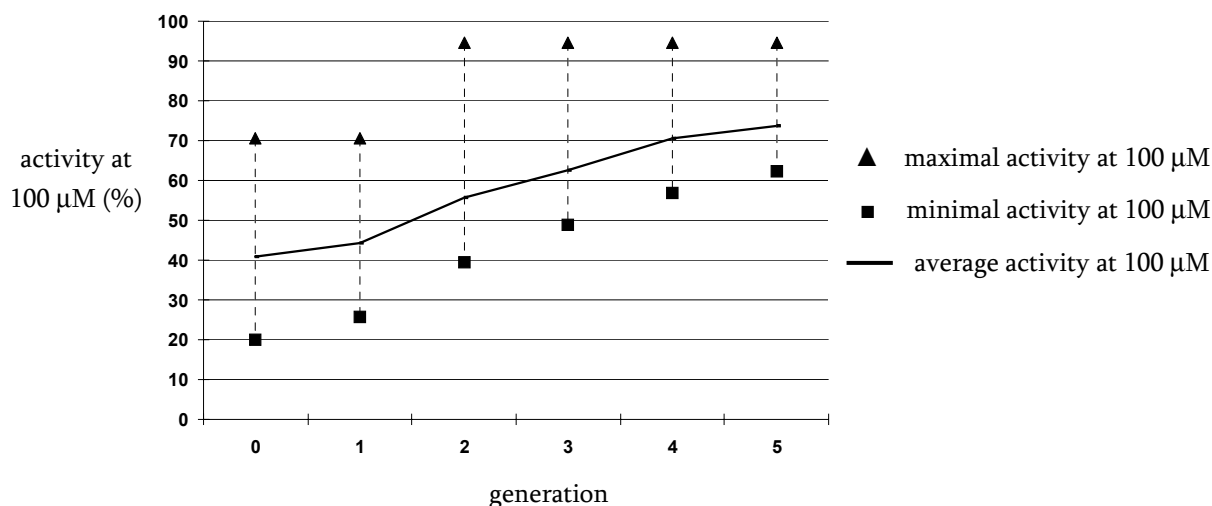
Figure 7.2 Average inhibitory percentage of the 16 best inhibitors per generation depicted with spreading of inhibitory percentage value of the best (▲) and worst (■) inhibitor.

The efficacy of the iterative optimisation procedure was evaluated by calculation of the average inhibitory percentage of the 16 best inhibitors of a generation (Figure 7.2). As can be seen in Figure 7.2, the average inhibitory percentage first increased rapidly with already in the second generation (Table 7.1a), compound **A03B02C14D16** was found to inhibit PGGT-1 for ≈95% at 100 μM concentration. After 5 generations (Table 7.1a) no significant improvement (not depicted in Figure 7.2) was obtained. A different ranking of the 16 best inhibitors of generation 5 is obtained by looking at the percentage inhibition of the 10 μM concentration data points (Table 7.1a). Two additional generations (generations 6 and 7, Table 7.1b) were synthesised during which no increase in average inhibitory potency was observed. A slightly more potent inhibitor **A03B10C14D16** holds the first place in this ranking with 97% inhibition of enzyme activity. The related compound **A03B02C14D16** is now second with 81% inhibition of PGGT-1 activity. The inaccurate pilot screening data could be replaced by the more accurate IC_{50}-values as selection criterium instead.[4] Therefore, the IC_{50} value of **A03B10C14D16** and **A03B02C14D16** against PGGT-1 was determined: **A03B02C14D16** and **A03B10C14D16** were found to inhibit PGGT-1 with an IC_{50}-value of 8.1±1.2 μM and 3.8±0.9 μM, respectively. In Scheme 7.3 the mutational pathway to these two compounds is shown and note that only the central two building blocks are transformed.

Chart 7.1 Set of A building blocks (A01-A24).[a]
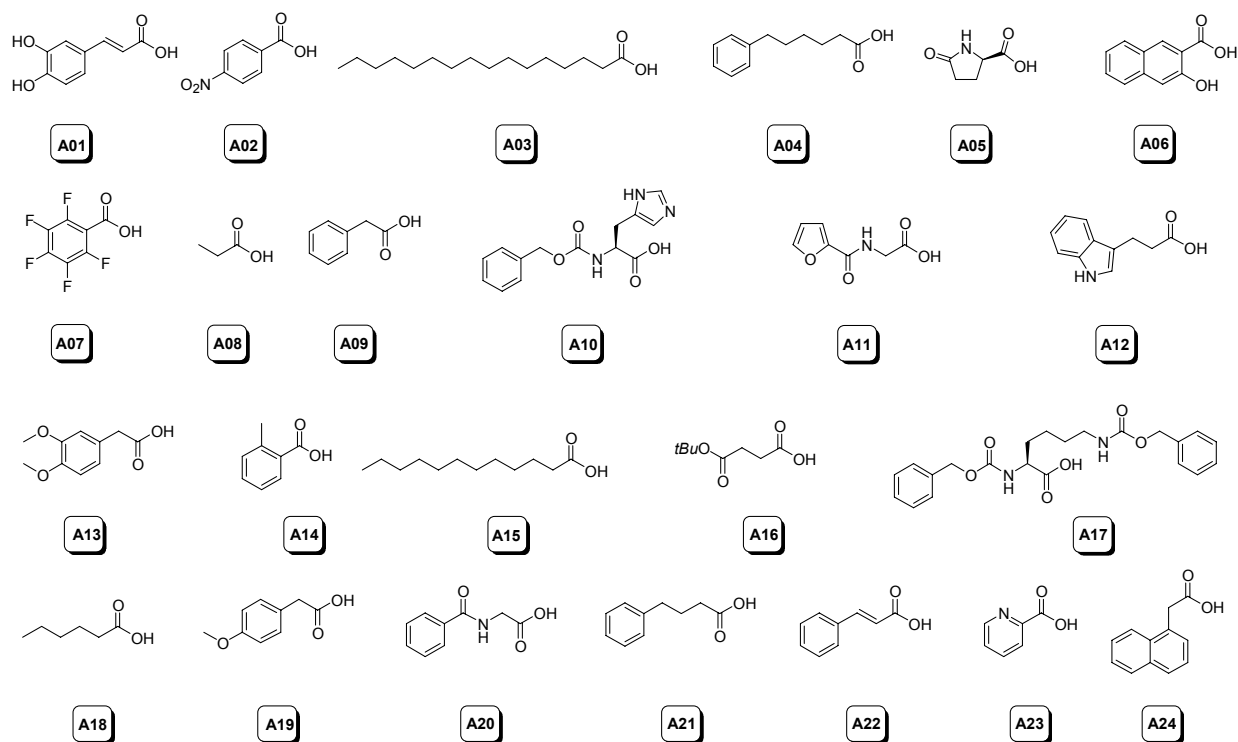
Chart 7.2 Set of B building blocks (B01-B22).[a]
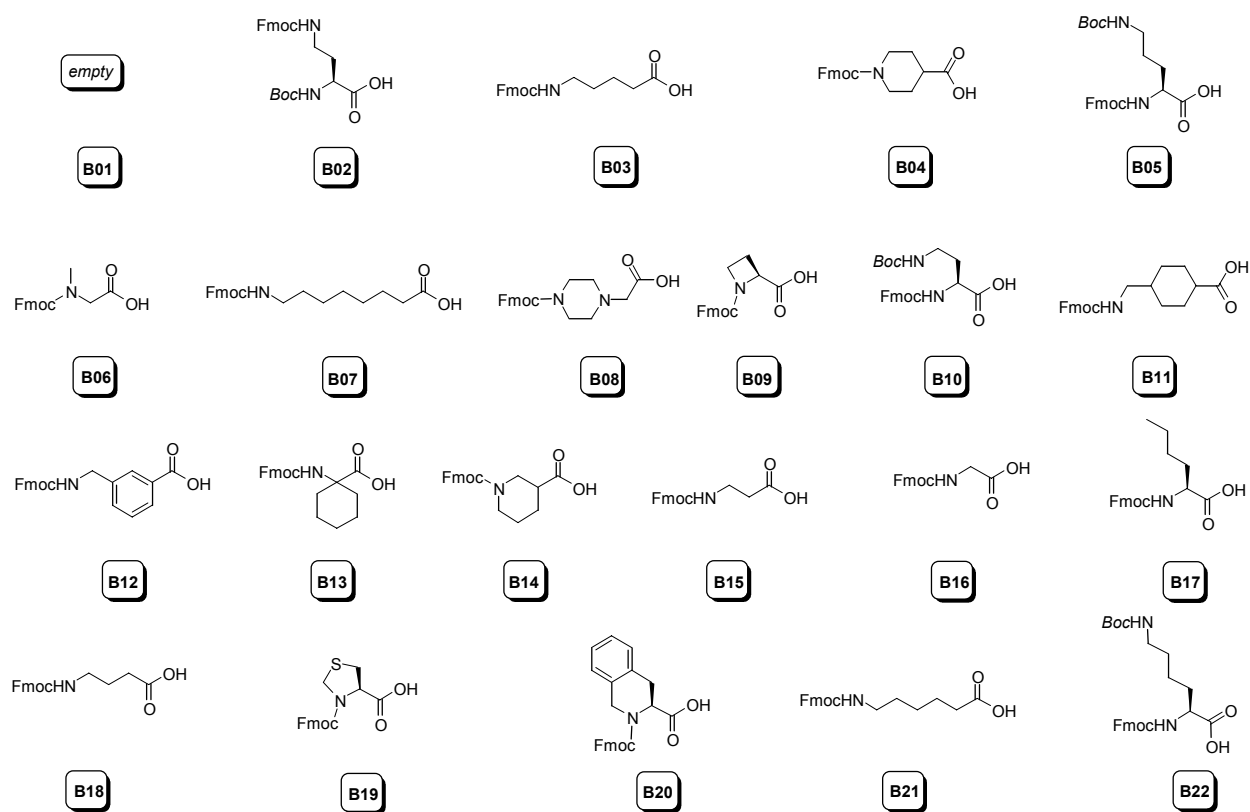
[a]Protective groups which are removed during the TFA mediated release of the product from the solid support are depicted in italic form.

