# Synthesis and Membrane Binding Properties of a Lipopeptide Fragment from Influenza Virus A Hemagglutinin

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Abstract: Hemagglutinin from influenza virus A is a S-palmitoylated lipoglycoprotein in which the lipid groups are thought to influence the interaction between cell membrane and capsid during budding of viral offspring as well as fusion processes of the viral membrane with the endosome after entry of the viral particle into the cell. The paper describes the development of a method for the synthesis of characteristic lipidated hemagglutinin derived peptides which additionally carry the fluorescent 7-nitrobenz-2oxa-1,3-diazole (NBD) group. To achieve this goal the enzyme-sensitive para-phenylacetoxybenzyloxycarbonyl (PAOB) ester was developed. It is cleaved from the peptides and lipidated peptides under very mild conditions and with complete selectivity by treatment with the enzyme penicillin G acylase; this results in the formation of a phenolate. This intermediate spontaneously undergoes fragmentation thereby releasing the desired carboxylates. The combined use of this enzymelabile fragmenting ester with the acidlabile Boc group, the Pd<sup>0</sup>-sensitive allyl ester and the corresponding Aloc urethane gave access to a mono-S-palmitoylated and a doubly S-palmitoylated NBD-labelled hemagglutinin peptide. The binding of these lipopeptides to model membranes was analyzed in a biophysical setup monitoring the transfer of fluorescent-labelled lipopeptide from vesicles containing the non-ex-

**Keywords:** enzyme catalysis • hemagglutinin • lipidated peptides • peptides • protecting groups changeable fluorescence quencher Rho-DHPE to quencher-free vesicles. The experiments demonstrate that one lipid group is not sufficient for quasiirreversible membrane insertion of lipidated peptides. This is, however, achieved by introduction of the bis-palmitoyl anchor. The intervesicle transfer always implies release of peptides localized at the outer face of the vesicles into solution followed by diffusion to and insertion into acceptor vesicles. For peptides bound at the inner face of the vesicle membrane, however, an additional flip-flop diffusion to the outer face has to occur beforehand. The kinetics of these processes were estimated by fast chemical quench of the outside fluorophores by sodium dithionite.

## Introduction

The infection of healthy cells by viruses is a complex multistep process which is decisively influenced and determined by posttranslationally modified proteins embedded in the viral

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lipid-bilayer. For instance hemagglutinin from influenza virus A is glycosylated in the extracellular domain (Figure 1)<sup>[1, 2]</sup> and the glycoprotein part is responsible for initiation of viral infection through selective binding to sialic acid receptors on the surface of the host cell. In addition, the protein is S-palmitoylated next to the transmembrane region (Figure 1);<sup>[3, 4]</sup> the lipid residues are required for the interaction between the cell membrane and the free capsid during budding of viral offspring.<sup>[5, 6]</sup> They are also thought to mediate protein-protein and protein-lipid interactions in the viruses<sup>[7]</sup> and may play an important role in fusion processes of the viral membrane with the endosome after entry of the viral particle into the cell.<sup>[8, 9]</sup> However, this proposal is controversial, since other investigations indicated that the lipidated cytoplasmic tail of the complex viral lipoglycoproteins is not essential for its membrane fusion activity.[10]

Also, the orchestration of fatty acid attachment to the viral glycoproteins is not well-known. Thus, until today a consensus sequence for acylation by a putative palmitoyl transferase



Figure 1. Schematic drawing of glycosylated and lipidated influenza virus A hemagglutinin and doubly palmitoylated target peptide **1**.

from several palmitoylated viral proteins could not be identified.<sup>[11]</sup> One of the few related facts known is that fatty acylation obviously takes place after exit from the endoplasmatic reticulum.<sup>[12]</sup>

For the study of these and related processes in molecular detail, lipidated peptides which represent the characteristic linkage region between the protein backbone and the lipid groups and which additionally carry a marker by which they can be traced in biological systems, that is a fluorescent group or a biotin unit, may be employed as efficient molecular probes.<sup>[13]</sup> However, their synthesis is complicated by the pronounced base lability of the palmitic acid thioesters which hydrolyse spontaneously at pH >7 or undergo base-mediated  $\beta$ -elimination reactions (Scheme 1). For the synthesis of sensitive lipidated peptides a combination of classical acidlabile, noble-metal sensitive, and enzymatically cleavable protecting groups may provide efficient solutions. These three types of protecting functions can be combined in such a way that they are orthogonally stable.<sup>[12, 14]</sup> However, an enzymelabile carboxy protecting group, which meets these demands and is also compatible with the sensitivity of the palmitic acid thioester, is lacking. In this paper we report on the development of the p-phenylacetoxybenzyl (PAOB) ester, a new enzyme-labile carboxy protecting group, and its application in the construction of a fluorescent-labelled lipopeptide 1 from influenza virus A hemagglutinin.<sup>[15]</sup>



Scheme 1. Lability of palmitoylated peptides in the presence of bases and nucleophiles. The cysteines can be separated by one or more peptide residues  $(a_x)_n$ , n = 0, 1, 2, ...

### **Results and Discussion**

In developing an enzyme-labile ester group which can be removed selectively under conditions that are mild enough for lipopeptide synthesis we resorted to the principle of fragmentation after cleavage of a suitable enzyme-labile bond. To this end, N-protected dipeptide PAOB esters **4** were synthesized (Schemes 2 and 3). The PAOB group embodies a phenyl-



Scheme 2. Principle of PAOB-ester-deprotection by enzyme-initiated fragmentation.

acetate which is specifically recognized and cleaved by the enzyme penicillin G acylase. Upon enzymatic hydrolysis a phenolate **5** is formed that fragments spontaneously to a quinone methide **7** and the desired carboxylic acids **6**. The quinone methide is trapped by water to give *p*-hydroxybenzyl alcohol **8** or by added nucleophiles. This principle of deprotection by enzyme-induced fragmentation has been used for the development of enzyme-labile urethanes before.<sup>[16, 17]</sup> However, already in the case of the urethanes the success of the enzyme-mediated fragmentation at pH 6–8 was rather surprising. Non-enzymatic induction of the fragmentation reaction requires the use of strong bases like ammonia<sup>[18]</sup> whereas in the presence of the biocatalysts the unmasking occurs already at neutral pH. In addition, in the case of the urethane protecting groups the entire unmasking is

driven by the liberation of  $CO_2$ , thereby shifting the equilibrium to the product side. This driving force is no longer operative in the case of an ester blocking function. Thus, it was highly questionable whether this principle could be transferred successfully from an amino to a carboxy protecting group.

The synthesis of PAOB esters 4 commenced with the esterification of Boc-protected amino acids 9 with p-phenyl-acetoxybenzyl alcohol (10), subsequent acid-mediated cleavage of the Boc group and coupling of the resulting amino acid PAOB esters 12 with differently masked amino acids (Scheme 3). In order to achieve high yields in the coupling reactions it is necessary to preactivate the N-terminally protected amino acid first followed by addition of the PAOB amino acid esters.

Upon treatment of dipeptide PAOB esters **4** with penicillin G acylase at pH 7 and room temperature the desired selective C-terminal deprotection occurred smoothly. The enzyme saponifies the phenylacetate, even at pH 7, and without the additional driving force of  $CO_2$  liberation the intermediary formed phenolates **5** undergo fragmentation to the desired unmasked dipeptides **6a**-**e** which were obtained in high yield (Scheme 3). Quinone methide **7** is efficiently



Scheme 3. Synthesis and selective enzymatic deprotection of *N*-protected dipeptide PAOB esters **6**. i) DIC/DMAP(cat.),  $CH_2Cl_2$ , 64-90%; ii) HCl/ Et<sub>2</sub>O or TFA/CH<sub>2</sub>Cl<sub>2</sub>, 73-98%; iii) PG-AA<sup>1</sup>-OH **9**, EEDQ, NEt<sub>3</sub>,  $CH_2Cl_2$  or DIC/HOBt, NEt<sub>3</sub>,  $CH_2Cl_2$ , 71-87%; iv) phosphate buffer (pH 7), 10% methanol, penicillin G acylase; for **6e**: phosphate buffer (pH 7), 30% acetone, penicillin G acylase. DIC: *N*,*N*'-diisopropylcarbodiimide; DMAP: 4-(dimethylamino)-pyridine; EEDQ: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; HOBt: 1-hydroxybenzotriazole; TFA: trifluoroacetic acid.

trapped by water, addition of stronger nucleophiles is not necessary. Penicillin G acylase is a readily available (immobilized, native, and as cross-linkes enzyme crystals) and very stable enzyme with a broad substrate tolerance which does not attack peptide bonds or urethanes. In addition, in the PAOB group the site of the enzymes' attack is remote from the differing amino acids of the substrates. Consequently, the efficiency of the enzyme-induced deprotection is nearly independent of steric bulk and structure (i.e., D- or L-amino acid, acyclic or cyclic amino acid). This is apparent from the differing steric demand of the C-terminal amino acids incorporated into 6. Thus, 6a - d are obtained in similar yields and the cyclic amino acid proline is tolerated at the C-terminus without any problem. In this context it should be noted that from peptides terminating in a proline amide<sup>[19]</sup> or a proline heptyl ester<sup>[20]</sup> the C-terminal enzyme-labile protecting group could not be removed by means of amidaseor lipase-catalyzed hydrolysis. The low yield obtained for the C-terminal deprotection of 4e is not a result of the steric demand of the C-terminal phenylalanine (compare the results for 4d and e which both terminate in this amino acid). Rather it reflects the very limited solubility of the hydrophobic dipeptide 4e in the buffer/cosolvent mixtures employed. This observation was already indicative of the problems encountered later on in the enzymatic unmasking of unpolar lipidated peptides (see below).

On the other hand the Boc- and the Aloc groups can be removed selectively from dipeptide esters **4** without harm to the PAOB esters, that is the protecting groups are orthogonally stable to each other (data not shown, see also below).

The full capacity of the enzyme-labile PAOB ester became evident in the synthesis of fluorescent labelled derivatives of virus hemagglutinin peptide **1**. This synthesis is complicated by the base lability of the thioesters and the need to additionally protect and deprotect the basic arginine side chain functionality. Thus, a set of three orthogonally stable protecting groups is required, whereby the use of base-labile and hydrogenolytically removable blocking groups is not permitted. This problem was overcome by using the acidlabile Boc group for the N-terminus, the enzymatically removable PAOB ester for the C-terminus and the Pd<sup>0</sup>sensitive Aloc group for the arginine guanidino side chain function.