Chart 7.3 Set of C building blocks (C01-C24).[a]
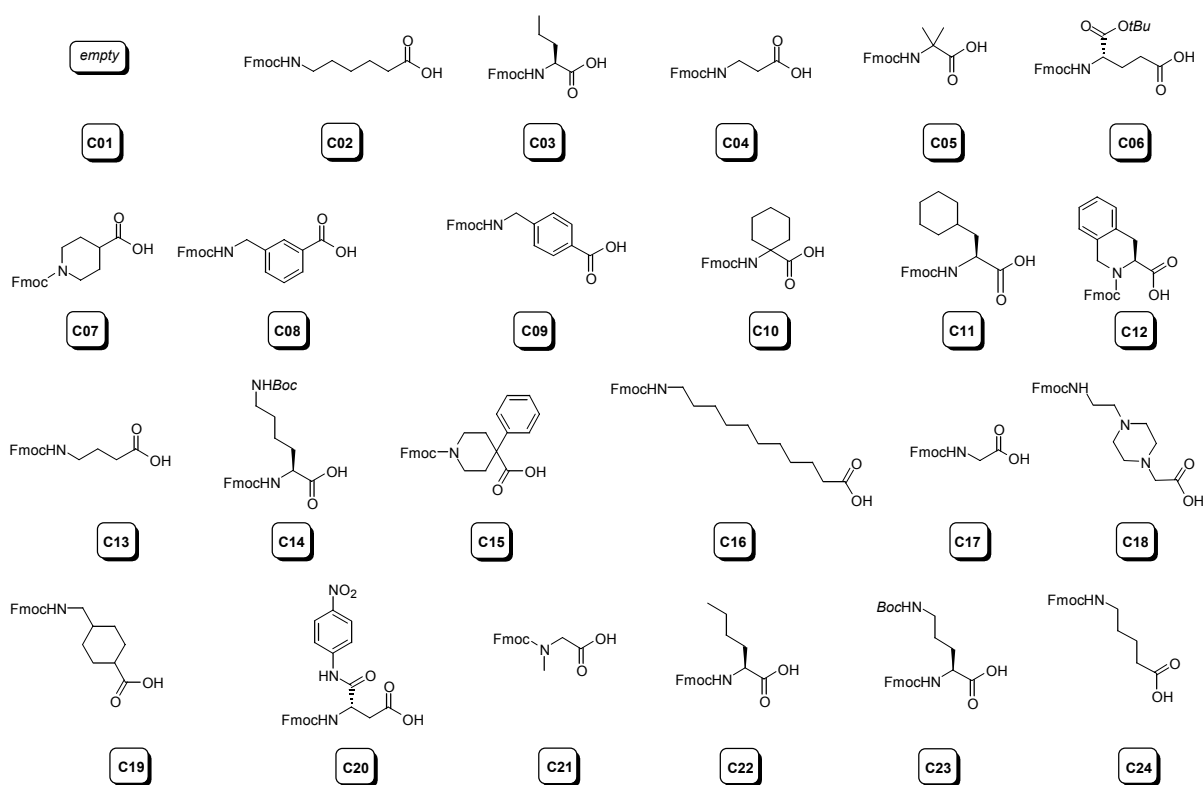
Chart 7.4 Set of D building blocks (D01-D18).[a]
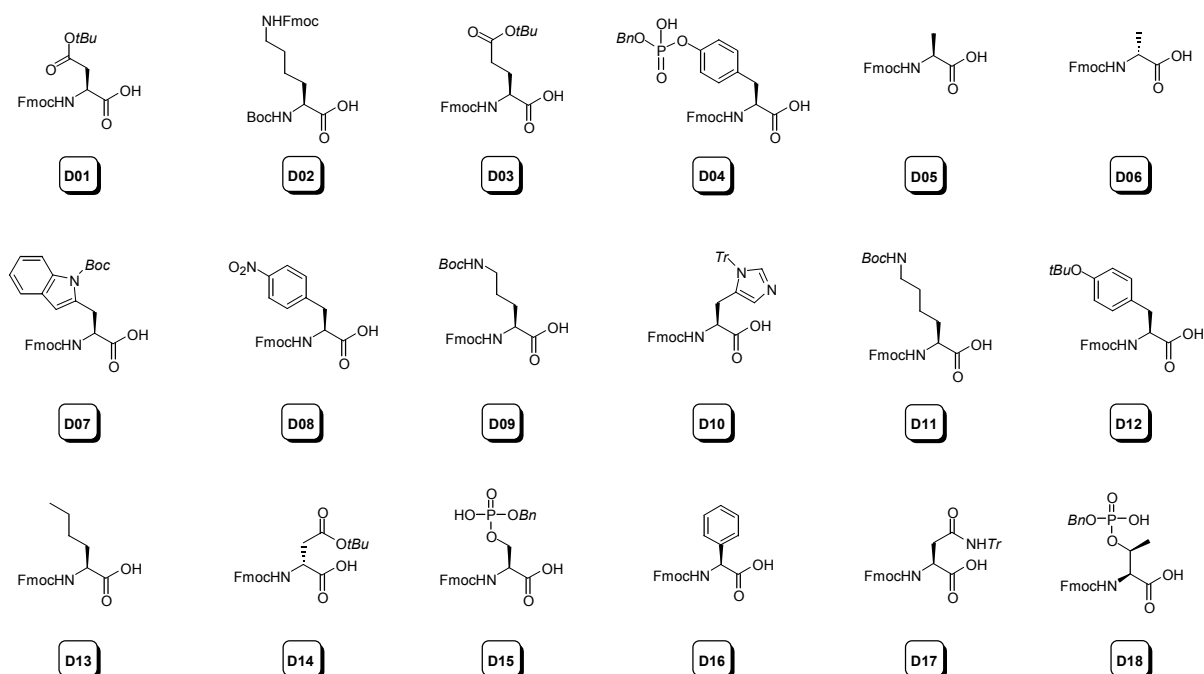
[a]Protective groups which are removed during the TFA mediated release of the product from the solid support are depicted in italic form.

	Best 16 of generation 0				Mutants (G1)	
Code	ABCD-code	A (%) at 100μM	→	Code	ABCD-code	A (%) at 100μM
G0-01	A21B21C01D15	71	→	G1-01	A21B21C01*D09*	0
G0-02	A04B07C05D11	63	→	G1-02	A04*B17*C05D11	10
G0-03	A19B12C01D15	55	→	G1-03	A19B12C01*D12*	24
G0-04	A02B07C24D01	50	→	G1-04	A02*B17*C24D01	0
G0-05	A02B06C21D01	49	→	G1-05	*A18*B06C21D01	7
G0-06	A03B02C07D07	49	→	G1-06	A03B02C14D07	39
G0-07	A10B03C04D15	48	→	G1-07	A10B03C04*D06*	26
G0-08	A21B01C02D18	41	→	G1-08	A21*B22*C02D18	0
G0-09	A21B21C01D04	40	→	G1-09	A21B21*C24*D04	48
G0-10	A07B07C07D01	30	→	G1-10	A07B07C07*D08*	0
G0-11	A16B11C21D11	29	→	G1-11	A16*B05*C21D11	32
G0-12	A07B11C24D05	25	→	G1-12	A07B11*C22*D05	0
G0-13	A04B03C05D11	23	→	G1-13	A04B03*C17*D11	11
G0-14	A15B03C10D01	21	→	G1-14	A15B03*C05*D01	19
G0-15	A10B04C05D03	20	→	G1-15	A10B04C05*D08*	41
G0-16	A24B01C23D01	20	→	G1-16	A24B01C23*D08*	0

	Best 16 after 1 generation				Mutants (G2)	
Code	ABCD-code	A (%) at 100μM	→	Code	ABCD-code	A (%) at 100μM
G0-01	A21B21C01D15	71	→	G2-01	A21B21*C20*D15	62
G0-02	A04B07C05D11	63	→	G2-02	A04B07*C27*D11	22
G0-03	A19B12C01D15	55	→	G2-03	A19B12C01*D09*	0
G0-04	A02B07C24D01	50	→	G2-04	A02B07*C12*D01	22
G0-05	A02B06C21D01	49	→	G2-05	A02B06*C15*D01	29
G0-06	A03B02C07D07	49	→	G2-06	A03B02*C12*D07	53
G0-07	A10B03C04D15	48	→	G2-07	A10*B13*C04D15	0
G1-09	A21B21C24D04	48	→	G2-08	A21*B02*C24D04	0
G1-15	A10B04C05D08	41	→	G2-09	A10*B14*C05D08	75
G0-08	A21B01C02D18	41	→	G2-10	*A05*B01C02D18	51
G0-09	A21B21C01D04	40	→	G2-11	A21*B20*C01D04	4
G1-06	A03B02C14D07	39	→	G2-12	A03B02C14*D16*	95
G1-11	A16B05C21D11	32	→	G2-13	A16B05*C03*D11	0
G0-10	A07B07C07D01	30	→	G2-14	A07B07*C10*D01	18
G0-11	A16B11C21D11	29	→	G2-15	A16B11*C09*D11	0
G1-07	A10B03C04D06	26	→	G2-16	A10*B06*C04D06	0

	Best 16 after 2 generations				Mutants (G3)	
Code	ABCD-code	A (%) at 100μM	→	Code	ABCD-code	A (%) at 100μM
G2-12	A03B02C14D16	95	→	G3-01	A03*B08*C14D16	57
G2-09	A10B14C05D08	75	→	G3-02	A10*B11*C05D08	20
G0-01	A21B21C01D15	71	→	G3-03	A21*B11*C01D15	0
G0-02	A04B07C05D11	63	→	G3-04	A04B07C05*D01*	59
G2-01	A21B21C20D15	62	→	G3-05	A21*B10*C20D15	21
G0-03	A19B12C01D15	55	→	G3-06	A19B12C01*D16*	44
G2-06	A03B02C12D07	53	→	G3-07	*A10*B02C12D07	73
G2-10	A05B01C02D18	51	→	G3-08	A05B01*C04*D18	22
G0-04	A02B07C24D01	50	→	G3-09	A02B07C24*D02*	0
G0-05	A02B06C21D01	49	→	G3-10	A02B06C21*D18*	2
G0-06	A03B02C07D07	49	→	G3-11	A03*B16*C07D07	20
G0-07	A10B03C04D15	48	→	G3-12	A10*B16*C04D15	62
G1-09	A21B21C24D04	48	→	G3-13	A21B21C24*D02*	0
G1-15	A10B04C05D08	41	→	G3-14	*A07*B04C05D08	0
G0-08	A21B01C02D18	41	→	G3-15	A21B01C02*D10*	65
G0-09	A21B21C01D04	40	→	G3-16	A21B21*C12*D20	62

	Best 16 after 3 generations				Mutants (G4)	
Code	ABCD-code	A (%) at 100μM	→	Code	ABCD-code	A (%) at 100μM
G2-12	A03B02C14D16	95	→	G4-01	A03*B13*C14D16	0
G2-09	A10B14C05D08	75	→	G4-02	A10B14*C04*D08	73
G3-07	A10B02C12D07	73	→	G4-03	A10B02*C07*D07	41
G0-01	A21B21C01D15	71	→	G4-04	A21*B20*C01D15	71
G3-15	A21B01C02D10	65	→	G4-05	A21*B05*C02D10	0
G0-02	A04B07C05D11	63	→	G4-06	*A24*B07C05D11	7
G2-01	A21B21C20D15	62	→	G4-07	*A13*B21C20D15	87
G3-12	A10B16C04D15	62	→	G4-08	A10*B14*C04D15	87
G3-16	A21B21C12D04	62	→	G4-09	*A20*B21C12D04	0
G3-04	A04B07C05D01	59	→	G4-10	*A22*B07C05D01	0
G3-01	A03B08C14D16	57	→	G4-11	A03*B22*C14D16	0
G0-03	A19B12C01D15	55	→	G4-12	A19B12*C16*D15	67
G2-06	A03B02C12D07	53	→	G4-13	A03B02C12*D03*	26
G2-10	A05B01C02D18	51	→	G4-14	A05B01*C16*D18	0
G0-04	A02B07C24D01	50	→	G4-15	A02B07*C06*D01	0
G0-05	A02B06C21D01	49	→	G4-16	A02*B19*C21D01	21

	Best 16 after 4 generations				Mutants (G5)			Best 16 after 5 generations		
Code	ABCD-code	A (%) at 100μM	→	Code	ABCD-code	A (%) at 100μM	Code	ABCD-code	A (%) at 100μM	A (%) at 10μM
G2-12	A03B02C14D16	95	→	G5-01	A03*B10*C14D16	68	G2-12	A03B02C14D16	95	81
G4-07	A13B21C20D15	87	→	G5-02	*A17*B21C20D15	72	G4-07	A13B21C20D15	87	53
G4-08	A10B14C04D15	87	→	G5-03	A10B14C04*D09*	0	G4-08	A10B14C04D15	87	26
G2-09	A10B14C05D08	75	→	G5-04	A10B14*C15*D08	73	G5-07	A02B20C01D15	80	60
G3-07	A10B02C12D07	73	→	G5-05	*A09*B02C12D07	58	G2-09	A10B14C05D08	75	37
G4-02	A10B14C04D08	73	→	G5-06	A10*B10*C04D08	1	G5-04	A10B14C15D08	73	0
G4-04	A21B20C01D15	71	→	G5-07	*A02*B20C01D15	80	G3-07	A10B02C12D07	73	22
G0-01	A21B21C01D15	71	→	G5-08	A21*B10*C01D15	48	G4-02	A10B14C04D08	73	26
G4-12	A19B12C16D15	67	→	G5-09	A19*B04*C16D15	50	G5-02	A17B21C20D15	72	64
G3-15	A21B01C02D10	65	→	G5-10	A21B01*C03*D10	17	G4-04	A21B20C01D15	71	39
G0-02	A04B07C05D11	63	→	G5-11	A04B07*C19*D11	10	G0-01	A21B21C01D15	71	40
G2-01	A21B21C20D15	62	→	G5-12	A21B21*C04*D15	43	G5-01	A03B10C14D16	68	97
G3-12	A10B16C04D15	62	→	G5-13	A10B16*C18*D15	35	G4-12	A19B12C16D15	67	32
G3-16	A21B21C12D04	62	→	G5-14	A21*B05*C12D04	54	G3-15	A21B01C02D10	65	18
G3-04	A04B07C05D01	59	→	G5-15	A04B07C05*D07*	0	G0-02	A04B07C05D11	63	40
G3-01	A03B08C14D16	57	→	G5-16	*A20*B08C14D16	16	G2-01	A21B21C20D15	62	29

Table 7.1a Results of the one-building mutation procedure for generation 1-5. Activity (A) of PGGT-1 at 100 μM or 10 μM of compound: expressed as % of control activity.

Mutants (G6)			Best 16 after 6 generations				Mutants (G7)			Best 16 after 7 generations		
Code	ABCD-code	A (%) at 10μM	Code	ABCD-code	A (%) at 10μM		Code	ABCD-code	A (%) at 10μM	Code	ABCD-code	A (%) at 10μM
G6-01	A03B10C24D16	27	G5-01	A03B10C14D16	97	→	G7-01	A03B10C02D16	0	G5-01	A03B10C14D16	97
G6-02	A03B02C13D16	38	G2-12	A03B02C14D16	81	→	G7-02	A03B14C14D16	0	G2-12	A03B02C14D16	81
G6-03	A17B21C20D01	5	G5-02	A17B21C20D15	64	→	G7-03	A02B21C20D15	9	G5-02	A17B21C20D15	64
G6-04	A02B20C12D15	0	G5-07	A02B20C01D15	60	→	G7-04	A20B20C01D15	36	G5-07	A02B20C01D15	60
G6-05	A11B08C14D16	0	G3-01	A03B08C14D16	57	→	G7-05	A03B08C14D05	0	G3-01	A03B08C14D16	57
G6-06	A13B17C20D15	18	G4-07	A13B21C20D15	53	→	G7-06	A13B16C20D15	23	G4-07	A13B21C20D15	53
G6-07	A21B21C01D17	6	G0-01	A21B21C01D15	40	→	G7-07	A21B14C01D15	1	G7-16	A03B22C23D16	44
G6-08	A04B07C22D11	1	G0-02	A04B07C05D11	40	→	G7-08	A06B07C05D11	0	G0-01	A21B21C01D15	40
G6-09	A21B20C01D05	0	G4-04	A21B20C01D15	39	→	G7-09	A10B20C01D15	0	G0-02	A04B07C05D11	40
G6-10	A07B07C10D15	14	G2-14	A07B07C10D01	39	→	G7-10	A07B07C11D01	0	G4-04	A21B20C01D15	39
G6-11	A10B14C05D10	9	G6-02	A03B02C13D16	38	→	G7-11	A03B02C13D03	0	G2-14	A07B07C10D01	39
G6-12	A11B04C16D15	0	G2-09	A10B14C05D08	37	→	G7-12	A10B14C21D08	28	G6-02	A03B02C13D16	38
G6-13	A21B21C09D15	0	G5-09	A19B04C16D15	37	→	G7-13	A19B04C15D15	0	G2-09	A10B14C05D08	37
G6-14	A19B09C16D15	0	G5-12	A21B21C04D15	33	→	G7-14	A21B21C24D15	0	G5-09	A19B04C16D15	37
G6-15	A03B22C02D16	0	G4-12	A19B12C16D15	32	→	G7-15	A19B18C16D15	0	G7-04	A20B20C01D15	36
G6-16	A21B21C12D15	18	G4-11	A03B22C14D16	32	→	G7-16	A03B22C23D16	44	G5-12	A21B21C04D15	33

Table 7.1b Results of the one-building mutation procedure for generations 6 and 7. Activity (A) of PGGT-1 at 10 μM of compound: expressed as % of control activity.
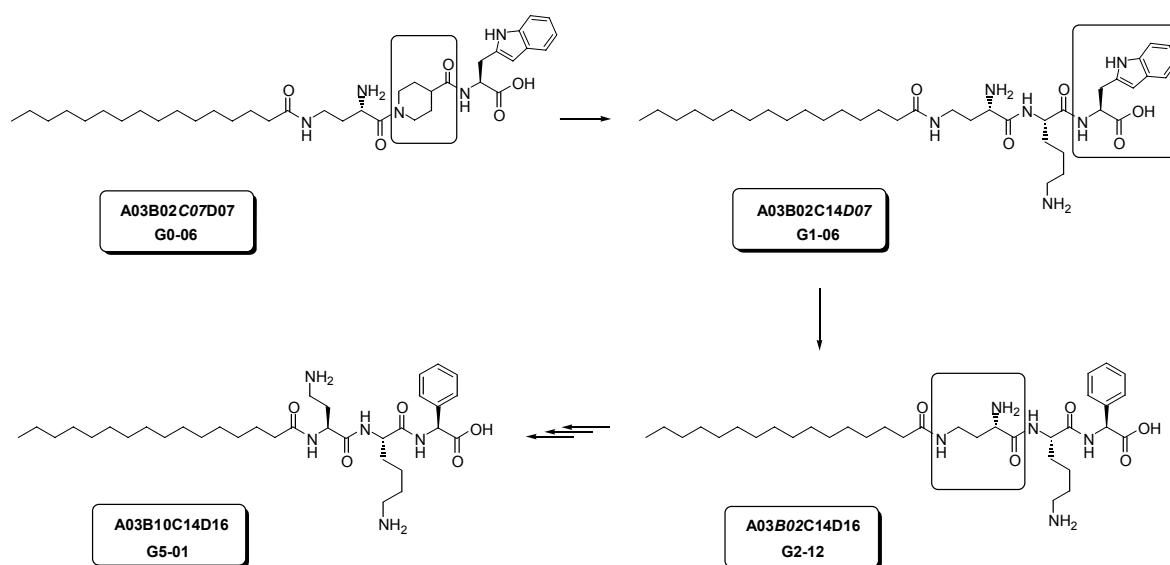
Scheme 7.3 Mutational development of A03B02C14D16 and A03B10C14D16.

Although the mode of action of **A03B02C14D16** and **A03B10C14D16** is not known at the moment, it is not excluded that these compounds act as a bisubstrate inhibitor in which the lipid part functions as isoprenyl mimic.[5] The presence of two amines in these compounds (*i.e.* in building blocks **B10**, **B02** and **C14**) indicates the possibility of H-bonding in the active site of PGGT-1.[6]

7.3 Conclusions

The *in silico* optimisation procedure (Schemes 7.1 and 7.2) presented in this chapter gave a cut-off inhibition value after 5 of the total 7 synthesised generations of ambiphilic peptides (Tables 7.1a and 7.1b). The most potent PGGT-1 inhibitors found in these 7 generations, **A03B02C14D16** and **A03B10C14D16**, have IC_{50}-values of 8.1±1.2 μM and 3.8±0.9 μM, respectively.[7] Thus by synthesizing a fraction ($\approx$ 0.06%)* of the total possible library population, already lead compounds which exhibit low micromolar inhibitory potency against PGGT-1 are obtained. Obviously, it is not known whether more active inhibitors of PGGT-1 can be found with the given set of building blocks A_w-D_z (Charts 7.1–7.4), however, the library obtained here can be further optimised by either introducing new building blocks or increasing the number of mutations. Altogether, the peptide analogs described in this chapter represent novel lead compounds for the construction of a conventional library using rational design. Because both selective and dual inhibitors[8] of PFT and PGGT-1 show promise as therapeutics or as tools to investigate the biological functioning of isoprenylating processes (proteomics, see Chapter 6), the selectivity of PGGT-1 inhibitors of the type presented in this chapter may also be used as a selection criterium. This can either be based on selecting for high selectivity (selective inhibitors) or low selectivity (dual inhibitors).[9]

7.4 Experimental Section

7.4.1 General – ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AV-400 (^{1}H-NMR: 400, ^{13}C-NMR: 100 MHz) or a Bruker DMX-600 (^{1}H-NMR: 600, ^{13}C-NMR: 150 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane (^{1}H-NMR δ= 0 ppm; ^{13}C-NMR middle resonance δ= 77.0 ppm) as internal standard. Mass spectra were recorded with a Perkin Elmer/SCIEX API 165 mass instrument and HR-MS spectra were recorded with a API QSTAR^{tm} Pulsar (Applied Biosystems). RP-HPLC analysis was performed on a Jasco HPLC system equipped with an Alltima C18 100Å 5 μm column (4.6×150 mm). Purifications were performed on a BioCad Vision (Applied Biosystem) HPLC system equipped with an Alltima C18 100Å 5 μm column (10×150 mm). The applied eluent systems were **I**: H_2O (eluent A), linear gradient of CH_3CN (eluent B), 1% aq. TFA (effective 0.1%, eluent C) or **II**: H_2O (eluent A), linear gradient of CH_3CN (eluent B), 0.1M NH_4Ac (effective 0.01M, eluent C). In the case of compounds containing building blocks **D04**, **D15** or **D18**, the best results were obtained with system **II**. All solvents were of HPLC quality (Biosolve). All employed building blocks (Charts 7.1-7.4) were purchased from commercial suppliers and were of the highest quality available. The SPPS was performed on a LaMOSS2 (*Labotec Modular Organic Synthesis System 2*) robotic synthesiser using standard Fmoc chemistry and Wang solid support (loading 0.5–1.1 mmol g^{-1}, Novabiochem, 100-200 mesh).

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* Total library= 24×22×24×18= 228096 compounds; 30+6×16= 126 compounds synthesised= $\approx$0.04%.

7.4.2 *General procedure for manual coupling of building blocks D01, D03, D04, D15 and D18:* 1.0 g Wang resin (0.81 mmol) was coevaporated 3× with anh. 1,4-dioxane (10 mL) and treated with a solution of the amino acid (2.0 equiv., 1.6 mmol) in DCM/DMF (3/1 v/v; *c*= 0.1–0.15M), DIC (2.4 equiv., 1.9 mmol, 0.3 mL) and DMAP (0.04 equiv., 5 mg). After shaking the mixture under argon for 6 h, the resin was washed with DCM, DMF, MeOH, DCM and finally Et$_2$O. A second coupling step was performed employing 1.0 equiv. of amino acid and the reaction mixture was shaken overnight. The resin was washed (DCM and DMF), capped (0.5M Ac$_2$O, 0.125M DIPEA and 0.015M HOBt in NMP), washed (DMF, MeOH, DCM and Et$_2$O) and dried *in vacuo*. The loading of the resin (0.3–0.5 mmol g^{-1}) was determined as follows: to a sample of the resin (1–2 mg) in a volumetric flask (10 mL) was added a solution of piperidine/DMF (1/4 v/v, 1.0 mL) and the mixture was left for 15 min. Next, the volume was adjusted to 10 mL by addition of EtOH (HPLC grade) and the UV absorption was measured between 250 and 350 nm. The loading could then be calculated using formula **A** (A$_{300}$= absorption at 300 nm; EtOH as reference); V= volume of sample (= 10 mL); wt= weight of employed resin (= 1–2 mg):

$$\text{loading (mmol g}^{-1}\text{)} = \frac{A_{300} \times V}{7.8 \times wt} \tag{A}$$

7.4.3 General synthetic protocol LaMOSS2 robot.

[a] *Coupling building block D:* Wang resin (50 µmol) was swollen with 2×2 mL DCM and treated with 5.0 equiv. of a building block D (0.25M solution in NMP, 1.0 mL), 5.0 equiv. DIC (0.5 mL, 0.5M solution in DCM) and 0.25 equiv. DMAP (0.5 mL, 0.025M solution in NMP). The reaction mixture was flushed with N$_2$ for 3 h after which the reagents were removed. This procedure was repeated, however this time the reaction mixture was allowed to react for 16 h instead of 3 h. After a washing with NMP (1×3 and 3×2 mL), the resin was capped with 2 mL of 0.5M Ac$_2$O, 0.125M DIPEA and 0.015M HOBt in NMP (2×5 min) and washed with NMP (1×3 and 3×2 mL).