It was planned to generate the base-labile palmitic acid thioesters at the dipeptide stage employing peptides with C-terminal cysteine residues to avoid a possible  $S \rightarrow N$  acyl migration which may occur after N-terminal unmasking of S-acylated cysteine peptides.<sup>[21]</sup> Intermediary reversible protection of the thiol functions was avoided by synthesizing cystine peptides as intermediates. Thus, Boc-cystine 9g was esterified with alcohol 10 to yield ester 11e which was selectively deprotected at the N-terminus (Scheme 4). The resulting cystine ester 12e was then condensed with Bocisoleucine and Boc/Aloc-protected arginine to yield fully masked dipeptides 13a and 13b, respectively. Reduction of the disulfide bonds incorporated into these intermediates with dithiothreitol (DTT) and subsequent S-palmitoylation gave peptides 14a and 14b from which the Boc group could be removed selectively in high yields. Finally, the peptide chain was elongated by coupling with Boc-threonine and Bocmethionine to yield lipidated tripeptide esters 16a and 16b, respectively.



Scheme 4. Synthesis of palmitoylated tripeptide PAOB esters 16a and 16b. i) DIC/DMAP (cat.), CH2Cl2, 86%; ii) HCl/Et2O, 95%; iii) Boc-Ile-OH, EEDQ, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 78%; iv) Boc-Arg(Aloc)<sub>2</sub>-OH, DIC/HOBt, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 62 %; v) a) DTT, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, b) 2 Pal-Cl, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 14a: 83%, 14b: 81%, two steps; vi) TFA/CH2Cl2, quant.; vii) Boc-Thr-OH, EEDQ, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 74%; viii) Boc-Met-OH, DIC/HOBt, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 65 %: Pal: palmitovl.

It was planned to enzymatically deprotect 16a and 16b in order to obtain two key intermediates for the final assembly of the desired heptapeptide target. C-terminal deprotection of 16a and subsequent elongation of the peptide chain by an isoleucine ester would yield the (still N-masked) C-terminal tetrapeptide unit of the target peptide. Enzymatic unmasking of 16b would yield the N-terminal tripeptide unit of the final target compound. Enzyme-catalyzed deprotection of lipophilic tripeptide PAOB ester 16a with immobilized penicillin G acylase was attempted in various water/cosolvent (methanol, acetone, DMF, THF, dioxane) mixtures but due to the low solubility of the substrate in these solvents an appreciable cleavage of the ester could not be observed. Also the application of cross-linked enzyme crystals<sup>[22]</sup> or the use of organic solvents saturated with water did not improve this situation.

Finally, the use of dimethyl- $\beta$ -cyclodextrin as solubilizing agent was successful. This cyclic hexasaccharide most probably slips over the hydrophobic fatty acid group<sup>[16, 23]</sup> thereby rendering the substrates soluble. After addition of the  $\beta$ cyclodextrin and ultrasonication lipidated peptide 16a dissolved well in phosphate buffer (pH 7) and treatment of this solution with penicillin G acylase at 25 °C resulted in a smooth and completely selective removal of the C-terminal enzymelabile protecting function (Scheme 5). Similarly, from S-pal-



Scheme 5. Enzymatic deprotection of palmitoylated peptides. i) penicillin G acylase, dimethyl-β-cyclodextrin, 0.05 M phosphate buffer (pH 7), 25 °C, 16a: 81%, 16b: isolation not possible, 14b: 77%.

mitoylated dipeptide PAOB ester 14b the enzyme labile blocking group was cleaved without any undesired side reaction. The mildness of the reaction conditions and the substrate specificity of the biocatalyst guarantee that neither the thioester nor the bis-acylated guanidino group are attacked, and the selectively unmasked S-palmitoylated peptides were isolated in high yields.

Also, tripeptide 16b was readily soluble in phosphate buffer/dimethyl- $\beta$ -cyclodextrin and deprotection with penicillin G acylase proceeded smoothly and to completeness. However, separation of lipotripeptide 17b from the cyclodextrin could not be achieved. Thus, although the results detailed above clearly demonstrated that the conditions required for enzymatic removal of the PAOB ester group are fully compatible with the demands of lipopeptide synthesis, the strategy for the synthesis of hemagglutinin peptide **1** had to be modified.

To this end, S-palmitoylated tripeptide carboxylic acid 17a was condensed with isoleucine allyl ester and the Boc group was removed to deliver lipotetrapeptide 20 in high overall yield (Scheme 6). Compound 20 was blocked as allyl ester at the C-terminus to allow for simultaneous Pd<sup>0</sup>-catalyzed deprotection of both the arginine side chain function and the C-terminal carboxylic acid towards the end of the synthesis. C-terminally deprotected and S-palmitoylated dipeptide building block 18 and N-terminally unmasked lipotetrapeptide 19 were then condensed to give doublepalmitoylated lipohexapeptide 23. The N-terminal Boc group was removed from 23 and the fluorescent 7-nitrobenz-2-oxo-1,3-diazole (NBD) label was introduced by coupling with the NBD aminocaproyl amide of methionine (NBAca-Met) 26 to obtain a high degree of convergency in the synthesis. Finally, the three allyl-type blocking functions were cleaved by Pd<sup>0</sup>catalyzed allyl transfer to N,N'-dimethylbarbituric acid as the accepting C-nucleophile.<sup>[24]</sup> Similarly, introduction of the NBD label followed by selective removal of the allyl ester yielded lipopeptide 22. The desired fluorescent and palmitoylated influenza virus hemagglutinin lipopeptides 22 and 28 were obtained in high overall yield.



Scheme 6. Final steps of the synthesis of fluorescent labeled lipopeptides **22** and **28**. i) H-Ile-OAll • HOTos, EEDQ, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 71%; ii) TFA/CH<sub>2</sub>Cl<sub>2</sub>, quant.; iii) NBDAca-OH, EDC/HOBt, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 86%; iv) [Pd(PPh<sub>3</sub>)<sub>4</sub>], DMB, THF, 86%; v) EEDQ, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 46%; vi) TFA/CH<sub>2</sub>Cl<sub>2</sub>, quant.; vii) [Pd(PPh<sub>3</sub>)<sub>4</sub>], DMB, THF, 94%; viii) EDC/HOBt, NEt<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 72%; ix) [Pd(PPh<sub>3</sub>)<sub>4</sub>], DMB, THF, 96%. NBDAca: *N*-(7-Nitrobenz-2-oxa-1,3-diazole-4-yl)-aminocaproyl; EDC: *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide hydrochloride; DMB: dimethyl-barbituric acid.

Fluorescent lipidated model peptides such as mono-palmitoylated tetrapeptide 22 and doubly palmitoylated heptapeptide 28 may serve as efficient tools to gain insight into the membrane binding properties of the parent lipoproteins.

In order to demonstrate this potential the binding of the lipidated peptides **22** and **28** to liposome model membranes was analysed in a biophysical setup.<sup>[25]</sup> Here, the lipopeptides were incorporated into lipid vesicles containing a suitable, non-exchangeable fluorescence quencher molecule that absorbs the fluorescence signal of the excited lipopeptide fluorophore as long as it is in sufficient vicinity. Addition of an excess of pure vesicles without quencher leads to an increase in fluorescence signal (see Figure 2).

In our assay methanolic solutions of the fluorescent-labeled lipopeptides **22** and **28** were mixed with a hundredfold molar excess of lipid (here: palmitoyl oleoyl phosphatidylcholine, POPC) and a twofold excess of a nonexchangeable fluorescence quencher (N-(lissamine rhodamine sulfonyl)phosphatidylethanolamine, Rho-DHPE). Both, lipid and quencher were dissolved in methanol. Buffer was added to generate mixed vesicles corresponding to a POPC concentration of 1 mm. Vesicles are trimmed by freeze-thaw cycles and extruder treatment<sup>[26]</sup> to generate vesicles of a defined size distribution (approximately 100 nm Ø). If the NBD fluorophor of the lipopeptide incorporated in such a vesicle is excited at 460 nm its fluorescence emission around 520-540 nm is directly absorbed by the rhodamine dye of the quencher when Rho-DHPE is close to the lipopeptide. Mixing those vesicles with an excess of pure POPC-vesicles allows mobile lipopeptides to leave their original environment. They enter the quencher-free vesicles where their NBD fluorescence is not quenched any longer resulting in an increase in fluorescence emission at 525 nm. Figure 2b shows the fluorescence emission spectra of vesicles loaded with Rho-DHPE and lipopeptide 22 before addition of empty vesicles ( $\blacktriangle$ ) and after the end of the exchange reaction  $(\times)$ . Note that the quencher is a fluorophore itself with an emission maximum at 585 nm. Excitation wavelength was 465 nm.

Mixed vesicles were generated containing 1 mol% of peptide 22 and 28, respectively and 2 mol% of Rho-DHPE. Only tetrapeptide 22 with a single palmitoyl-thioester showed an increase in fluorescence in two hours observation time after mixing a solution of  $5 \mu M$  POPC (mixed vesicles) with a 40-fold excess of pure POPC-vesicles (Figure 3). This indicates the transfer of the lipopeptide to the vesicles free of Rho-DHPE and shows that one lipid modification is not sufficient for irreversible membrane insertion. In contrast the NBD emission of the heptapeptide 28 with two palmitoyl-residues was not affected by the dilution with quencher-free vesicles. This observation demonstrates that two C16 anchors are sufficient to fix the lipopeptide in its original environment.

The intervesicle transfer of lipopeptide 22 is composed of two separate processes. Peptides anchored at the outer face of the vesicle can directly migrate to the acceptor vesicle by diffusion. This step can be described as an irreversible first order mechanism if the acceptor vesicles are present in high excess. Depending on their distribution between inner and outer face of the vesicles the intravesicular lipopeptides have to perform a reversible flip-flop diffusion to appear on the outer face of the vesicles. In a first estimation we fitted the overall change in fluorescence for the tetrapeptide by a monoexponential function including a linear term for the flip-flop process. Best fits were obtained for five independent experiments by a dissociation rate of  $1.1 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$  (n = 5) and a linear drift of  $1.0 \times 10^2$  units of fluorescence s<sup>-1</sup>. The drift corresponds to approximately 40% of the fitted amplitude.

To obtain more information about the slow phase of the kinetics which putatively reflects the flip-flop diffusion we performed a long-term measurement over 16 h (Figure 4). Again no fluorescence increase could be observed for the vesicles containing the doubly palmitoylated lipopeptide **28**. The curve for lipopeptide **22** could be best fitted with a double exponential function with a fast rate constant of  $6.6 \times 10^{-4}$  s<sup>-1</sup> and a slow rate constant of  $8.7 \times 10^{-5}$  s<sup>-1</sup>. Here, the amplitude of the fast reaction was about 40% of the total amplitude.



intervesicle transfer experiment (Figure 4). In contrast to the latter, the kinetics of the consecutive fluorescence decay when fitted as a monoexponential function showed an apparent rate constant of  $2.1 \times 10^{-3} \text{ s}^{-1}$  which was significantly faster than the slow phase of the intervesicle transfer. This accelerated decay therefore might reflect a leakage of the vesicles rather than the true flip-flop reaction.

A second finding of the dithionite experiment was the different amplitude of the fast fluorescence shift for lipopeptide 22 and 28. While dithionite reduces the fluorescence signal of the mono-palmitoylated lipopeptide 22 by 40% (see above) the lipopeptide with two palmitoylation sites shows a decay of 80% (Figure 5b). We interpret this finding as an indication of an asymmetric distribution of lipopeptide 28 between the inner and the outer surface of the vesicles.