[b] *Removal Fmoc group:* the resin was treated with 2 mL 20% piperidine in NMP (4×2 min) and washed with NMP (1×3 and 3×2 mL).[10]

[c] *Coupling of a building block B or C:* to the resin were added 5.0 equiv. of building block B or C (0.25M in NMP, 1.0 mL), 5.0 equiv. BOP/HOBt (1/1, 0.5 mL, 0.5M in NMP) and 10 equiv. DMAP (0.5 mL, 1.0M in NMP). The reaction mixture is flushed with N$_2$ for 45 min after which the reagents are removed. In the case of building blocks which are known to couple difficult (*e.g.* **B06** or **C05**) the coupling procedure was repeated. The resin was washed (1×3 and 3×2 mL NMP), capped with 2×2 mL of 0.5M Ac$_2$O, 0.125M DIPEA and 0.015M HOBt in NMP and washed again (1×3 and 3×2 mL NMP).

[d] *Coupling building block A:* to the resin were added 5.0 equiv. of a building block A (0.25M solution in NMP, 1.0 mL), 5.0 equiv. BOP/HOBt (0.5 mL, 0.5M solution in DCM) and 10 equiv. DMAP (0.5 mL, 1.0M solution in NMP). After flushing with N$_2$ for 45 min all reagents are removed and the resin was washed (1×3 mL and 3×2 mL NMP), capped (2×2 mL 0.5M Ac$_2$O, 0.125M DIPEA and 0.015M HOBt in NMP) and washed again (2 mL DCM and 2 mL MeOH (3×); 1×3 mL and 3×2 mL DCM).

[e] *Cleavage of product from resin:* to the resin was added 3 mL TFA/H$_2$O/*i*Pr$_3$SiH (95/4/1 v/v/v) under N$_2$ flushing. After 2 h the TFA solution was collected in a tube and the resin was rinsed 2× with 2 mL TFA/H$_2$O/*i*Pr$_3$SiH (95/4/1 v/v/v).

[f] *Work-up procedure:* The filtrate was concentrated *in vacuo*, dissolved in 4 mL H$_2$O/CH$_3$CN/*t*BuOH (1/1/1 v/v/v), analysed by LC-MS and purified by RP-HPLC (Tables 7.3–7.5 list LC-MS data for compounds of generation 1–5).

compound	A (%) at 100μM	compound	A (%) at 100μM	compound	A (%) at 100μM
A21B21C01D15	71	A16B11C21D11	29	A24B21C04D18	10
A04B07C05D11	63	A07B11C24D05	25	A04B13C24D01	9
A19B12C01D15	55	A04B03C05D11	23	A04B03C19D11	9
A02B07C24D01	50	A15B03C10D01	21	A01B03C24D01	4
A02B06C21D01	49	A10B04C05D03	20	A21B12C01D03	3
A03B02C07D07	49	A24B01C23D01	20	A19B15C02D10	0
A10B03C04D15	48	A21B16C09D01	19	A15B02C21D01	0
A21B01C02D18	41	A21B03C24D01	16	A07B11C05D13	0
A21B21C01D04	40	A16B03C19D11	16	A12B03C10D03	0
A07B07C07D01	30	A10B02C24D03	14	A24B01C08D03	0

Table 7.2 Initial pool of compounds (16 most potent compounds= generation 0), synthesised *via* the general procedures described in **§7.4.2** and **§7.4.3** using a diverse range of building blocks (Chart 7.1–7.4). A= activity of enzyme at 100 μM of compound: expressed as % of control activity.

7.4.5 Tables 7.3-7.5 list MS (ESI, *m/z*) data and R_t values of compounds from generation 1– 5.

compound (G1)	[M+H]+	R_t (min)[a]	yield (%)[b]	compound (G2)	[M+H]+	R_t (min)[c]	yield (%)[b]
A21B21C01D09	392,2	8,2	42	A21B21C20D15	680,4	10,2	1
A04B17C05D11	519,4	9,8	54	A04B07C21D11	533,4	12,2[a]	98
A19B12C01D12	463,2	9,0	56	A19B12C01D09	414,2	8,6	94
A02B17C24D01	495,3	10,2	89	A02B07C12D01	583,5	13,6	34
A18B06C21D01	374,1	7,9	28	A02B06C15D01	541,4	10,9	79
A03B02C14D07	671,8	13,8	38	A03B02C12D07	702,6	25,9[a]	22
A10B03C04D06	531,3	10,6	38	A10B13C04D15	853,6	6,9	1
A21B22C02D18	587,5	8,9	1	A21B02C24D04	607,4	8,0	35
A21B21C24D04	463,2	10,2	1	A10B14C05D08	678,4	11,5	60
A07B07C07D08	657,7	12,4	45	A05B01C02D18	424,1	2,3	28
A16B05C21D11	432,2	2,4	98	A21B20C01D04	567,2	9,3	14
A07B11C22D05	536,2	11,5	49	A03B02C14D16	618,6	12,7[a]	66
A04B03C17D11	477,3	8,5	42	A16B05C03D11	460,2	2,1	89
A15B03C05D01	500,4	20,5	68	A07B07C10D01	594,4	15,5	38
A10B04C05D08	678,3	8,4	48	A16B11C09D11	519,4	7,9	71
A24B01C23D08	493,3	8,8	43	A10B06C04D06	503,3	8,5	94

Table 7.3 LC-MS data (R_t and [M+H]+) of compounds from generation 1 (G1) and 2 (G2). [a]Linear gradient B 05→90%, 26 min. [b]Non-optimised yields. All compounds were ≥95% pure as determined by LC-MS. [c]Unless stated otherwise: linear gradient B 05→50%, 26 min.

compound (G3)	[M+H]⁺	R_t(min)[a]	yield (%)[b]	compound (G4)	[M+H]⁺	R_t(min)[a]	yield (%)[b]
A03B08C14D16	644,5	18,8	30	A03B13C14D16	643,3	22.6	98
A10B11C05D08	706,4	13,1	81	A10B14C04D08	664,2	12.2	98
A21B11C01D15	471,3	11,8	6	A10B02C07D07	687,5	11.2	60
A04B07C05D01	534,3	17,5	89	A21B20C01D15	491,2	13.0	70
A21B10C20D15	667,3	13,1	4	A21B05C02D10	529,5	9.9	98
A19B12C01D16	433,1	15,6	62	A24B07C05D11	541,3	12.7	98
A10B02C12D07	735,4	12,6	37	A13B21C20D15	712,5	11.1	6[c]
A05B01C04D18	382,1	1,9	35	A10B14C04D15	639,2	8.8	31
A02B07C24D02	536,2	12,4	98	A20B21C12D04	695,4	11.7	98
A02B06C21D18	491,1	9,0	34	A22B07C05D01	490,2	13.9	98
A03B16C07D07	615,5	27,0	64	A03B22C14D16	646,5	16.7	54
A10B16C04D15	585,2	9,0	14	A19B12C16D15	650,3	12.0	13
A21B21C24D02	505,3	11,8	51	A03B02C12D03	645,4	20.1	80
A07B04C05D08	601,3	16,9	81	A05B01C16D18	494,2	10.4	15
A21B01C02D10	416.1	12.0	37	A02B07C06D01	553,3	12.6	98
A21B21C12D20	680,4	13,9	48	A02B19C21D01	469,1	11.6	37

**Table 7.4** LC-MS data (R_t and [M+H]⁺) of compounds from generation 3 (G3) and 4 (G4). [a]Linear gradient B 05→90%, 26 min. [b]Non-optimised yields. All compounds were ≥95% pure as determined by LC-MS.

| compound	[M+H]⁺	R_t(min)[a]	yield (%)[b]	compound	[M+H]⁺	R_t(min)[a]	yield (%)[b]
A03B10C14D16	618,6	17.5	76	A19B04C16D15	628,5	14.9	7
A17B21C20D15	930,5	13.7	4	A21B01C03D10	401,1	10.6	98
A10B14C04D09	586,3	9.6	98	A04B07C19D11	601,5	14.0	97
A10B14C15D08	780,4	15.0	96	A21B21C04D15	516,2	11.4	10
A09B02C12D07	582,3	14.1	17	A10B16C18D15	683,4	8.9	26
A10B10C04D08	653,6	11.3	98	A21B05C12D04	681,4	11.1	18
A02B20C01D15	494,1	10.8	7	A04B07C05D07	605,5	19.2	13
A21B10C01D15	432,1	9.2	11	A20B08C14D16	567,3	8.7	98

Table 7.5 LC-MS data [R_t and (M+H)⁺] of compounds from generation 5 (G5).[a]Linear gradient B 05→90%, 26 min. [b]Non-optimised yields. All compounds were ≥95% pure as determined by LC-MS.

7.4.6 Spectroscopic and spectrometric data of compounds from the pool of synthesised compounds.

A03B02C14D16 (G02-12): ¹H-NMR (400 MHz, DMSO-*d6*): δ 8.86 (d, 1H, *J*= 7.6 Hz), 8.73 (m, 1H), 8.03 (d, *J*= 4.0 Hz), 7.81 (m), 7.36 (m, 5H, Ph), 7.07 (d, 1H, *J*= 8.0 Hz), 6.96 (d, 1H, *J*= 8.4 Hz), 6.82 (d, 1H, *J*= 6.8 Hz), 6.62 (d, 1H, *J*= 8.4 Hz), 5.36 (d, 1H, *J*= 7.6 Hz), 5.29 (d, 1H, *J*= 7.2 Hz), 4.47 (m, 1H), 4.16–3.96 (m, 6H), 3.72 (m, 1H), 3.32, 3.12 (2×s), 2.76–2.67 (2×m), 2.49 (s), 2.06 (dd, 2H, *J*= 6.8 and 7.2 Hz), 1.76 (m, 3H), 1.56, 1.50 (2×m), 1.22 (bs), 1.10 (s), 0.86 (apparent t, 3H, *J*= 6.0 and 6.8 Hz). Purity >95%, 20.4 mg (66% yield). LC-MS analysis: R$_t$= 12.7 min (linear gradient B 05→90%, 26 min), *m/z* 618.5 (M+H)⁺. HR-MS: calc. for [C$_{34}$H$_{59}$N$_5$O$_5$+H]⁺ 618.45945, found 618.45972.
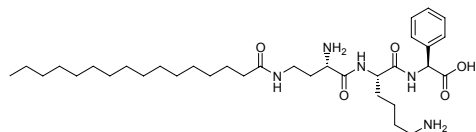
A03B10C14D16 (G05-01): ¹H-NMR (400 MHz, DMSO-*d6*): δ 8.80 (d, 1H, *J*= 8.0 Hz), 8.66 (d, 1H, *J*= 7.2 Hz), 8.07 (m, 4H), 7.81 (m), 7.35 (m, 5H^{Ph}), 7.07 (d, 1H, *J*= 8.0 Hz), 6.96 (d, 1H), 6.82 (d, 1H, *J*= 7.2 Hz), 6.64 (d, 1H, *J*= 8.4 Hz), 5.35 (d, 1H, *J*= 8.0 Hz), 5.27 (d, 1H, *J*= 7.2 Hz), 4.37 (m, 2H), 4.17–3.96 (m, 3H), 3.34 and 3.16 (2×s), 2.88–2.67 (m, 4H), 2.49 (s), 2.09 (m, 2H), 1.90–1.71 (m, 3H), 1.54–1.36 (m), 1.22 (bs), 1.10 (s), 0.84 (apparent t, 3H, *J*= 6.0 and 6.8 Hz). Purity >95%, 23.5 mg, (76% yield). LC-MS analysis: R$_t$= 17.5 min (linear gradient B 05→90%, 26 min), *m/z* 618.6 (M+H)⁺. HR-MS: calc. for [C$_{34}$H$_{59}$N$_5$O$_5$+H]⁺ 618.45945, found 618.45953.
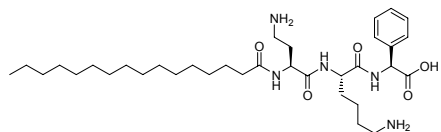
A10B11C05D08 (G03-02): ¹H-NMR (400 MHz, DMSO-*d6*): δ 8.93 (s), 8.10 (d, *J*= 8.4 Hz), 7.94 (apparent t, *J*= 5.6 and 6.0 Hz), 7.71 (s), 7.60 (dd, *J*= 8.0 and 8.4 Hz), 7.47 (d, *J*= 8.8 Hz), 7.38–7.28 (m), 5.00 (2×d, 2H, *J*= 12.8 Hz), 4.47 (dt, *J*= 4.8, 8.0 and 8.4 Hz), 4.30 (m), 3.23 (dd, *J*= 4.4 and 4.8 Hz), 3.09 (m), 2.96–2.85 (m), 2.02 (apparent bt, *J*= 11.6 and 12.0 Hz), 1.63 (bt, *J*= 14.4 and 15.2 Hz), 1.26 (s), 1.17 (m), 1.11 (s), 0.78 (dd, *J*= 12.4 and 12.8 Hz). Purity >95%, 28.6 mg, (81% yield). LC-MS analysis: R$_t$= 13.1 min (linear gradient B 05→90%, 26 min), *m/z* 706.4 (M+H)⁺.
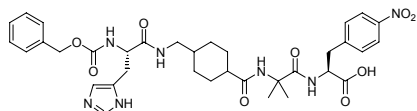
A21B21C24D02 (G03-13): ¹H-NMR (400 MHz, DMSO-*d6*): δ 7.76 (m), 7.27 (m), 7.17 (m), 6.54 (s), 3.00 (m), 2.52 (m), 2.04 (m), 1.77 (m), 1.45 (m), 1.37 (m), 1.20 (m). Purity >95%, 12.9 mg (51% yield). LC-MS analysis: R$_t$= 11.8 min (linear gradient B 05→90%, 26 min), *m/z* 505.3 (M+H)⁺.
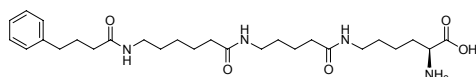
A07B04C05D08 (G03-14): ¹H-NMR (400 MHz, DMSO-*d6*): δ 8.10 (2×d, 2H, *J*= 2.0 Hz), 7.85 (d, 1H, *J*= 5.6 Hz), 7.62 (dd, 1H, *J*= 8.0 and 12.8 Hz), 7.47 (dd, 2H, *J*= 3.2 and 8.4 Hz), 4.50–4.39 (m, 2H), 3.53 (d, 1H, *J*= 13.6 Hz), 3.22 (m, 1H), 3.08 (m, 2H), 2.91 (m, 1H), 2.44 (m, 1H), 1.78 (bd, *J*= 13.2 Hz), 1.63 (bt, *J*= 10.4 Hz), 1.44–1.30 (m, 8H), 1.10 (s, 1H); ¹³C-NMR (100 MHz, DMSO-*d6*): δ 174.4, 173.6, 172.9, 156.2, 146.6, 131.1, 123.5, 56.2, 53.3, 46.3, 41.6, 41.5, 36.8, 29.3, 28.5, 25.5, 25.2. Purity >95%, 24.3 mg (81% yield). LC-MS analysis: R$_t$= 16.9 min (linear gradient B 05→90%, 26 min), *m/z* 601.3 (M+H)⁺.
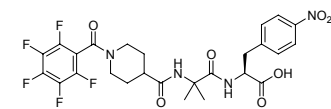
A02B17C24D01 (G01-04): ¹H-NMR (400 MHz, DMSO-*d6*): δ 8.76 (d, 1H, *J*= 8.0 Hz), 8.30 (d, 2H, *J*= 7.2 Hz), 8.12 (d, 3H, *J*= 7.2 Hz), 8.00 (t, 1H, *J*= 5.6 Hz), 4.49 (dd, 1H, *J*= 6.4 and 6.8 Hz), 4.38 (m, 1H), 3.10–3.01 (m, 2H), 2.65 (2×d, 1H, *J*= 6.0 Hz), 2.50 (m, obscured by solvent peak), 2.10 (t, 2H, *J*= 7.2 Hz), 1.74 (m, 2H), 1.51–1.20 (m, 9H), 0.85 (apparent t, 3H, *J*= 6.0 and 6.8 Hz); ¹³C-NMR (100 MHz, DMSO-*d6*): δ 173.0, 172.2, 171.8, 165.2, 149.5, 140.4, 129.6, 123.8, 54.2, 49.0, 38.8, 36.7, 35.1, 31.9, 29.0, 28.4, 23.0, 22.3, 14.4. Purity >95%, 22.0 mg (89% yield). LC-MS analysis: R$_t$= 10.2 min (linear gradient B 05→90%, 26 min), *m/z* 495.3 (M+H)⁺.
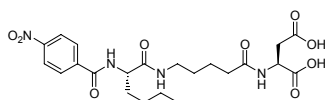
A16B11C09D11 (G02-15): ^{1}H-NMR (400 MHz, DMSO-*d6*): δ 8.53 (d, 1H, *J*= 8.0 Hz), 8.33 (t, 1H, *J*= 6.0 Hz), 7.82 (d, 3H, *J*= 8.4 Hz), 7.70 (bs, 3H), 7.30 (d, 2H, *J*= 8.0 Hz), 4.35 (m, 1H), 4.30 (d, 2H, *J*= 5.6 Hz), 2.89 (apparent t, 2H, *J*= 6.0 and 6.4 Hz), 2.78 (bs, 2H), 2.41 (apparent t, 2H, *J*= 6.4 and 7.2 Hz), 2.31 (apparent t, 2H, *J*= 6.4 and 7.2 Hz), 2.12 (bt, 1H, *J*= 12.0 Hz), 1.79 (m, 6H), 1.59 (m, 2H), 1.52–1.25 (m, 6H), 0.85 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-*d6*): δ 174.4, 174.2, 171.3, 166.8, 144.0, 132.8, 128.0, 127.1, 52.7, 45.2, 44.5, 42.0, 37.6, 30.5, 30.1, 29.7, 29.3, 27.0, 23.3. Purity >95%, 18.4 mg (71% yield). LC-MS analysis: R$_t$= 7.9 min (linear gradient B 05→50%, 26 min), *m/z* 519.4 (M+H)[+].
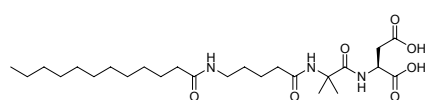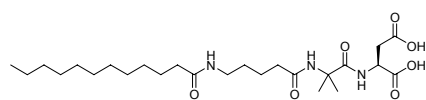
A15B03C05D01 (G01-14): ^{1}H-NMR (400 MHz, DMSO-*d6*): δ 7.92 (s, 1H), 7.73 (apparent t, 1H, *J*= 4.8 and 5.2 Hz), 7.52 (d, 1H, *J*= 8.0 Hz), 4.42 (d, 1H, *J*= 5.6 Hz), 3.00 (m, 2H), 2.60 (m, 2H), 2.04 (m, 4H), 1.46 (m, 5H), 1.40–1.10 (m), 0.86 (apparent t, 3H, *J*= 6.0 and 6.8 Hz); ^{13}C-NMR (100 MHz, DMSO-*d6*): δ 174.1, 173.0, 172.6, 172.4, 56.1, 48.9, 38.6, 35.9, 35.5, 31.8, 29.5, 29.2, 25.8, 25.1, 23.0, 22.6, 14.1. Purity >95%, 17.0 mg (68% yield). LC-MS analysis: R$_t$= 20.5 min (linear gradient B 05→90%, 26 min), *m/z* 500.4 (M+H)[+].

A16B05C21D11 (G01-11): ^{1}H-NMR (400 MHz, DMSO-*6*): δ 8.44 (d, 1H, *J*= 6.0 Hz), 8.23 (dd, 2H, *J*= 8.0 and 8.4 Hz), 7.76 (bs), 4.71 (m, 1H), 4.60 (m, 1H), 4.37 (d, 1H, *J*= 17.2 Hz), 4.17 (m, 2H), 4.10 (d, 1H, *J*= 16.0 Hz), 3.88 (dd, 2H, *J*= 16.0 and 17.2 Hz), 3.02 (s, 3H), 2.38 (m), 1.76–1.20 (m). Purity >95%, 21.1 mg (98% yield). LC-MS analysis: R$_t$= 2.4 min (linear gradient B 05→90%, 26 min), *m/z* 432.2 (M+H)[+].
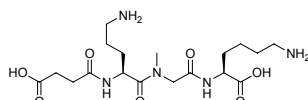
7.4.5 Procedure Pilot Assay[11,†] – Determination of PGGT-1 activity was performed by using a sepharose-coupled octapeptide as substrate. The amino acid sequence of the peptide was Met-Gly-Leu-Pro-Cys-Val-Val-Leu containing the *C*-terminal Ca$_1$a$_2$L-box, which is the consensus sequence for geranylgeranylation by PGGT-1. This substrate has been designated as pepCsep. PepDsep, another sepharose-coupled peptide, which is non-isoprenylatable by replacing Cys by Ala, was used as control to measure non-specific association of radiolabeled GGPP. A partial purified PGGT-1 enzyme preparation, isolated from bovine brain,[12] was used in the assay. The incubation mixture (25 μL) contained 2.5 μL of pepCsep or pepDsep (1 nmol of peptide), 3 μL of PGGT-1 (bovine brain), 1 μM of [3H]-GGPP (spec. radioactivity 15 Ci mmol^{-1}, American Radiolabeled Chemicals, USA), 50 μM ZnCl$_2$, 0.5 mM MgCl$_2$, 1 mM DTT, 0.004% Triton X-100, 50 mM Tris-HCl (pH 7.4). For the determination of the inhibitory potencies of the various compounds, three different concentrations were used (in duplo) in the mixture (generations 0 – 4: 10, 100 and 1000 μM; generation 5 – 7: 3, 10 and 100 μM). The incubation was performed at 37°C for 40 min under continuous shaking. The reaction was terminated by addition of 1 mL of 2% (w/v) of SDS, the beads were spun down and washed successively 3× with 2% (w/v) SDS under shaking for 45 min at 50°C. The remaining adhering radioactivity was counted in a Liquid Scintillation Counter. For the calculation of PGGT-1 activity the [3H]-counts bound to pepDsep were subtracted from the counts bound to pepCsep. For the determination of the IC$_{50}$-values of the test compounds the assay was repeated at least two times in the presence of the various concentrations of the compounds and the concentration at 50% inhibition was determined using a mathematical function fitting to the concentration/inhibition curves.

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† This protocol is a minor modification of the assay described in reference 11.

7.5 References and Notes

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(10)   The yields of compounds containing building block **D15** were found to be low in some cases. LC-MS analysis showed that a major side-product was formed during the deprotection step with 20% piperidine in DMF. The *m/z* ratio of the side-product indicates that the monobenzyl protected phosphate group has been displaced by a piperidine moiety. Although no attempts were undertaken to optimise the yield, using the non-nucleophilic base DBU may solve this problem. See (a) J. D. Wade, J. Bedford, R. C. Sheppard, G. W. Tregear *Pept. Res.* **1991**, *4*, 194. (b) S. A. Kates, N. A. Solé, M. Beyermann, G. Barany, F. Albericio *Pept. Res.* **1996**, *9,* 106.