The apparent rate constant for the intervesicle transfer of tetrapeptide **22** and heptapeptide **28** between POPC vesicles can be compared with data for

lipopeptides with a single farnesyl modification or two hydrophobic residues (farnesyl thioether and palmitoyl thioester).<sup>[27]</sup> Here, a half-life of 21 s for a peptide with the sequence NBD-GCMGLPC(Far)-OMe and 155 h for NBD-GC(Pal)MGLPC(Far)-OMe were calculated for experiments at 37 °C, while tetrapeptide **20** has a half-life of about 11 min at 20 °C. Thus, in comparison to a farnesyl thioether a single





Figure 2. a) General principle of intervesicle transfer detection; b) emission spectra of NBD in the presence ( $\blacktriangle$ ) and absence ( $\times$ ) of the quencher Rho-DHPE.



Figure 3. Intervesicle transfer of single palmitoylated tetrapeptide 22.

A separate analysis of the flip – flop exchange is possible by monitoring the fluorescence of vesicles loaded with lipopeptides after treatment with sodium dithionite. The addition of sodium dithionite resulted in fast reduction of all accessible NBD fluorophors at the outer face of the vesicles. This first loss of emission signal was followed by a slower decay, indicating the flip – flop of the intravesicular lipopeptides to the outside (Figure 5).

For lipopeptide 22 the fluorescence drops by 40% after dithionite addition, which corresponds to the amplitude of the fast phase double exponential fit of the long time range

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Figure 5. Time course of fluorescence of vesicles containing lipotetrapeptide **22** (a) or lipoheptapeptide **28** (b) after dithionite reduction.

palmitoyl group confers a significantly enhanced stability of membrane insertion, however, desorption from the model membrane still occurs at a relatively fast rate. Combination of a farnesyl thioether with a palmitic acid thioester results in a very slow but still detectable intervesicle transfer, but in the presence of two palmitoyl groups desorption of membranebound lipidated peptides cannot be detected at all. Thus, a bispalmitoyl membrane anchor will lead to quasi irreversible membrane anchoring of doubly palmitoylated proteins which can only be reversed by hydrolysis of the palmitic acid thioester bonds.

These values correspond well to data recorded for model peptides which are derived from other lipidated proteins (such as Rho) and which have been used to predict the membrane binding properties of their parent proteins.<sup>[27, 28]</sup> Thus, determining a full set of data for several different hemagglutinin-derived lipopeptides in a detailed biophysical analysis should allow for an extrapolation to the membrane-binding properties of this lipoglycoprotein as well.

## Conclusion

We have developed a new enzyme-labile carboxy protecting group which can be removed by treatment with penicillin G acylase under very mild conditions and with complete selectivity. The PAOB ester meets all demands posed by sensitive lipidated peptides. It was successfully applied in the synthesis of differently lipidated and fluorescent-labelled peptides which represent the characteristic linkage region between the protein backbone and the lipid groups of influenza virus A hemagglutinin. The analysis of the membrane binding properties of these model peptides in a biophysical setup led to the conclusion that one palmitoyl group is not sufficient for quasi-irreversible membrane localization of the lipidated molecules. Rather a bis-palmitoylated membrane anchor is required for this purpose. The availability of such hemagglutinin-derived lipopeptides and their application in a more detailed biophysical analysis should allow for an extrapolation to the membrane-binding properties of the parent lipoglycoprotein. This knowledge will further our understanding of the biological processes influenced by hemagglutinin.

## **Experimental Section**

**General procedures:** <sup>1</sup>H und <sup>13</sup>C NMR spectra were recorded on Bruker AC-250, Bruker AM-400 and Bruker DRX-500 spectrometers. EI and FAB mass spectra were measured on a Finnigan MAT MS 70 Workstation. Specific rotations were measured with a Perkin–Elmer polarimeter 241. Elementary analyses were performed on a Heraeus CHN-Rapid apparatus. Melting points were determined in open capillaries using a Büchi 530 apparatus and are uncorrected.

**Materials**: Analytical chromatography was performed on E. Merck silica gel 60  $F_{254}$  coated plates. Flash chromatography was performed on Baker silica gel (40–64 µm). Size-exclusion chromatography was performed on Pharmacia Sephadex LH 20. Penicillin G acylase (E.C. 3.5.1.11) was obtained in immobilized form on Eupergit C from Roche (Boehringer Mannheim). Penicillin G acylase CLEC were obtained from Altus Biologics. All solvents were distilled using standard procedures. Commercial reagents were used without further purification. Where indicated the reactions were performed under argon. Several compounds were prepared according to literature methods: 4-(phenylacetoxy)benzyl alcohol (10),<sup>[17]</sup> Boc-Arg(Aloc)<sub>2</sub>-OH (9h).<sup>[29, 30]</sup>

Synthesis of Boc-protected amino acid PAOB-esters: DIC (0.570 g, 4.10 mmol) was added to a solution of Boc-protected amino acid (4.10 mmol) 9a-d, 4-(phenylacetoxy)benzyl alcohol 10 (1 g, 4.10 mmol) and DMAP (0.010 g, 0.10 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (150 mL) and the mixture was stirred at room temperature for 16 h. Then the solution was extracted with acetic acid (5%, 2 × 50 mL) and water (2 × 50 mL), the organic layer was dried with MgSO<sub>4</sub> and the solvent was removed under reduced pressure. The products were purified by flash chromatography on silica gel using solvent mixtures as indicated.

**Boc-Ala-OPAOB** (11a): Purification by flash chromatography using *n*-hexane/ethyl acetate 5:2 yielded the title compound as a colorless solid (1.53 g, 90%). M.p. 76 °C;  $[α]_D^{20} = -10.5$  (c = 1.0 in CHCl<sub>3</sub>);  $R_f = 0.41$  (*n*-hexane/ethyl acetate 5:2); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 7.40 - 7.24$  (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHC*H*, aromatic), 7.06–7.03 (m, 2H, OCC*H*, aromatic), 5.16 (d, <sup>2</sup>*J* = 12.4 Hz, 1H, CH<sub>2a</sub>-O), 5.09 (d, <sup>2</sup>*J* = 12.4 Hz, 1H, CH<sub>2b</sub>-O), 5.10 (br, 1H, NH), 4.35–4.32 (m, 1H,  $\alpha$ -CH, Ala), 3.84 (s, 2H, CH<sub>2</sub>-COO), 1.43 (s, 9H, *t*Bu), 1.36 (d, <sup>3</sup>*J* = 7.2 Hz, 3H, β-CH<sub>3</sub>, Ala); <sup>13</sup>C NMR (100.6 MHz, CDCl<sub>3</sub>):  $\delta = 173.10$ , 169.81 ( $2 \times C=0$ , ester), 155.06 (C=O, carbamate), 150.63 (C<sub>q</sub>-O, aromatic), 133.28, 133.08 ( $2 \times C_q$ , aromatic), 129.31, 129.26, (28.71, 127.36, 121.61 (CH, aromatic), 79.78 (C<sub>q</sub>, *H*u), 66.21 (CH<sub>2</sub>-O), 49.32 ( $\alpha$ -CH), 41.33 (CH<sub>2</sub>-COO), 28.29 (CH<sub>3</sub>, *H*u), 18.46 ( $\beta$ -CH<sub>3</sub>); MS (EI): *m*/z: calcd: 413.184; found: 413.185; elemental analysis calcd (%) for C<sub>23</sub>H<sub>27</sub>NO<sub>6</sub> (413.5): C 66.81, H 6.58, N 3.39; found: C 66.73, H 6.56, N 3.40.

**Boc-IIe-OPAOB (11b)**: Purification by flash chromatography using *n*-hexane/ethyl acetate 4:1 yielded a colorless oil (1.16 g, 64%).  $[\alpha]_D^{2D} = -1.2$  (c = 1.0 in CHCl<sub>3</sub>);  $R_f = 0.50$  (*n*-hexane/ethyl acetate 4:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 7.39 - 7.29$  (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.07 - 7.03 (m, 2H, OCCH, aromatic), 5.18 (d, <sup>2</sup>J = 12.4 Hz, 1H, CH<sub>2a</sub>-O),

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5.09 (d,  ${}^2J$  = 12.4 Hz, 1 H, CH<sub>2b</sub>-O), 5.02 (d,  ${}^3J$  = 8.8 Hz, 1 H, NH), 4.31–4.29 (m, 1 H, α-CH, IIe), 3.86 (s, 2 H, CH<sub>2</sub>-COO), 1.90–1.82 (m, 1 H, β-CH, IIe), 1.44 (s, 9 H, *t*Bu), 1.38–1.31 (m, 1 H, γ-CH<sub>2a</sub>, IIe), 1.16–1.10 (m, 1 H, γ-CH<sub>2b</sub>, IIe), 0.90–0.85 (m, 6 H, 2 × CH<sub>3</sub>, IIe);  ${}^{13}$ C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta$  = 172.19, 169.82 (2 × C=O, ester), 155.54 (C=O, carbamate), 150.69 (C<sub>q</sub>-O, aromatic), 133.31, 133.10 (2 × C<sub>q</sub>, aromatic), 129.50, 129.28, 128.74, 127.39, 121.61 (CH, aromatic), 79.77 (C<sub>q</sub>, *t*Bu), 66.12 (CH<sub>2</sub>-O), 57.93 (α-CH), 41.38 (CH<sub>2</sub>-COO), 38.02 (β-CH), 28.31 (CH<sub>3</sub>, *t*Bu), 24.93 (γ-CH<sub>2</sub>), 15.55, 11.38 (2 × CH<sub>3</sub>); MS (EI): *m*/*z*: calcd: 455.231; found: 455.229; elemental analysis calcd (%) for C<sub>26</sub>H<sub>33</sub>NO<sub>6</sub> (455.5): C 68.55, H 7.30, N 3.07; found: C 69.10, H 7.41, N 3.27.

**Boc-Pro-OPAOB (11 c)**: Purification by flash chromatography using *n*-hexane/ethyl acetate 3:1 yielded a colorless oil (1.56 g, 89 %).  $[a]_{20}^{20} = -21.4$  (*c* = 1.0 in CHCl<sub>3</sub>);  $R_{\rm f}$ =0.41 (*n*-hexane/ethyl acetate 3:1); <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>):  $\delta$ =7.40–7.31 (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.06 (d,  ${}^{3}J$ =7.9 Hz, 2H, OCCH, aromatic), 5.20 (d,  ${}^{2}J$ =12.2 Hz, 1H, CH<sub>2a</sub>-O), 5.08 (d,  ${}^{2}J$ =12.2 Hz, 1H, CH<sub>2b</sub>-O), 4.24 (dd,  ${}^{3}J_{1}$ =7.8 Hz,  ${}^{3}J_{2}$ =5.3 Hz, 1H, *α*-CH, Pro), 3.88 (s, 2H, CH<sub>2</sub>-COO), 3.61–3.38 (m, 2H, *δ*-CH<sub>2</sub>, Pro), 2.30–2.13 (m, 1H, *β*-CH<sub>2a</sub>, Pro), 2.02–1.81 (m, 3H, *β*-CH<sub>2b</sub>, *γ*-CH<sub>2</sub>, Pro), 1.43 (s, 9H, *t*Bu); MS (EI): *m/z*: calcd for C<sub>25</sub>H<sub>29</sub>NO<sub>6</sub>: 439.1995; found: 439.2021.