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Chapter 8
Summary and Future Prospects

Since the observation that oncogenic small G-proteins belonging to the Ras family play a major role in tumorogenesis, the blocking of the functioning of these oncoproteins has emerged as one of the main strategies in the field of anti-cancer research. In this context, prevention of maturation of Ras proteins by inhibition of the required post-translational isoprenylation mediated by the transferases PFT (protein:farnesyl transferase) and PGGT-1 (protein:geranylgeranyl transferase-1) is recognised as a promising approach to prevent oncogenic Ras functioning. Since the early 90's numerous PFT and PGGT-1 inhibitors have been reported and tested on their capacity to function as anti-cancer agents. Besides this, some inhibitors of PFT and/or PGGT-1 show potency against a range of pathological disorders (multiple sclerosis, osteoporosis, atherosclerosis and restenosis) and parasitic infections such as malaria. The major part of the research described in this Thesis was devoted to the development of PFT and PGGT-1 inhibitors, the design of which was based on the individual substrates of PFT and PGGT-1 (Chapters 2 – 5 and 7). Chapter 6 presents the development of a novel methodology to label and isolate farnesylated proteins from cells as a potential proteomic analytical tool.[1]

In **Chapter 1** an introduction is presented on the isoprene metabolism, a pathway which accounts for the biosynthesis of numerous biologically important compounds. After an overview of some therapies which are based on the inhibition of some key enzymes of the isoprene metabolism, attention is focused on the isoprenyl transferases PFT and PGGT-1. The mode of action, the molecular basis for substrate specificity and the structural design behind the development of PFT and PGGT-1 inhibitors is described. As four PFT inhibitors of PFT are and were the subject of ongoing clinical trials, these inhibitors are highlighted separately.
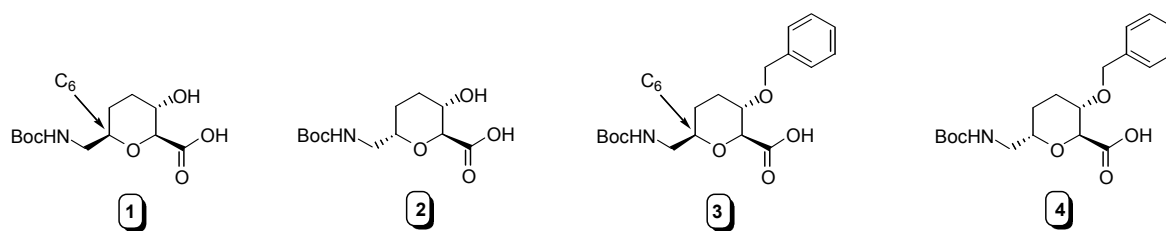
Figure 8.1 Sugar amino acid building blocks **1 – 4**.

Chapter 2 presents a novel route toward two dideoxy sugar amino acids (SAAs **1** and **2**, Figure 8.1) which were used in the construction of a set of Ca_1a_2L based peptidomimetics as potential PGGT-1 inhibitors. In the design the characteristic cysteine and leucine residues were preserved for recognition by PGGT-1 and the a_1a_2 dipeptide part was replaced by an isosteric SAA. To probe the influence of the nature of the cysteine and leucine residues on inhibition potency against PGGT-1, both the D and L

enantiomers were used. Two Ca_1a_2L analogs, which structurally differ in the configuration of both the C_6 position of the central SAA residue (Figure 8.1) and the C_α of the cysteine residue were found to inhibit PGGT-1 with equal potency (IC_{50}= 68 µM). As these results indicated that the developed SAA building blocks showed potential to function as dipeptide isosteres in the development of PGGT-1 inhibitors, this strategy was explored further.

Chapter 3 describes the synthesis of a set of Ca_1a_2L and Ca_1a_2M based analogs in which the a_1a_2 dipeptide was replaced by hydrophobic sugar amino acids. As hydrophobic interactions govern the binding of the central dipeptide part a_1a_2 in the active site of the isoprenyl transferases, it was envisioned that masking of the hydroxyl group of the SAAs presented in **Chapter 2** with a benzyl group (**3** and **4**, Figure 8.1), could increase inhibitory potency and selectivity. In order to increase the stability against proteolytic degradation, Ca_1a_2L and Ca_1a_2M analogs in which the amide bond between the SAA and the *C*-terminal X-residue was replaced by an amine were constructed. It appeared that the C_6 stereochemistry of the SAA residue had a pronounced effect on both inhibitory potency and selectivity. Furthermore, incorporation of an amine bond between the dipeptide isostere and the X amino acid proved to be detrimental for the inhibition of both enzymes, in particular for PFT. From the Ca_1a_2M analogs a potent and selective PFT inhibitor (IC_{50}= 250±20 nM) was obtained. The corresponding methylester of this PFT inhibitor exhibited *in vivo* inhibitory potency against PFT. From the Ca_1a_2L analogs, a modest dual inhibitor of PFT and PGGT-1 was obtained (IC_{50}= 14 µM). The a_1a_2 binding cavity in PGGT-1 is smaller and less aromatic,[2] and therefore it is envisioned that introduction of a smaller and non-aromatic group (such as Me or Et) at C-3 may be a potential improvement of the presented SAA based Ca_1a_2L analogs (**7**, Scheme 8.1).
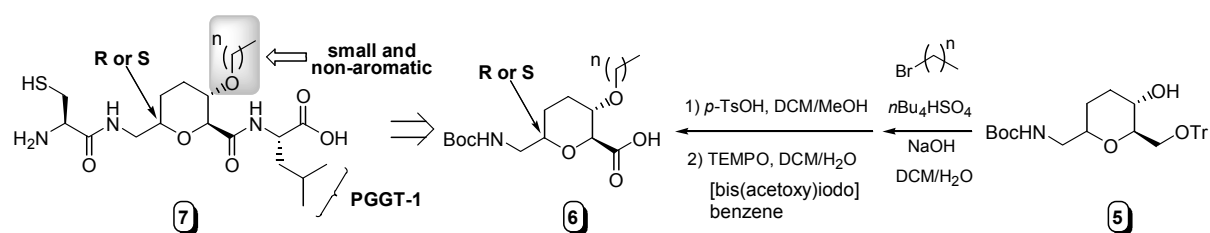
Scheme 8.1 Alternative modification of central SAA in Ca_1a_2L analogs presented in Chapter 2.

Reduction of the amide bond between the dipeptide isostere and the cysteine residue (Scheme 8.2) is another promising modification aimed at enhancing potency and selectivity. It is known from crystallographic studies that in the parent Ca_1a_2X tetrapeptide sequence this amide bond is not involved in any interaction with the enzyme. Two elegant approaches toward construction of the amine can be envisioned. First, by reductive amination of amine **8** with known amino cysteine aldehyde **9**[3] (Scheme 8.2). Second, by the Fukuyama/Mitsunobu glycosylation of ortho-nitrobenzenesulfonamides **11** and readily available cysteinol **12**.[4]
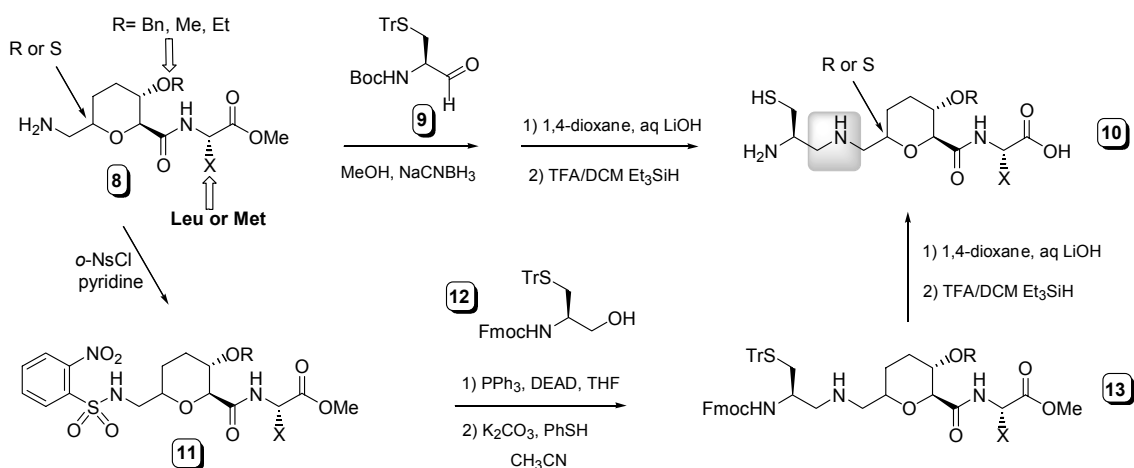
Scheme 8.2 Reduction of amide bond between cysteine and SAA residue in Ca_1a_2X analogs of Chapter 3.

Chapter 4 describes the design, synthesis and evaluation of lipophilic Ca_1a_2L analogs as potential bisubstrate inhibitors of PGGT-1. On the basis of the finding that isoprenyl transferases exhibit an unusual high affinity for their two substrates and especially for the turnover product, Ca_1a_2L analogues presented in **Chapter 2** were connected either directly or *via* a linker (C_2: glycine or C_4: 4-aminobutyric acid) to the fatty acids lauric (C_{12}) or palmitic acid (C_{16}). Biological evaluation of the lipophilic Ca_1a_2L analogues against PGGT-1 showed that the introduced lipophilicity had a positive effect on the inhibition potency. The gain in potency was found to depend on the type of SAA residue incorporated in the Ca_1a_2L part. Similarly to the PGGT-1 inhibitors of Chapters 2 and 3 it is not excluded that the inhibitory action of these lipophilic Ca_1a_2L analogs can be improved by executing the set of modifications depicted in Figure 8.2.
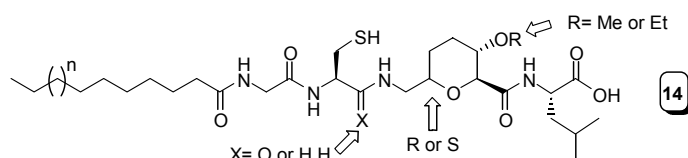
Figure 8.2 General structure of potential bisubstrate inhibitors of PGGT-1.

Chapter 5 describes the evaluation of tetrazole as potential carboxyl bioisostere in the design of Ca_1a_2M based PFT inhibitors. The potent PFT inhibitor arising from **Chapter 3** and the parent PFT Ca_1a_2X sequence CVIM were used as lead compounds. A properly protected tetrazole analog of methionine was synthesised and used for the construction of the target compounds. Biological evaluation revealed that tetrazole is a suitable carboxylic acid isostere in SAA based PFT inhibitors. Unfortunately, the tetrazole analog of leucine could not be synthesised following a similar synthetic protocol. An alternative synthesis toward the targeted leucine tetrazole is shown in Scheme 8.3. With iodine and aqueous ammonia the properly protected leucine **15** is transformed into the corresponding nitrile

(**16**).[5] Subsequent treatment with zinc bromide and sodium azide under reflux conditions should subsequently furnish tetrazole **17**.[6] Although unprotected tetrazoles have been used under peptide coupling conditions,[7] subsequent protection of **17** would be advantageous during the following synthetic steps. Alternatively, the introduction of the tetrazole could also take place at a later stage of the synthesis (**16** → **18** → **19**).
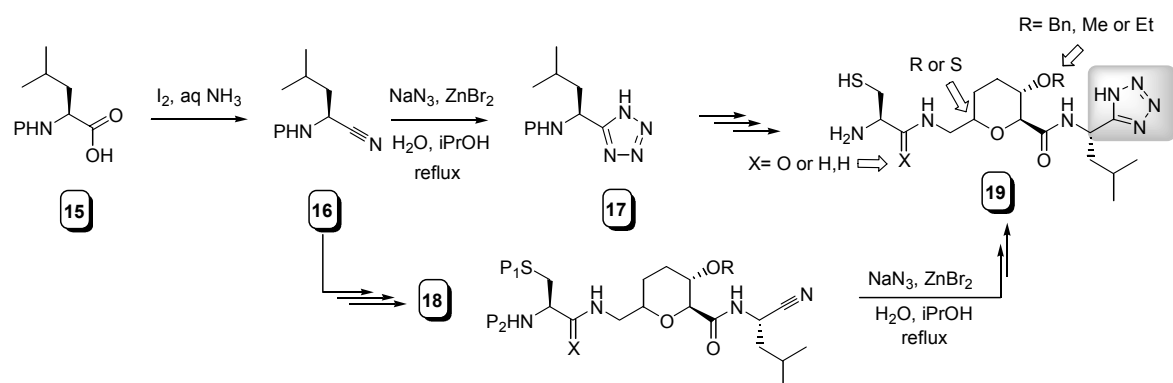
Scheme 8.3 Alternative synthetic routes toward the synthesis of Ca_1a_2L of type **19**.