**Boc-Phe-OPAOB (11d)**: Purification by flash chromatography using *n*-hexane/ethyl acetate 4:1 yielded a colorless solid (1.63 g, 81 %). M.p. 66 °C;  $[a]_D^{20} = -1.1$  (*c* = 1.0 in CHCl<sub>3</sub>);  $R_f = 0.38$  (*n*-hexane/ethyl acetate 4:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 7.38 - 7.21$  (m, 10H, aromatic), 7.04 - 7.02 (m, 4H, aromatic), 5.11 (d, <sup>2</sup>*J* = 12.4 Hz, 1H, CH<sub>2a</sub>-O), 5.04 (d, <sup>2</sup>*J* = 12.4 Hz, 1H, CH<sub>2a</sub>-O), 5.04 (d, <sup>3</sup>*J* = 7.2 Hz, 1H, NH), 4.61 - 4.59 (m, 1H, *a*-CH, Phe), 3.85 (s, 2H, CH<sub>2</sub>-COO), 3.06 - 3.04 (m, 2H, β-CH<sub>2</sub>, Phe), 1.41 (s, 9H, *t*Bu); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta = 171.76$ , 169.69 (2 × C=O, ester), 155.15 (C=O, carbamate), 150.81 (C<sub>q</sub>-O, aromatic), 132.85, 133.28, 133.08 (3 × C<sub>q</sub>, aromatic), 129.78, 129.35, 128.81, 128.61, 127.46, 121.68 (CH, aromatic), 80.04 (C<sub>q</sub>, *t*Bu), 66.42 (CH<sub>2</sub>-O), 54.53 (*a*-CH), 41.44 (CH<sub>2</sub>-COO), 38.2 (β-CH<sub>2</sub>), 28.35 (CH<sub>3</sub>, *t*Bu); MS (EI): *m*/*z*: calcd: 489.2151; found: 489.2171; elemental analysis calcd (%) for C<sub>29</sub>H<sub>21</sub>NO<sub>6</sub> (489.6): C 71.15, H 6.38, N 2.86; found: C 71.02, H 6.44, N 2.55.

Synthesis of N-terminally deprotected amino acid PAOB esters: TFA (3.00 mL, 40.0 mmol) was added at 0 °C to a solution of *N-tert*-butyloxy-carbonyl-L-amino acid-4-phenylacetoxybenzyl ester 11a-d (2.0 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (30 mL) and the mixture was stirred at room temperature for 2 h. TFA and the solvent were coevaporated with toluene under reduced pressure. The residue was triturated five times with diethyl ether to yield the desired compound.

**H-Ala-OPAOB · TFA (12a)**: Colorless solid (0.760 g, 89%); m.p. 69°C;  $[\alpha]_D^{20} = -2.0 \ (c = 1.0 \ in CHCl_3); R_f = 0.22 \ (n-hexane/ethyl acetate 1:4 with 2 vol.% triethylamine); <sup>1</sup>H NMR (250 MHz, CDCl_3): <math>\delta = 8.23$  (br, 3 H, NH<sub>3</sub>), 7.37 – 7.24 (m, 7 H, C<sub>6</sub>H<sub>5</sub>, OCCH*CH*, aromatic), 7.04 – 6.99 (m, 2 H, OCC*H*, aromatic), 5.12 (d, <sup>2</sup>*J* = 15.0 Hz, 1 H, CH<sub>2a</sub>-O), 5.07 (d, <sup>2</sup>*J* = 15.0 Hz, 1 H, CH<sub>2b</sub>-O), 4.00 (q, <sup>3</sup>*J* = 7.1 Hz, 1 H, α-CH, Ala), 3.85 (s, 2 H, CH<sub>2</sub>-COO), 1.51 (d, <sup>3</sup>*J* = 7.1 Hz, 3 H, β-CH<sub>3</sub>, Ala); MS (FAB, 3-NBA/TFA): *m/z*: calcd for C<sub>20</sub>H<sub>20</sub>F<sub>3</sub>NO<sub>6</sub>: 314.139, found: 314.146 [*M*]<sup>+</sup>.

**H-IIe-OPAOB·TFA** (12b): Colorless solid (0.680 g, 73%); m.p. 65 °C;  $[\alpha]_{10}^{20} = +2.3$  (*c* = 1.0 in CHCl<sub>3</sub>); *R*<sub>f</sub> = 0.31 (*n*-hexane/ethyl acetate 1:2 with 2 vol. % triethylamine); <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.23 (br, 3 H, NH<sub>3</sub>), 7.37 – 7.27 (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHC*H*, aromatic), 7.06 – 6.99 (m, 2H, OCC*H*, aromatic), 5.18 (d, <sup>2</sup>*J* = 10.0 Hz, 1H, CH<sub>2a</sub>-O), 5.07 (d, <sup>2</sup>*J* = 10.0 Hz, 1H, CH<sub>2b</sub>-O), 3.95 – 3.92 (m, 1H, α-CH, IIe), 3.85 (s, 2H, CH<sub>2</sub>-COO), 2.06 – 1.92 (m, 1H, β-CH, IIe), 1.49 – 1.18 (m, 2H, γ-CH<sub>2</sub>, IIe), 0.95 – 0.84 (m, 6H, 2× CH<sub>3</sub>, IIe); MS (EI): *m/z*: calcd for C<sub>23</sub>H<sub>26</sub>F<sub>3</sub>NO<sub>6</sub>: 356.186; found: 356.184 [*M*]<sup>+</sup>.

**H-Phe-OPAOB · TFA (12 d)**: Colorless solid (0.917 g, 91%); m.p. 117 °C;  $[\alpha]_{10}^{20} = +5.5$  (*c* = 1.0 in CH<sub>3</sub>OH); *R*<sub>f</sub> = 0.67 (*n*-hexane/ethyl acetate 1:2 with 2 vol.% triethylamine); <sup>1</sup>H NMR (250 MHz, CD<sub>3</sub>OD):  $\delta$  = 7.40–7.26 (m, 10H, aromatic), 7.17–7.03 (m, 4H, aromatic), 5.25 (d, <sup>2</sup>*J* = 14.4 Hz, 1H, CH<sub>2a</sub>-O), 5.18 (d, <sup>2</sup>*J* = 14.4 Hz, 1H, CH<sub>2b</sub>-O), 4.43 (t, <sup>3</sup>*J* = 6.8 Hz, 1H, α-CH, Phe), 3.92 (s, 2H, CH<sub>2</sub>-COO), 3.19 (d, <sup>3</sup>*J* = 6.8 Hz, 2H, β-CH<sub>2</sub>, Phe); MS (EI): *m/z*: calcd: 390.171; found: 390.169; elemental analysis calcd (%) for C<sub>26</sub>H<sub>24</sub>F<sub>3</sub>NO<sub>6</sub> (504.6): C 62.03, H 4.80, N 2.78; found C 62.06, H 4.82, N 2.42.

**H-Pro-OPAOB · HCl (12 c)**: A saturated solution of HCl in diethyl ether (10 mL) was added at  $0^{\circ}$ C to a solution of Boc-Pro-OPAOB (**11 c**; 0.530 g, 1.20 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (30 mL) and the mixture was stirred at  $0^{\circ}$ C for

45 min and at room temperature for 1 h. Then the excess HCl and the solvent were removed under reduced pressure. The residue was triturated three times with diethyl ether (10 mL) to yield the desired compound as a colorless solid (0.532 g, 98%). M.p. 131 °C;  $[a]_{D}^{20} = -28.2$  (c = 1.0 in CH<sub>3</sub>OH); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta = 7.44$  (d, <sup>3</sup>J = 8.6 Hz, 2 H, OCCHCH, aromatic), 7.38–7.27 (m, 5H, C<sub>6</sub>H<sub>5</sub>, aromatic), 7.09 (d, <sup>3</sup>J = 8.6 Hz, 2H, OCCHCH, aromatic), 5.29 (d, <sup>2</sup>J = 12.1 Hz, 1H, CH<sub>2a</sub>-O), 5.26 (d, <sup>2</sup>J = 12.1 Hz, 1H, CH<sub>2b</sub>-O), 4.45 (dd, <sup>3</sup> $J_1 = 8.8$  Hz, <sup>3</sup> $J_2 = 7.1$  Hz, 1H,  $\alpha$ -CH, Pro), 3.89 (s, 2H, CH<sub>2</sub>-COO), 3.41–3.32 (m, 2H,  $\delta$ -CH<sub>2</sub>, Pro), 2.45–2.38 (m, 1H,  $\beta$ -CH<sub>2a</sub>, Pro), 2.14–2.01 (m, 3H,  $\beta$ -CH<sub>2b</sub>,  $\gamma$ -CH<sub>2</sub>, Pro); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta = 171.77$ , 169.88 (2 × C=O, ester), 152.59 (C<sub>q</sub>-O, aromatic), 135.08, 134.03 (2 × C<sub>q</sub>, aromatic), 131.02, 130.43, 129.68, 128.32, 122.95 (CH, aromatic), 68.71 (CH<sub>2</sub>-O), 60.74 (a-CH, Pro), 47.14 ( $\delta$ -CH<sub>2</sub>, Pro), 41.84 (CH<sub>2</sub>-COO), 29.27 ( $\beta$ -CH<sub>2</sub>, Pro), 24.45 ( $\gamma$ -CH<sub>2</sub>, Pro), 7.14 ( $\delta$ -CH<sub>2</sub>, N-R), m/z: calcd for C<sub>20</sub>H<sub>22</sub>NO<sub>4</sub>: 340.1549; found: 340.1576 [M - CI]<sup>+</sup>.

Synthesis of N-terminally protected dipeptide-4-phenylacetoxybenzyl esters: EEDQ (1.2 equiv) was added at  $0^{\circ}$ C to a solution of N-terminally protected amino acid (1 equiv) 9 in CH<sub>2</sub>Cl<sub>2</sub> (30 mL). Then L-amino acid 4-phenylacetoxybenzyl ester protected trifluoroacetate (1 equiv) 12 and triethylamine (1 equiv) were added and the mixture was stirred at room temperature for 16 h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using solvent mixtures as indicated.

Aloc-Val-Ala-OPAOB (4a): Purification by flash chromatography using nhexane/ethyl acetate 2:1 yielded a colorless solid (0.354 g (1 equiv = 1.00 mmol), 71 %). M.p. 138 °C;  $[\alpha]_{\rm D}^{20} = -17.5 \ (c = 1.0 \text{ in CHCl}_3); R_{\rm f} = 0.26$ (*n*-hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 7.37 - 7.31$ (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.06 - 7.04 (m, 2H, OCCH, aromatic), 6.71 (br, 1H, NH, amide), 5.94-5.84 (m, 1H, CH=CH<sub>2</sub>), 5.50 (br, 1H, NH, carbamate), 5.31-5.18 (m, 2H, CH=CH<sub>2</sub>), 5.16 (d, <sup>2</sup>J = 12.4 Hz, 1H, CH<sub>2a</sub>-O, benzyl), 5.09 (d,  ${}^{2}J = 12.4$  Hz, 1 H, CH<sub>2b</sub>-O, benzyl), 4.64–4.55 (m, 3 H, CH2-O, allyl, α-CH, Ala), 4.03-4.01 (m, 1 H, α-CH, Val), 3.85 (s, 2 H, CH2-COO), 2.07 – 2.04 (m, 1 H,  $\beta$ -CH, Val), 1.38 (d,  ${}^{3}J = 7.2$  Hz, 3 H, CH<sub>3</sub>, Ala), 0.94 (d,  ${}^{3}J = 6.7$  Hz, 3 H, CH<sub>3</sub>, Val), 0.91 (d,  ${}^{3}J = 6.7$  Hz, 3 H, CH<sub>3</sub>, Val); <sup>13</sup>C NMR (100.6 MHz, CDCl<sub>3</sub>):  $\delta = 172.45$ , 171.00 (2 × C=O, ester), 169.87 (C=O, amide), 156.28 (C=O, carbamate), 150.74 (C<sub>a</sub>-O, aromatic), 133.28, 132.60 (2 × C<sub>q</sub>, aromatic), 132.91 (CH, allyl), 129.43, 129.28, 128.75, 127.41, 121.69 (CH, aromatic), 117.78 (CH=CH<sub>2</sub>), 66.47 (CH<sub>2</sub>-O, benzyl), 65.84 (CH2-O, allyl), 60.08 (a-CH, Ala), 48.07 (a-CH, Val), 41.38 (CH2-COO), 31.31 (β-CH, Val), 19.11 (CH<sub>3</sub>, Ala), 18.04, 17.79 (2 × CH<sub>3</sub>, Val); MS (EI): m/z: calcd: 496.221; found: 496.220; elemental analysis calcd (%) for C<sub>27</sub>H<sub>32</sub>N<sub>2</sub>O<sub>7</sub> (496.6): C 65.31, H 6.50, N 5.64; found: C 65.27, H 6.55, N 5.55.

Aloc-Ser-Ile-OPAOB (4b): Purification by flash chromatography using nhexane/ethyl acetate 3:2 yielded a colorless oil (0.270 g (1 equiv = 0.60 mmol), 81%).  $[\alpha]_D^{20} = -31.3$  (c = 1.0 in CHCl<sub>3</sub>);  $R_f = 0.22$  (n-hexane/ ethyl acetate 3:2); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 7.41 - 7.29$  (m, 7 H,  $C_6H_5$ , OCCHCH, aromatic), 7.21 (d,  ${}^{3}J = 7.7$  Hz, 1 H, NH, amide), 7.06 – 7.04 (m, 2H, OCCH, aromatic), 5.67 (d,  ${}^{3}J = 7.2$  Hz, 1H, NH, carbamate), 5.18 (d,  ${}^{2}J = 12.3$  Hz, 1H, CH<sub>2a</sub>-O), 5.09 (d,  ${}^{2}J = 12.3$  Hz, 1H, CH<sub>2b</sub>-O), 4.57–4.54 (m, 1 H,  $\alpha$ -CH), 4.18 (br, 1 H,  $\beta$ -CH<sub>2a</sub>, Ser), 4.02 (d,  ${}^{3}J = 10.0$  Hz, 1 H, β-CH<sub>2b</sub>, Ser), 3.85 (s, 2 H, CH<sub>2</sub>-COO), 3.62-3.60 (m, 1 H, α-CH), 3.49 (br, 1 H, OH), 1.93-1.90 (m, 1 H, β-CH, Ile), 1.44 (s, 9 H, tBu), 1.37-1.29 (m, 1H,  $\gamma$ -CH<sub>2a</sub>, Ile), 1.17–1.08 (m, 1H,  $\gamma$ -CH<sub>2b</sub>, Ile), 0.92–0.80 (m, 6H,  $2 \times CH_3$ , Ile); <sup>13</sup>C NMR (100.6 MHz, CDCl<sub>3</sub>):  $\delta = 171.60$ , 171.47 ( $2 \times C=O$ , ester), 169.86 (C=O, amide), 156.14 (C=O, carbamate), 150.71 (Cq-O, aromatic), 133.26, 132.90 (2  $\times$   $C_q,$  aromatic), 129.56, 129.27, 128.73, 127.38 (CH, aromatic), 80.39 (C<sub>q</sub>, tBu), 66.36 (CH<sub>2</sub>-O), 62.67 (β-CH<sub>2</sub>, Ser), 56.75 (α-CH, Ile), 54.52 (α-CH, Ser), 41.35 (CH<sub>2</sub>-COO), 37.41 (β-CH, Ile), 28.25 (CH<sub>3</sub>, tBu), 24.83 ( $\gamma$ -CH<sub>2</sub>, Ile), 15.57, 11.50 (2 × CH<sub>3</sub>, Ile); MS (EI): m/z: calcd for C29H38N2O8: 542.263; found: 542.264.

**Boc-Thr-Phe-OPAOB (4d)**: Purification by flash chromatography using *n*-hexane/ethyl acetate 1:1 yielded a colorless solid (0.230 g (1 equiv = 0.39 mmol), 85 %). M.p. 141 °C;  $[\alpha]_{10}^{20} = -61.3$  (*c* = 1.0 in CHCl<sub>3</sub>);  $R_t$ = 0.43 (*n*-hexane/ethyl acetate 1:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.40 – 7.30 (m, 7 H, C<sub>6</sub>H<sub>5</sub>, PAOB, CCH, Phe, aromatic), 7.26 (d, <sup>3</sup>J = 7.6 Hz, 2 H, OCCHCH, aromatic), 7.06 – 7.03 (m, 5 H, OCCH, PAOB, Phe, aromatic), 5.42 (d, <sup>3</sup>J = 7.7 Hz, 1 H, NH, carbamate), 5.12 (d, <sup>2</sup>J = 12.2 Hz, 1 H, CH<sub>2a</sub>-O), 5.07 (d, <sup>2</sup>J = 12.2 Hz, 1 H, CH<sub>2b</sub>-O), 4.61 – 4.59 (m, 1 H, *α*-CH, Phe), 4.28 – 4.26 (m, 1 H, *β*-CH, Thr), 4.06 (d, <sup>3</sup>J = 7.2 Hz, 1 H, *β*-CH<sub>2a</sub>, Phe), 3.03 (dd, <sup>2</sup>J = 13.8 Hz, <sup>3</sup>J = 6.7 Hz, 1 H, *β*-CH<sub>2b</sub>, Phe), 1.43 (s, 9 H, *t*Bu), 1.12 (d,

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<sup>3</sup>*J* = 6.4 Hz, 3 H, γ-CH<sub>3</sub>, Thr); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta$  = 171.00, 169.83 (3 × C=O, ester, amide), 156.24 (C=O, carbamate), 150.85 (C<sub>q</sub>-O, aromatic), 135.55, 133.31, 132.67 (3 × C<sub>q</sub>, aromatic), 129.79, 129.29, 129.23, 128.76, 128.61, 127.42, 127.17, 121.68 (CH, aromatic), 80.29 (C<sub>q</sub>, *t*Bu), 66.88 (β-CH, Thr), 66.60 (CH<sub>2</sub>-O), 58.09 (α-CH, Phe), 53.34 (α-CH, Thr), 41.40 (CH<sub>2</sub>-COO), 37.84 (β-CH<sub>2</sub>, Phe), 28.28 (CH<sub>3</sub>, *t*Bu), 18.07 (γ-CH<sub>3</sub>, Thr); MS (FAB, 3-NBA): *m/z*: calcd: 591.2706; found: 591.2732 [*M*+H]<sup>+</sup>; elemental analysis calcd (%) for C<sub>33</sub>H<sub>39</sub>N<sub>2</sub>O<sub>8</sub> (590.7): C 67.10, H 6.48, N 4.74; found: C 66.72, H 6.47, N 4.63.

Aloc-Val-Phe-OPAOB (4e): Purification by flash chromatography using nhexane/ethyl acetate 2:1 yielded a colorless solid (0.770 g (1 equiv = 2.00 mmol), 67 %). M.p. 144 °C;  $[\alpha]_{D}^{20} = -4.0$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{f} = 0.20$ (*n*-hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 7.39 - 7.17$ (m, 10 H, aromatic), 7.05 - 6.98 (m, 4 H, aromatic), 6.45 (d, <sup>3</sup>J = 7.6 Hz, 1 H, NH, amide), 5.94-5.86 (m, 1H, CH=CH<sub>2</sub>), 5.37-5.19 (m, 3H, CH=CH<sub>2</sub>, NH, carbamate), 5.11 (d,  ${}^{2}J = 12.2$  Hz, 1 H, CH<sub>2a</sub>-O, benzyl), 5.03 (d,  ${}^{2}J =$ 12.2 Hz, 1 H, CH<sub>2b</sub>-O, benzyl), 4.90 (dt,  ${}^{3}J_{1} = 7.6$  Hz,  ${}^{3}J_{2} = 6.1$  Hz, 1 H,  $\alpha$ -CH, Phe), 4.58-4.49 (m, 2H, CH<sub>2</sub>-O, allyl), 4.02-3.99 (m, 1H, α-CH, Val), 3.86 (s, 2 H, CH<sub>2</sub>-COO), 3.07 (d,  ${}^{3}J = 6.1 \text{ Hz}, \beta$ -CH<sub>2</sub>, Phe), 2.07 – 2.01 (m, 1 H,  $\beta$ -CH, Val), 0.90 (d,  ${}^{3}J = 6.7$  Hz, 3H, CH<sub>3</sub>, Val), 0.85 (d,  ${}^{3}J = 6.7$  Hz, 3H, CH<sub>3</sub>, Val); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta = 171.03$ , 170.91 (2 × C=O, ester), 169.81 (C=O, amide), 156.15 (C=O, carbamate), 150.81 (C<sub>q</sub>-O, aromatic), 135.38, 133.28, 132.59 (3 × C<sub>q</sub>, aromatic), 132.63 (CH, allyl), 129.79, 129.28, 129.22, 128.75, 128.60, 127.40, 127.15, 121.65 (CH, aromatic), 117.77 (CH=CH<sub>2</sub>), 66.54 (CH<sub>2</sub>-O, benzyl), 65.83 (CH<sub>2</sub>-O, allyl), 60.10 (a-CH, Phe), 53.12 (α-CH, Val), 41.37 (CH<sub>2</sub>-COO), 37.89 (β-CH<sub>2</sub>, Phe), 31.09 (β-CH, Val), 19.09, 17.68 (2 × CH<sub>3</sub>, Val); MS (EI): *m*/*z*: calcd: 572.252; found: 572.251; elemental analysis calcd (%) for C33H36N2O7 (572.7): C 69.21, H 6.34, N 4.89; found C 68.98, H 6.33, N 4.73.