A promising substitution for the thiol group[8] in the SAA based Ca_1a_2X analogs, presented in this Thesis, is the imidazole group. The zinc-chelating properties of the imidazole group have been successfully exploited in the design of inhibitors for several Zn^{2+}-containing enzymes,[9] including PFT and PGGT-1 (see **Chapter 1**). Combining all proposed structural prospects of this Thesis offers an interesting opportunity to construct SAA based Ca_1a_2X analogs with a high peptidomimetic character (**23a**, Scheme 8.4). Note that the amine functionality does not present an essential feature of the Ca_1a_2X box and may be deleted (**23b**).[10]
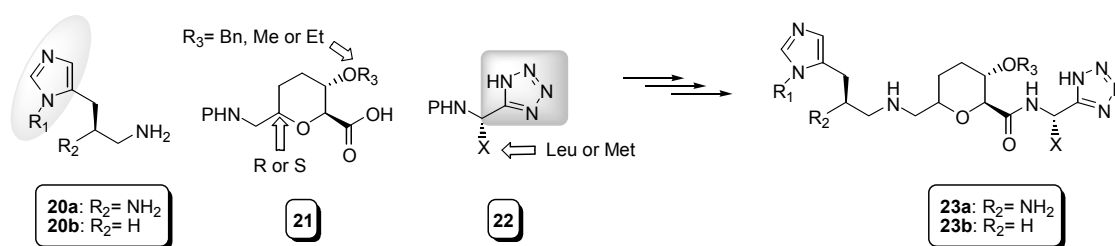
Scheme 8.4 Imidazole as replacement for the thiol function in SAA based Ca_1a_2X analogs (**22**).

Chapter 6 describes a novel labeling approach which enables the identification of farnesylated proteins in living cells. After addition of azidofarnesyl pyrophosphate to living cells, PFT recognises this farnesyl pyrophosphate analog as substrate and consequently modifies proteins susceptible to farnesylation. After lysis of the cells a biotinylated phosphine reagent (**25**, Scheme 8.5) is added, resulting in selective biotinylation through a Bertozzi-Staudinger reaction between the azide and phosphine.

Next, by using SDS-PAGE analysis and avidin-horseradish peroxidase (HRP) chemiluminescence, the isolated proteins could be visualised. When selective inhibitors of PFT were added to the cell culture, a decrease in labeling efficacy was observed. Analogously, labeling and isolation of PGGT-1 transformed proteins can be attained using azidogeranylgeranyl pyrophosphate (**25**, Scheme 8.5) as potential alternative substrate for PGGT-1. Ultimately, by employing current available proteomic tools,[11] the here presented labeling methodology allows the analysis of PFT and/or PGGT-1 substrates.
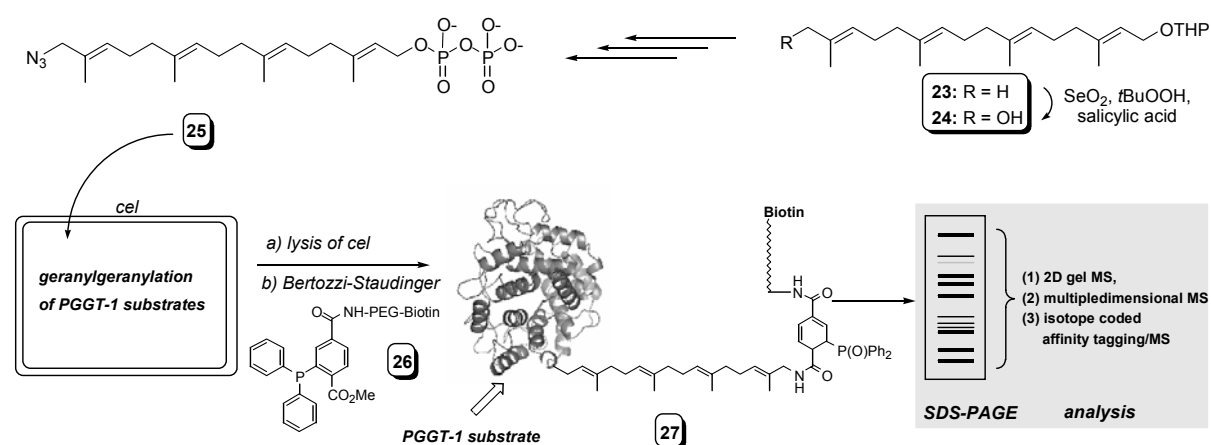
Scheme 8.5 Azidogeranylgeranyl pyrophosphate for the *in vivo* labeling of geranylgeranylated proteins.

Chapter 7 describes a combinatorial approach toward a library of ambiphilic peptide-based compounds as potential inhibitors of PGGT-1. To circumvent the synthesis of large numbers of compounds, an *in silico* iterative optimization procedure was used. This comprises the arbitrarily rearrangement of individual building blocks of a set of compounds. The newly formed compounds were then synthesised and evaluated for their inhibitory activity against PGGT-1. After each synthesis and evaluation cycle, the 16 most potent inhibitors were selected for the next optimization procedure. A total of 7 generations of compounds were constructed and a progressive improvement of the average inhibitory activity was observed for 5 generations. IC_{50}-values of the most active compounds were found to reside in the low micromolar range. The obtained lead compounds can be further developed using a classical structure based approach or the *in silico* iterative optimization procedure can be continued. This option requires an enhancement of the number of structurally divers building blocks. Additionally, diversity may also be enhanced by increasing the number of altered building blocks from one to two compounds per cycle. This means that per cycle two building blocks are replaced by a different building block.

Finally, molecular modeling techniques represent, besides structure-activity relationship studies and X-ray crystallography, an important alternative tool for the understanding and improvement of the peptidomimetic design of isoprenyl transferase

inhibitors presented in this Thesis.[12] As the isoprenyl transferases bind two different substrates in a relative big active site, kinetic analysis can be used in order to determine whether a compound is competitive for the Ca_1a_2X part, the isoprenyl part or both (bisubstrate inhibitor). In the case of a Ca_1a_2X analog, it is important to take into account that the isoprenyl substrate binds prior to the Ca_1a_2X moiety and thus should be included as a part of the active site. In general, two strategies can be envisioned during the modeling process (Figure 8.3). During the first and most widely used approach, the thiol group is "fixed" to the active site by its interaction with the Zn^{2+} ion while the remainder of the molecule is allowed to find an optimal binding mode. In the second approach, only the active site residues are fixed and the whole peptidomimetic inhibitor is allowed to find an optimal binding mode. In the case of a prenyl analog the amount of spacial freedom is larger and constraintment can be introduced at the pyrophosphate part.
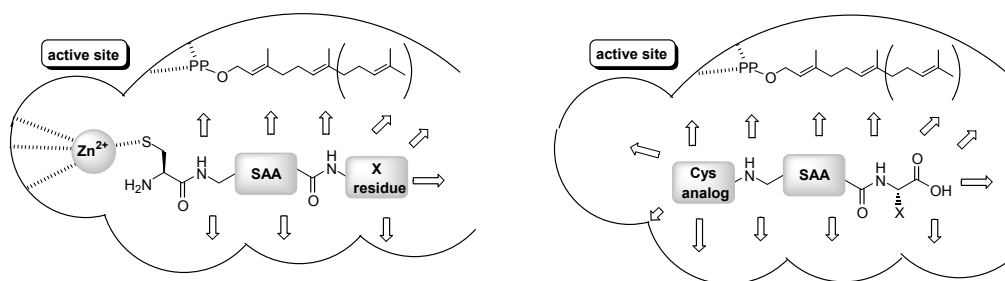

Figure 8.3 Approaches toward molecular modeling of SAA based Ca_1a_2X analogs.

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Samenvatting

"Design, Synthesis and Biological Evaluation of Peptidomimetic Prenyl Transferase Inhibitors"

In elke cel vervullen signaaltransductie-eiwitten een zeer belangrijke rol. Een bekende familie van signaaltransductie-eiwitten zijn G-eiwitten. De term G-eiwit is afgeleid van het feit dat deze eiwitten GTP (guanosine trifosfaat) binden en hydrolyseren. Deze membraangebonden eiwitten kunnen beschouwd worden als schakelaars en zijn betrokken bij het reguleren van talrijke belangrijke processen zoals celgroei, celdifferentiatie en transport. Begin jaren '80 werd voor het eerst in ratten ontdekt dat bepaalde gemuteerde G-eiwitten betrokken zijn bij het ontwikkelen van kanker. Deze gemuteerde G-eiwitten bleken niet meer in staat om stop gezet te worden. Op grond van dit gegeven werden verscheidene onderzoeken gestart om deze gemuteerde G-eiwitten te remmen en op deze wijze anti-kanker middelen te ontwikkelen. In dit kader werd ontdekt dat G-eiwitten als precursor eiwitten worden gesynthetiseerd in het cytosol en door post-translationele veranderingen actief worden. De eerste essentiële stap in deze reeks van veranderingen is isoprenylering, een modificatie die nodig is voor aanhechting van de G-eiwitten aan (de binnenzijde van) membranen waar zij hun biologische rol kunnen uitoefenen. Bij isoprenylering brengen de enzymen protein:farnesyl transferase (PFT) of protein:geranylgeranyl transferase-1 (PGGT-1) een covalente binding tot stand tussen een farnesyl (F) of geranylgeranyl (GG) koolstofketen en de *C*-terminus van het nog onvolwassen G-eiwit. De primaire structuur van de laatste vier *C*-terminale aminozuren bepaalt of een G-eiwit wordt gefarnesyleerd of gegeranylgeranyleerd. Deze keten van vier aminozuren is bekend als de Ca_1a_2X-box en is voldoende om door PFT of PGGT-1 als substraat te worden herkend. In de Ca_1a_2X-box staat C voor de cysteine waaraan de isopreen wordt verbonden, a_1a_2 is een dipeptide keten die over het algemeen hydrofoob van karakter is. X staat voor het laatste *C*-terminale aminozuur en de stelregel is dat wanneer dit een methionine of serine is wordt het G-eiwit gefarnesyleerd. Wanneer X een leucine is wordt het G-eiwit gegeranylgeranyleerd. Farnesyl pyrofosfaat (FPF) en geranylgeranyl pyrofosfaat (GGPF), de substraten die de enzymen PFT en PGGT-1 in deze reactie gebruiken, zijn op hun beurt producten van het isopreen metabolisme in de cel. Een veel onderzochte benadering om gemuteerde G-eiwitten te blokkeren is gericht op het voorkomen van de localizatie van deze eiwitten in het celmembraan door het remmen van PFT en/of PGGT-1. Het belang van het ontwikkelen van PFT en PGGT-1 remmers is de laatste jaren toegenomen omdat er duidelijke aanwijzingen zijn dat naast anti-kanker therapieën, andere therapeutische toepassingen in het vooruitzicht liggen. Hierbij gaat het om bestrijding van botontkalking (osteoporose), vernauwingen in het vasculaire systeem (atherosclerose/restenose), malaria en multiple sclerose.

De algemene inleiding (**Hoofdstuk 1**) begint met een korte uiteenzetting van het isopreen metabolisme en zijn produkten. Verschillende therapieën, die zijn gebaseerd op het ingrijpen in enzymatische processen aan het begin van het isopreen metabolisme worden opgesomd. Hierbij worden de enzymen PFT en PGGT-1 in detail besproken en wordt een selectie gegeven van relevante PFT en PGGT-1 remmers, waaronder vier verbindingen die momenteel klinisch getest worden. Aan de hand van de gekozen remmers worden de verschillende strategieën, die gebruikt worden om PFT en PGGT-1 remmers te ontwikkelen, uiteengezet. Een van deze strategieën maakt gebruik van het gegeven dat tetrapeptiden gebaseerd op de Ca_1a_2X-box keten PFT en/of PGGT-1 kunnen remmen. Korte peptidefragmenten zijn echter niet geschikt voor therapeutische doeleinden, omdat zij in het lichaam meestal snel enzymatisch worden afgebroken en bovendien in het algemeen slecht door cellen worden opgenomen. De nadelen van natuurlijk voorkomende peptiden kunnen worden ondervangen door verbindingen die de structuur van peptiden nabootsen, de zogenaamde peptidomimetica.