Boc-Ala-Pro-OPAOB (4c): HOBt (63 mg, 0.40 mmol) and DIC (60 µL, 0.33 mmol) were added at 0°C to a solution of Boc-Ala-OH (9a; 63 mg, 0.33 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL). Then H-Pro-OPAOB · HCl (12c; 125 mg, 0.33 mmol) and triethylamine (50 µL, 0.33 mmol) were added and the mixture was stirred at room temperature for 16 h. Then the solution was extracted with hydrochloric acid (1M, 10 mL) and water (10 mL) and the organic layer was dried with MgSO4. After filtration, the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using n-hexane/ethyl acetate 1:1 to yield a colorless oil (0.148 g, 87 %).  $[a]_{D}^{20} = -56.8 (c = 1.0 \text{ in CHCl}_{3}); R_{f} = 0.59 (n-1)$ hexane/ethyl acetate 1:1); <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>):  $\delta = 7.39 - 7.30$  (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.06 (d,  ${}^{3}J = 8.6$  Hz, 2H, OCCH, aromatic), 5.38 (d,  ${}^{3}J = 7.2$  Hz, 1 H, NH, carbamate), 5.19 (d,  ${}^{2}J = 13.8$  Hz, 1 H, CH<sub>2a</sub>-O), 5.07 (d,  ${}^{2}J = 13.8$  Hz, 1 H, CH<sub>2b</sub>-O), 4.59 (dd,  ${}^{3}J_{1} = 8.5$  Hz,  ${}^{3}J_{2} = 6.9$  Hz, 1 H,  $\alpha$ -CH, Pro), 4.52 – 4.40 (m, 1 H,  $\alpha$ -CH, Ala), 3.84 (s, 2 H, CH<sub>2</sub>-COO), 3.75-3.68 (m, 1H, δ-CH<sub>2a</sub>, Pro), 3.62-3.53 (m, 1H, δ-CH<sub>2b</sub>, Pro), 2.27–2.16 (m, 1 H, β-CH<sub>2a</sub>, Pro), 2.04–1.91 (m, 3 H, β-CH<sub>2b</sub>, γ-CH<sub>2</sub>, Pro), 1.43 (s, 9H, *t*Bu), 1.79 (d,  ${}^{3}J = 7.3$  Hz, 3H,  $\beta$ -CH<sub>3</sub>, Ala); C<sub>28</sub>H<sub>34</sub>N<sub>2</sub>O<sub>7</sub> (510.4)

Enzymatic removal of the PAOB-ester group from the model dipeptides: Immobilized penicillin G acylase was added to a suspension of N-terminally protected dipeptide-4-phenylacetoxybenzyl ester 4a - e in a mixture of phosphate buffer (0.05 M, pH 7) and cosolvent and the reaction mixture was shaken at room temperature for 24 h. After the enzyme was filtered off and washed with the cosolvent and water, the organic solvent was removed under reduced pressure. The resulting aqueous phase was washed with  $CH_2Cl_2$  (3 × 50 mL), adjusted to pH 2 and extracted with ethyl acetate (5 × 50 mL). The combined ethyl acetate layers were dried with MgSO<sub>4</sub>, filtered and the solvent was removed under reduced pressure.

Aloc-Val-Ala-OH (6a): Aloc-Val-Ala-OPAOB (4a; 0.248 g, 0.50 mmol) was suspended in phosphate buffer (90 mL) and methanol (10 mL) and immobilized penicillin G acylase (300 U) was added. The crude product was purified by recrystallisation using *n*-hexane/ethyl acetate to yield a colorless solid (0.109 g, 80 %). M.p.  $163 \,^{\circ}$ C;  $[\alpha]_D^{\circ} = -39.2$  (c = 1.0 in CH<sub>3</sub>OH);  $R_t = 0.16$  (*n*-hexane/ethyl acetate 2:1 with 2 vol.-% acetic acid); <sup>1</sup>H NMR (400 MHz, [D<sub>6</sub>]DMSO):  $\delta = 12.43$  (br, 1H, COOH), 8.19 (d, <sup>3</sup>J = 6.9 Hz, 1H, NH, amide), 7.16 (d, <sup>3</sup>J = 5.5 Hz, 1H, NH, carbamate), 5.95 - 5.85 (m, 1H, CH=CH<sub>2</sub>), 5.31 - 5.16 (m, 2H, CH=CH<sub>2</sub>), 4.47 - 4.46 (m, 2H, CH<sub>2</sub>-O), 4.22 - 4.15 (m, 1H,  $\alpha$ -CH, Ala), 3.89 - 3.85 (m, 1H,  $\alpha$ -CH, Val), 1.99 - 1.91 (m, 1H,  $\beta$ -CH, Val), 1.27 (d, <sup>3</sup>J = 6.7 Hz, 3H, CH<sub>3</sub>, Ala), 0.89 (d, <sup>3</sup>J = 6.7 Hz, 3H, CH<sub>3</sub>, Val), 0.84 (d, <sup>3</sup>J = 6.7 Hz, 3H, CH<sub>3</sub>, Val); <sup>13</sup>C NMR (100.6 MHz, [D<sub>6</sub>]DMSO):  $\delta = 173.98$  (C=O, carboxylic acid), 170.94 (C=O,

amide), 155.86 (C=O, carbamate), 133.63 (CH, allyl), 116.87 (CH=CH<sub>2</sub>), 64.36 (CH<sub>2</sub>-O, allyl), 59.65 ( $\alpha$ -CH, Ala), 47.43 ( $\alpha$ -CH, Val), 30.48 ( $\beta$ -CH, Val), 19.11 (CH<sub>3</sub>, Ala), 18.04, 17.79 (2 × CH<sub>3</sub>, Val); MS (EI): m/z: calcd: 272.137; found: 272.139; elemental analysis calcd (%) for C<sub>12</sub>H<sub>20</sub>N<sub>2</sub>O<sub>5</sub> (272.3): C 52.96, H 7.41, N 10.29; found C 52.81, H 7.38, N 10.38.

Boc-Ser-Ile-OH (6b): Aloc-Ser-Ile-OPAOB (4b; 0.150 g, 0.28 mmol) was suspended in phosphate buffer (90 mL) and methanol (10 mL) and immobilized penicillin G acylase (300 U) was added. The crude product was purified by flash chromatography using n-hexane/ethyl acetate 1:1 with 2 vol.- % acetic acid to yield a colorless oil (0.070 g, 80 %).  $[a]_{\rm D}^{20} = -6.6 (c =$ 0.5 in CH<sub>3</sub>OH);  $R_f = 0.15$  (*n*-hexane/ethyl acetate 1:1 with 2 vol.-% acetic acid); <sup>1</sup>H NMR (400 MHz, [D<sub>6</sub>]DMSO):  $\delta = 7.69$  (d, <sup>3</sup>J = 8.4 Hz, 1 H, NH, amide), 6.75 (d,  ${}^{3}J = 8.1$  Hz, 1 H, NH, carbamate), 4.22 – 4.18 (m, 1 H,  $\alpha$ -CH, Ile), 4.03 - 3.98 (m, 1 H,  $\alpha$ -CH, Ser), 3.55 (dd,  ${}^{2}J = 11.0$  Hz,  ${}^{3}J = 4.9$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Ser), 3.49 (dd, <sup>2</sup>J = 11.0 Hz, <sup>3</sup>J = 6.7 Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Ser), 1.79 -1.72 (m, 1 H, β-CH, Ile), 1.42-1.40 (m, 1 H, γ-CH<sub>2a</sub>, Ile), 1.37 (s, 9 H, tBu),  $1.18 - 1.11 \text{ (m, 1 H, } \gamma\text{-CH}_{2b}\text{, Ile}\text{)}, 0.85 - 0.79 \text{ (m, 6 H, } 2 \times \text{CH}_{3}\text{, Ile}\text{)}; {}^{13}\text{C NMR}$ (100.6 MHz, [D<sub>6</sub>]DMSO): δ = 172.94 (C=O, carboxylic acid), 170.54 (C=O, amide), 155.44 (C=O, carbamate), 78.46 (C<sub>q</sub>, tBu), 61.79 (β-CH<sub>2</sub>, Ser), 56.27 (2×α-CH), 36.94 (β-CH, Ile), 28.24 (CH<sub>3</sub>, tBu), 24.73 (γ-CH<sub>2</sub>, Ile), 15.48, 11.39 (2 × CH<sub>3</sub>, Ile); MS (EI): m/z: calcd for C<sub>14</sub>H<sub>18</sub>N<sub>2</sub>O<sub>6</sub>: 318.179; found: 318.180

Boc-Ala-Pro-OH (6c): Boc-Ala-Pro-OPAOB (4c; 0.135 g, 0.26 mmol) was suspended in phosphate buffer (90 mL) and methanol (10 mL) and immobilized penicillin G acylase (500 U) was added. The crude product was purified by recrystallisation using n-hexane/ethyl acetate to yielld colorless solid (0.061 g, 81%). M.p.  $154^{\circ}C$ ;  $[\alpha]_{D}^{20} = -95.0$  (c = 1.0 in CH<sub>3</sub>OH);  $R_f = 0.11$  (*n*-hexane/ethyl acetate 1:1); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta = 4.48 - 4.45$  (m, 1 H,  $\alpha$ -CH, Pro), 4.37 (q,  ${}^{3}J = 7.0$  Hz, 1 H,  $\alpha$ -CH, Ala), 3.80-3.76 (m, 1H, δ-CH<sub>2a</sub>, Pro), 3.67-3.62 (m, 1H, δ-CH<sub>2b</sub>, Pro), 2.29-2.23 (m, 1 H, β-CH<sub>2a</sub>, Pro), 2.07-1.99 (m, 3 H, β-CH<sub>2b</sub>, γ-CH<sub>2</sub>, Pro), 1.43 (s, 9H, tBu), 1.29 (d,  ${}^{3}J = 7.0$  Hz, 3H,  $\beta$ -CH<sub>3</sub>, Ala);  ${}^{13}C$  NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta = 175.31$  (C=O, carboxylic acid), 174.04 (C=O, amide), 157.61 (C=O, carbamate), 80.50 (Ca, tBu), 60.31 (a-CH, Pro), 48.49 (α-CH, Ala), 48.01 (δ-CH<sub>2</sub>, Pro), 30.02 (β-CH<sub>2</sub>, Pro), 28.71 (CH<sub>3</sub>, tBu), 25.82 (γ-CH<sub>2</sub>, Pro), 17.12 (β-CH<sub>3</sub>, Ala); MS (EI): m/z: calcd: 286.1529; found: 286.1517; elemental analysis calcd (%) for C13H22N2O5.H2O (304.4): C 51.31, H 7.95, N 9.20; found: C 51.51, H 7.84, N 9.05.

Boc-Thr-Phe-OH (6d): Boc-Thr-Phe-OPAOB (4d; 0.100 g, 0.17 mmol) was suspended in phosphate buffer (90 mL) and methanol (10 mL) and immobilized penicillin G acylase (500 U) was added. The crude product was purified by flash chromatography using n-hexane/ethyl acetate 1:2 with 2 vol.-% acetic acid to yield a colorless oil (0.051 g, 82%).  $[\alpha]_{D}^{20} = +10.0$  $(c = 1.0 \text{ in CH}_3\text{OH}); R_f = 0.52$  (*n*-hexane/ethyl acetate 1:2 with 2 vol.-% acetic acid); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta = 7.26 - 7.17$  (m, 5H, Phe, aromatic), 4.68-4.66 (m, 1 H, α-CH, Phe), 4.08-4.06 (m, 1 H, β-CH, Thr), 4.00-3.98 (m, 1 H,  $\alpha$ -CH, Thr), 3.18 (dd,  ${}^{2}J = 13.8$  Hz,  ${}^{3}J = 5.3$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Phe), 3.04 (dd,  ${}^{2}J = 13.8$  Hz,  ${}^{3}J = 7.4$  Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Phe), 1.43 (s, 9H, tBu), 1.13 (d,  ${}^{3}J = 6.3$  Hz, 3H,  $\gamma$ -CH<sub>3</sub>, Thr);  ${}^{13}C$  NMR (125.7 MHz, CD<sub>3</sub>OD): δ = 174.49 (C=O, carboxylic acid), 172.88 (C=O, amide), 157.83 (C=O, carbamate), 138.10 (Cq, aromatic), 129.89, 129.37, 127.73 (CH, aromatic), 80.81 (C<sub>q</sub>, tBu), 68.47 (β-CH, Thr), 61.21 (α-CH, Phe), 55.10 (α-CH, Thr), 38.43 (β-CH<sub>2</sub>, Phe), 28.67 (CH<sub>3</sub>, tBu), 19.87 (γ-CH<sub>3</sub>, Thr); MS (FAB, 3-NBA): *m*/*z*: calcd for [*M*+H]+: 367.1869; found: 367.1894; elemental analysis calcd (%) for  $C_{18}H_{26}N_2O_6\boldsymbol{\cdot}H_2O$  (385.4): C 56.25, H 7.34, N 7.29; found: C 56.77, H 7.35, N 7.07.

Aloc-Val-Phe-OH (6e): Aloc-Val-Phe-OPAOB (4e; 0.286 g, 0.50 mmol) was suspended in phosphate buffer (70 mL) and acetone (30 mL) and immobilized penicillin G acylase (600 U) was added. The crude product was purified by recrystallisation using *n*-hexane/ethyl acetate to yield a colorless solid (0.068 g, 39%). M.p. 157°C;  $[\alpha]_D^{20} = -15.4$  (c = 0.5 in CH<sub>3</sub>OH);  $R_f = 0.32$  (*n*-hexane/ethyl acetate 1:1 with 2 vol.-% acetic acid); <sup>1</sup>H NMR (500 MHz, [D<sub>6</sub>]DMSO):  $\delta = 12.67$  (br, 1 H, COOH), 8.12 (d, <sup>3</sup>*J* = 7.8 Hz, 1 H, NH, amide), 7.26 – 7.17 (m, 5H, aromatic), 7.08 (d, <sup>3</sup>*J* = 9.1 Hz, 1 H, NH, carbamate), 5.93 – 5.86 (m, 1 H, CH=CH<sub>2</sub>), 5.30 – 5.16 (m, 2 H, CH=CH<sub>2</sub>), 4.50 – 4.41 (m, 3H, CH<sub>2</sub>-O, α-CH, Phe), 3.84 (dd, <sup>3</sup>*J*<sub>1</sub> = 9.0 Hz, <sup>3</sup>*J*<sub>2</sub> = 7.4 Hz, 1 H, α-CH, Val), 3.04 (dd, <sup>2</sup>*J* = 13.9 Hz, <sup>3</sup>*J* = 5.1 Hz, 1 H, β-CH<sub>2a</sub>, Phe), 2.89 (dd, <sup>3</sup>*J* = 13.9 Hz, <sup>3</sup>*J* = 9.0 Hz, 1 H, β-CH<sub>2b</sub>, NaR (125.7 MHz, [D<sub>6</sub>]DMSO):  $\delta = 172.75$  (C=O, carboxylic acid), 170.09 (C=O, amide), 155.72 (C=O, carbamate), 137.44 (C<sub>q</sub>, aromatic), 133.59

(CH, allyl), 129.07, 128.09, 126.34 (CH, aromatic), 116.86 (CH=CH<sub>2</sub>), 64.36 (CH<sub>2</sub>-O, allyl), 59.91 ( $\alpha$ -CH, Phe), 53.26 ( $\alpha$ -CH, Val), 36.71 ( $\beta$ -CH<sub>2</sub>, Phe), 30.99 ( $\beta$ -CH, Val), 19.10, 18.02 (2 × CH<sub>3</sub>, Val); MS (EI): m/z: calcd: 348.169, found: 348.170; elemental analysis calcd (%) for C<sub>18</sub>H<sub>24</sub>N<sub>2</sub>O<sub>5</sub> (348.4): C 62.05, H 6.94, N 8.04; found: C 61.76, H 6.84, N 7.32.

(Boc-Cys-OPAOB)<sub>2</sub> (11e): DIC (2.17 mL, 13.98 mmol) was added to a solution of bis(N-tert-butyloxycarbonyl)-L-cystine 9g (3.082 g, 6.99 mmol), 4-(phenylacetoxy)benzyl alcohol 10 (3.390 g, 13.98 mmol) and DMAP (0.005 g, 0.04 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and the mixture was stirred at room temperature for 18 h. Then the solution was extracted with hydrochloric acid (1M,  $2 \times 50$  mL) and water ( $2 \times 50$  mL), the organic layer was dried with MgSO4 and the solvent was removed under reduced pressure. The product was purified by flash chromatography on silica gel using nhexane/ethyl acetate 2:to yield a colorless solid (5.299 g, 86 %). M.p. 78  $^\circ\mathrm{C};$  $[a]_{D}^{20} = +42.6$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{f} = 0.43$  (n-hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 7.40 - 7.30$  (m, 10 H, C<sub>6</sub>H<sub>5</sub>, aromatic), 7.35  $(d, {}^{3}J = 8.4 \text{ Hz}, 4 \text{ H}, \text{ OCCHCH}, \text{ aromatic}), 7.05 (d, {}^{3}J = 8.4 \text{ Hz}, 4 \text{ H}, \text{ OCCH},$ aromatic), 5.42 (d,  ${}^{3}J = 7.8$  Hz, 2H, NH, carbamate), 5.14 (s, 4H, CH<sub>2</sub>-O), 4.61 - 4.59 (m, 2H,  $\alpha$ -CH), 3.86 (s, 4H, CH<sub>2</sub>-COO), 3.10 (d,  ${}^{3}J = 5.0$  Hz, 4H,  $\beta$ -CH<sub>2</sub>), 1.44 (s, 18H, *t*Bu); <sup>13</sup>C NMR (100.6 MHz, CDCl<sub>3</sub>):  $\delta$  = 170.44, 169.81 (2 × C=O, ester), 154.96 (C=O, carbamate), 150.69 (C<sub>a</sub>-O, aromatic), 133.21, 132.63 (2  $\times$  Cq, aromatic), 129.63, 129.22, 128.68, 127.33, 121.61 (CH, aromatic), 80.21 (Cq, tBu), 66.72 (CH2-O), 52.85 (a-CH), 41.28 (CH2-COO), 40.91 (β-CH<sub>2</sub>), 28.23 (CH<sub>3</sub>, tBu); MS (FAB, 3-NBA): m/z: calcd: 888.3; found: 887.8; elemental analysis calcd (%) for C<sub>46</sub>H<sub>52</sub>N<sub>2</sub>O<sub>12</sub>S<sub>2</sub> (889.1): C 62.15, H 5.90, N 3.15; found: C 62.09, H 5.99, N 3.21.

(H-Cys-OPAOB · HCl)2 (12e): A saturated solution of HCl in diethyl ether (100 mL) was added at 0°C to a solution of (Boc-Cys-OPAOB)<sub>2</sub> (11e; 4.501 g, 5.20 mmol) in diethyl ether (20 mL) and the mixture was stirred at 0°C for 45 min and at room temperature for 1 h. Then the excess HCl and the solvent were removed under reduced pressure. The residue was triturated three times with diethyl ether (20 mL) to yield the desired compound as a colorless solid (3.759 g, 95%). M.p. 140 °C;  $[\alpha]_{D}^{20} = -10.8$  $(c = 1.0 \text{ in CH}_3\text{OH})$ ; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 7.43$  (d, <sup>3</sup>J = 8.6 Hz, 4H, OCCHCH, aromatic), 7.37-7.28 (m, 10H, C<sub>6</sub>H<sub>5</sub>, aromatic), 7.06 (d, <sup>3</sup>J = 8.6 Hz, 4 H, OCCH, aromatic), 5.28 (d, <sup>2</sup>J = 12.1 Hz, 2 H, CH<sub>2a</sub>-O), 5.21 (d,  ${}^{2}J = 12.1$  Hz, 2H, CH<sub>2b</sub>-O), 4.40 (dd,  ${}^{3}J_{1} = 6.1$  Hz,  ${}^{3}J_{2} = 5.5$  Hz, 2H,  $\alpha$ -CH), 3.89 (s, 4H, CH<sub>2</sub>-COO), 3.31-3.19 (m, 4H,  $\beta$ -CH<sub>2</sub>); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta = 171.99$ , 168.76 (2 × C=O, ester), 152.56 (C<sub>a</sub>-O, aromatic), 135.02, 133.80 (2 × C<sub>q</sub>, aromatic), 131.39, 130.44, 129.69, 128.32, 122.99 (CH, aromatic), 68.78 (CH2-O), 52.81 (a-CH), 41.81 (CH2-COO), 37.52 ( $\beta$ -CH<sub>2</sub>); MS (FAB, 3-NBA): m/z: calcd for  $[M - 2HCl+H]^+$ : 689.1991; found: 689.2004; C<sub>36</sub>H<sub>36</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub> • 2 HCl (761.7).