In **Hoofdstuk 2** wordt beschreven dat (dideoxy) suikeraminozuren (SAAs= "sugar amino acids") als dipeptide isosteer kunnen dienen in de ontwikkeling van potentiële remmers van PGGT-1. Suikeraminozuren zijn monosaccharide derivaten die ten minste één amine- en één carbonzuurgroep bevatten. Suikeraminozuren verhogen na inbouw in een peptide de enzymatische stabiliteit en kunnen een bepaalde conformatie induceren. Twee verschillende SAA bouwstenen, met de amine en de carboxyl groep aan de pyranose suikerring in een cis of trans relatie, werden gesynthetiseerd en ingebouwd in de Ca_1a_2X-box ter vervanging van het centrale a_1a_2 dipeptide gedeelte. Naast de SAAs werden zowel de natuurlijke L-configuratie en de onnatuurlijke D-configuratie van de aminozuren cysteine (C) en leucine (L) in de Ca_1a_2L analoga ingebouwd. Twee van de 8 gesynthetiseerde verbindingen bleken actieve remmers van het enzym PGGT-1 te zijn.

De synthese en inbouw van hydrofobe SAAs in Ca_1a_2X analoga als potentiële PFT en PGGT-1 remmers wordt in **Hoofdstuk 3** besproken. Deze SAAs bevatten een extra benzylgroep in vergelijking met de SAAs van **Hoofdstuk 2**. Tevens werd de amide binding tussen het C aminozuur (cysteine) en het suikeraminozuur vervangen door een amine binding. Uit de biologische evaluatie bleek dat deze amine verbindingen veel minder actief waren en dat de amide band tussen de cysteine en SAA een belangrijke rol speelt. De Ca_1a_2X analoga met de gebenzyleerde SAAs bleken, na biologische evaluatie tegen PFT en PGGT-1, een goede remmende werking te vertonen. Opvallend was dat de activiteit

en selectiviteit (voor PFT of PGGT-1) van de Ca_1a_2X analoga sterk werd beïnvloed door de cis of trans relatie van de ingebouwde SAA bouwsteen. Ten slotte werd een sterke en selectieve remmer van PFT geselecteerd voor biologische evaluatie in een levende celcultuur. Daarvoor was het noodzakelijk om de eindstandige zuurgroep van het M aminozuur (methionine) om te zetten in een methylester. Door deze hydrofobe verandering passeert de remmer gemakkelijker het celmembraan om vervolgens in de cel door enzymen te worden gehydrolyseerd tot het oorspronkelijke zuur. Uit de testresultaten bleek de remmer ook in levende cellen als een potente PFT remmer te fungeren.

In **Hoofdstuk 4** wordt de bereiding van lipofiele Ca_1a_2L analoga als potentiële bisubstraat remmers van PGGT-1 gepresenteerd. PGGT-1 en PFT hebben een zeer grote affiniteit voor hun geïsoprenyleerde produkt. Door de remmers uit de **Hoofdstukken 2** en **3** aan het amino-uiteinde te voorzien van een alkylstaart worden lipofiele Ca_1a_2L analoga verkregen, die zowel met de peptide als isopreen pocket van het enzym interactie kunnen hebben. De lipofiele Ca_1a_2L analoga bleken een positieve invloed op de remmende werking te hebben. De toename in activiteit bleek wel afhankelijk van het type SAA. Er werd echter niet onomstotelijk bewezen dat de verbindingen echte bisubstraat remmers waren.

In **Hoofdstuk 5** wordt de vraag beantwoord of tetrazool gebruikt kan worden als vervanging van de carboxyl functie in PFT remmers. Eerst werd de tetrazool derivaat van methionine gesynthetiseerd en vervolgens ingebouwd in zowel de meest actieve PFT remmer uit **Hoofdstuk 3** als het referentie peptide CVIM. Uit de testresultaten bleek dat de activiteit van deze remmers slechts marginaal was afgenomen, waarmee is aangetoond dat tetrazool een geschikte vervanger is van de carboxyl functie. Een overeenkomstige procedure voor PGGT-1 remmers was niet succesvol, omdat de synthese van de tetrazool derivaat van leucine niet mogelijk bleek volgens de methode gepresenteerd in dit hoofdstuk.

In **Hoofdstuk 6** wordt aandacht besteed aan een nieuwe methode om gefarnesyleerde eiwitten in de levende cel te detecteren. Het aantonen van elke familie van eiwitten in levende cellen is van groot belang om biologische processen onder normale en pathologische omstandigheden te begrijpen alsmede om het effect van geneesmiddelen op deze processen te onderzoeken en kwantificeren. De gebruikte methode omvatte de synthese van een farnesyl pyrofosfaat derivaat dat eindstandig was voorzien van de

biologisch inerte azide groep. Het derivaat bleek niet alleen de celwand te passeren maar tevens te worden ingebouwd in gefarnesyleerde eiwitten. Dit kon na lysering van de cel worden bewezen omdat de azide functie met behulp van een selectieve reactie kan worden voorzien van een biotine-label. Na scheiding van de gemarkeerde eiwitten met behulp van SDS polyacrylamide gel electroforese worden de eiwitten op de gel zichtbaar gemaakt door middel van een "avidine-horseradish peroxidase" behandeling. Ook het effect van in dit proefschrift beschreven remmers op het farnesyleren van eiwitten in levende cellen kon zichtbaar worden gemaakt.

Ten slotte beschrijft **Hoofdstuk 7** een combinatoriële benadering om PGGT-1 remmers te ontwikkelen. De bindingsplaats van geranylgeranyl pyrofosfaat in het enzym werd als doel gekozen zodat de algemene structuur van de potentiële remmers lipofiel met een polair uiteinde moest zijn. De verbindingen werden met behulp van peptide chemie gemaakt via een vaste drager procedure. Om een grote diversiteit te krijgen werden vier verschillende groepen van commercieel verkrijgbare bouwstenen geselecteerd. Om het aantal te synthetiseren en te testen verbindingen te beperken werd tevens een rekenmethode gebruikt waarmee de doelverbindingen werden geselecteerd. Na vijf cycli van zestien verbindingen gesynthetiseerd en getest te hebben, bleek de gemiddelde activiteit van de remmers niet meer toe te nemen. In totaal werden zeven cycli uitgevoerd en bleken de beste remmers een laag micromolaire IC_{50} waarde te hebben voor PGGT-1 remming.

List of Publications

Synthesis and biological evaluation of protein:geranylgeranyl transferase-1 inhibitors based on the Ca_1a_2X box: incorporation of sugar amino acids as dipeptide isosters

Farid El Oualid, Leon Bruining, Ingrid M. Leroy, Louis H. Cohen, Jacques H. van Boom, Gijs A. van der Marel, Herman S. Overkleeft, Mark Overhand

Helv. Chim. Acta **2002**, *85*, 3455 − 3472

Synthesis and elaboration of functionalised carbohydrate-derived spiroketals

Peter A. V. van Hooft, Farid El Oualid, Herman S. Overkleeft, Gijsbert A. van der Marel, Jacques H. van Boom, Michiel A. Leeuwenburgh

Org. Biomol. Chem. **2004**, *2*, 1395 − 1403

Alkylated sugar amino acids: A new entry toward highly functionalized dipeptide isosters

Michael Raunkjær, Farid El Oualid, Gijs A. van der Marel, Herman S. Overkleeft, Mark Overhand

Org. Lett. **2004**, *6*, 3167 − 3170

Design, synthesis, and evaluation of sugar amino acid based inhibitors of protein prenyl transferases PFT and PGGT-1

Farid El Oualid, Brigitte E. A. Burm, Ingrid M. Leroy, Louis H. Cohen, Jacques H. van Boom, Hans van den Elst, Herman S. Overkleeft, Gijs A. van der Marel, Mark Overhand

J. Med. Chem. **2004**, *47*, 3920 − 3923

A practical synthesis of Gramicidin S and sugar amino acid containing analogues

Gijsbert M. Grotenbreg, Martijn Kronemeijer, Mattie S. M. Timmer, Farid El Oualid, Renate M. van Well, Martijn Verdoes, Emile Spalburg, Peter A. V. van Hooft, Albert J. de Neeling, Daan Noort, Jacques H. van Boom, Gijsbert A. van der Marel, Herman S. Overkleeft, Mark Overhand

J. Org. Chem. **2004**, *69*, 7851 − 7859

Synthesis and biological evaluation of lipophilic Ca$_1$a$_2$L analogues as potential bisubstrate inhibitors of protein:geranylgeranyl transferase-1

Farid El Oualid, Jayand Baktawar, Ingrid M. Leroy, Hans van den Elst, Louis H. Cohen, Gijs A. van der Marel, Herman S. Overkleeft, Mark Overhand

Bioorg. Med. Chem. **2005**, *13*, 1463 – 1475

A combinatorial approach toward the generation of ambiphilic peptide-based inhibitors of protein:geranylgeranyl transferase-1.

Farid El Oualid, Hans van den Elst, Ingrid M. Leroy, Elsbeth Pieterman, Louis H. Cohen, Brigitte E. A. Burm, Herman S. Overkleeft, Gijs A. van der Marel, Mark Overhand

Manuscript submitted

Curriculum Vitae

Farid El Oualid was born in Beni Chikar (Morocco) on the 28th of November 1976. After the completion of his secondary education in June 1995 at the Groen van Prinsterer Lyceum in Vlaardingen (The Netherlands), he started his academic studies in chemistry at Leiden University in September 1995. From March 1998 to June 1999 he conducted undergraduate research in the "Bio-organic Synthesis" group of the late Prof. dr. Jacques H. van Boom under the supervision of dr. Gijs A. van der Marel and dr. Peter A. V. van Hooft. His undergraduate thesis describes the stereoselective synthesis of carbohydrate derived spiroketals and their application in synthetic organic chemistry. Parts of the work described in the undergraduate thesis were presented on a poster during the PAC Symposium in Amsterdam (1999).

From September 1999 to March 2000, he was a member of the *Chemical and Analytical Technologies Department* of GlaxoWellcome (currently GlaxoSmithkline) in Stevenage (United Kingdom). Under the supervision of Dr. David J. Davies and Ian M. Campbell he was involved in a research project entailing azomethine-ylide chemistry and the development of a traceless solid phase linker.

After returning to Leiden, he obtained his doctorandus (Drs., Master of Science) degree in June 2000. Subsequently, he was affiliated as a Ph.D. student with Leiden University during the period of September 2000 to December 2004. The work described in this Thesis was conducted under the supervision of Prof. dr. Herman S. Overkleeft, Prof. dr. Jacques H. van Boom, dr. Gijs A. van der Marel and dr. Mark Overhand, in a close co-operation with dr. Louis H. Cohen, ing. Ingrid M. Leroy and ing. Elsbeth Pieterman of TNO Leiden (Gaubius Laboratory). Financial aid was from *Netherlands Technology Foundation* (STW) and *Netherlands Organization for Scientific Research* (NWO). He partook in the 3rd International and 28th European Peptide Symposium in Prague, Czech Republic (September 2004). Parts of the work described in this Thesis were presented at annual meetings of the Organic Chemistry Section (NWO) in Lunteren (2001, 2002, poster presentation) and the HRSMC meeting in Amsterdam (University of Amsterdam, March 2004, oral presentation).

Nawoord

Op deze plek wil ik een aantal mensen noemen die direct of indirect betrokken waren bij het tot een goed eind brengen van mijn promotie en de totstandkoming van het daarbij behorende proefschrift. De collega's van de BIOSYN vakgroep verdienen een bijzondere vermelding voor hun bijdrage aan de prettige werksfeer en het onderzoek in de afgelopen jaren en met name wil ik hierbij noemen: Peter van Hooft (voor zijn uitstekende begeleiding), Remy Litjens, Richard van den Berg, Dima Filippov, Leendert van den Bos, Michiel Leeuwenburgh, Peter de Visser, Kimberly Bonger, Lene Petersen, Cindy Kaltner, Rian van den Nieuwendijk, Karen Sliedregt-Bol, Jeroen Codée, Clara Comuzzi, Gijs Grotenbreg, Martijn de Koning, Mattie Timmer, Silvia Cavalli, Erwin Tuin, Bas Lastdrager en Tom Wennekes.

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