(Boc-Ile-Cys-OPAOB)<sub>2</sub> (13a): EEDQ (1.194 g, 4.83 mmol) was added at  $0^{\circ}$ C to a solution of Boc-Ile-OH (9b; 1.117 g, 4.83 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (75 mL). Then (H-Cys-OPAOB·HCl)<sub>2</sub> (12e; 1.840 g, 2.42 mmol) and triethylamine (0.67 mL, 4.83 mmol) were added and the mixture was stirred at room temperature for 18 h. The mixture was extracted with hydrochloric acid (1m, 50 mL), NaHCO<sub>3</sub> (1m, 50 mL) and water (50 mL) and the organic layer was dried with MgSO4. Then the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using n-hexane/ethyl acetate 2:1 to yield a colorless solid (2.111 g, 78%). M.p. 139°C;  $[\alpha]_{\rm D}^{20} = +25.8 \ (c = 1.0 \text{ in CHCl}_3); R_{\rm f} = 0.34 \ (n-1)^{10}$ hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 7.51$  (d, <sup>3</sup>J = 7.2 Hz, 2H, NH, amide), 7.36 – 7.28 (m, 10H,  $C_6H_5$ , aromatic), 7.31 (d,  ${}^{3}J =$ 8.4 Hz, 4H, OCCHCH, aromatic), 7.03 (d,  ${}^{3}J = 8.4$  Hz, 4H, OCCH, aromatic), 5.40 (d,  ${}^{3}J = 8.9$  Hz, 2H, NH, carbamate), 5.14 (d,  ${}^{2}J = 12.2$  Hz, 2H, CH<sub>2a</sub>-O), 5.08 (d,  ${}^{2}J = 12.2$  Hz, 2H, CH<sub>2b</sub>-O), 4.91–4.87 (m, 2H,  $\alpha$ -CH, Cys), 4.15-4.11 (m, 2H, α-CH, Ile), 3.84 (s, 4H, CH<sub>2</sub>-COO), 3.06-2.96 (m, 4H,  $\beta$ -CH<sub>2</sub>, Cys), 1.91 – 1.79 (m, 2H,  $\beta$ -CH, Ile), 1.59 – 1.52 (m, 2H,  $\gamma$ -CH<sub>2a</sub>, Ile), 1.39 (s, 18H, *t*Bu), 1.19–1.09 (m, 2H,  $\gamma$ -CH<sub>2b</sub>, Ile), 0.92 (d, <sup>3</sup>*J*=6.8 Hz, 6H,  $\gamma$ -CH<sub>3</sub>, Ile), 0.85 (t, <sup>3</sup>J = 7.2 Hz, 6 H,  $\delta$ -CH<sub>3</sub>, Ile); <sup>13</sup>C NMR (100.6 MHz, CDCl<sub>3</sub>):  $\delta = 172.27$  (C=O, amide), 169.79 (2 × C=O, ester), 156.43 (C=O, carbamate), 150.72 (Cq-O, aromatic), 133.24, 132.64 ( $2 \times Cq$ , aromatic), 129.59, 129.23, 128.69, 127.34, 121.61 (CH, aromatic), 79.69 (C<sub>a</sub>, tBu), 66.70 (CH2-O), 58.80 (α-CH, Cys), 52.42 (α-CH, Ile), 41.31 (CH2-COO), 39.27 (β-CH<sub>2</sub>, Cys), 37.07 (β-CH, Ile), 28.27 (CH<sub>3</sub>, tBu), 24.74 (γ-CH<sub>2</sub>, Ile), 15.26, 10.97 (2 × CH<sub>3</sub>, Ile); MS (FAB, 3-NBA): m/z: calcd for  $[M+Na]^+$ : 1137.5; found: 1136.9; elemental analysis calcd (%) for C58H74N4O14S2 (1115.4): C 62.46, H 6.69, N 5.02; found: C 62.77, H 6.50, N 4.68.

(Boc-Arg(Aloc)<sub>2</sub>-Cys-OPAOB)<sub>2</sub> (13b): HOBt (0.196 g, 1.45 mmol) and DIC (0.19 mL, 1.21 mmol) were added at 0°C to a solution of Boc-Arg(Aloc)<sub>2</sub>-OH (9h; 0.535 g, 1.21 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL). Then (H-Cys- $OPAOB \cdot HCl_2$  (12e; 0.460 g, 0.61 mmol) and triethylamine (0.17 mL, 1.21 mmol) were added and the mixture was stirred at room temperature for 18 h. The mixture was extracted with hydrochloric acid (1m, 40 mL). NaHCO3 (1M, 40 mL) and brine (40 mL) and the organic layer was dried with MgSO<sub>4</sub>. Then the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using nhexane/ethyl acetate 1:1 to yield a colorless oil (0.581 g, 62 %).  $[\alpha]_D^{20} = +7.7$  $(c=1.0 \text{ in CHCl}_3); R_f=0.44$  (*n*-hexane/ethyl acetate 1:1); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 9.40$  (br, 2H, NH, guanidino), 9.24 (br, 2H, NH, guanidino), 7.42 (d, <sup>3</sup>J = 6.7 Hz, 2 H, NH, amide), 7.36 - 7.28 (m, 10 H, C<sub>6</sub>H<sub>5</sub>, aromatic), 7.31 (d,  ${}^{3}J = 8.4$  Hz, 4H, OCCHCH, aromatic), 7.03 (d,  ${}^{3}J =$ 8.4 Hz, 4H, OCCH, aromatic), 6.00-5.87 (m, 4H, CH=CH2), 5.80 (d,  ${}^{3}J = 7.8$  Hz, 2H, NH, carbamate), 5.35 – 5.16 (m, 8H, CH=CH<sub>2</sub>), 5.12 (d,  $^{2}J = 12.4$  Hz, 2H, CH<sub>2a</sub>-O, benzyl), 5.08 (d,  $^{2}J = 12.4$  Hz, 2H, CH<sub>2b</sub>-O, benzyl), 4.81-4.76 (m, 2H, α-CH, Cys), 4.67 (d, <sup>3</sup>J=5.7 Hz, 4H, CH<sub>2</sub>-O, allyl), 4.64-4.52 (m, 4H, CH2-O, allyl), 4.32-4.30 (m, 2H, α-CH, Arg), 4.04-3.86 (m, 4H, δ-CH<sub>2</sub>, Arg), 3.84 (s, 4H, CH<sub>2</sub>-COO), 3.05-3.00 (m, 4H, β-CH<sub>2</sub>, Cys), 1.81-1.79 (m, 2H, β-CH<sub>2a</sub>, Arg), 1.72-1.65 (m, 6H, β-CH<sub>2b</sub>, γ-CH<sub>2</sub>, Arg), 1.41 (s, 18 H, tBu); <sup>13</sup>C NMR (100.6 MHz, CDCl<sub>3</sub>): δ = 172.21, 169.76 (3 × C=O, 2 × ester, amide), 163.47 ( $C_q$ , guanidino), 160.56, 155.68, 155.60 (3 × C=O, carbamate), 150.69 (C<sub>q</sub>-O, aromatic), 133.23, 132.99 (2 ×  $C_q$ , aromatic), 132.61, 131.01 (2 × CH=CH<sub>2</sub>), 129.51, 129.22, 128.67, 128.46, 127.32, 121.61 (CH, aromatic), 119.43, 117.70 (2 × CH=CH<sub>2</sub>), 79.69 (C<sub>a</sub>, tBu), 67.65, 66.73, 66.07 (CH<sub>2</sub>-O, 2 × allyl, 1 × benzyl), 53.70 (α-CH, Cys), 51.89 (α-CH, Arg), 44.05 (CH<sub>2</sub>-COO), 41.28 (δ-CH<sub>2</sub>, Arg), 39.66 (β-CH<sub>2</sub>, Cys), 28.93 (β-CH<sub>2</sub>, Arg), 28.28 (CH<sub>3</sub>, tBu), 24.60 (γ-CH<sub>2</sub>, Arg); MS (FAB, 3-NBA): m/z: calcd for  $[M+H]^+$ : 1538.7; found: 1538.4; elemental analysis calcd (%) for C74H92N10O22S2 (1537.7): C 57.80, H 6.03, N 9.11; found: C 57.86, H 6.04, N 8.79.

Boc-Ile-Cys(Pal)-OPAOB (14a): DTT (0.474 g, 3.08 mmol) and triethylamine (0.21 mL, 1.54 mmol) were added under argon to a solution of (Boc-Ile-Cys-OPAOB)<sub>2</sub> (13a; 0.858 g, 0.77 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and the mixture was stirred at room temperature for 90 min. Then the solution was extracted with hydrochloric acid (1M, 3 × 30 mL) and the organic layer was dried with MgSO<sub>4</sub> and filtered. At 0°C triethylamine (0.21 mL, 1.54 mmol) and palmitoyl chloride (0.845 g, 3.08 mmol) were added to the above solution and the mixture was stirred at room temperature for 2 h. Then the solvent was removed under reduced pressure and the product was isolated by flash chromatography on silica gel using n-hexane/ethyl acetate 4:1 to yield a colorless solid (1.017 g, 83%). M.p. 59°C;  $[\alpha]_{D}^{20} = -0.7$  (c = 1.0 in CHCl<sub>3</sub>);  $R_f = 0.42$  (*n*-hexane/ethyl acetate 4:1); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 7.38 - 7.30$  (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.06 (d, <sup>3</sup>J = 8.5 Hz, 2H, OCCH, aromatic), 6.72 (d, <sup>3</sup>J = 6.5 Hz, 1H, NH, amide), 5.11 (s, 2H, CH<sub>2</sub>-O), 5.05 (d,  ${}^{3}J = 8.2$  Hz, 1H, NH, carbamate), 4.79-4.74 (m, 1H, α-CH, Cys), 3.99-3.96 (m, 1H, α-CH, Ile), 3.86 (s, 2H, CH<sub>2</sub>-COO), 3.37 (dd,  ${}^{2}J = 13.1$  Hz,  ${}^{3}J = 6.1$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.32 (dd,  ${}^{2}J = 13.1$  Hz,  ${}^{3}J = 4.6$  Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.52 (t,  ${}^{3}J = 7.5$  Hz, 2 H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.93 – 1.81 (m, 1 H, β-CH, Ile), 1.68-1.57 (m, 3 H, γ-CH<sub>2a</sub>, Ile, β-CH<sub>2</sub>, Pal), 1.44 (s, 9H, tBu), 1.25 (s, 24H, CH<sub>2</sub>, Pal), 1.17-1.08 (m, 1H, γ-CH<sub>2b</sub>, Ile), 0.91- $0.86 (m, 9H, CH_3, Ile, CH_3, Pal); {}^{13}C NMR (100.6 MHz, CDCl_3): \delta = 199.37$ (C=O, thioester), 171.45 (C=O, amide), 169.82, 169.59 (2 × C=O, ester), 155.56 (C=O, carbamate), 150.80 (C<sub>q</sub>-O, aromatic), 133.28, 132.63 ( $2 \times C_q$ , aromatic), 129.73, 129.28, 128.74, 127.40, 121.68 (CH, aromatic), 79.83 (Cq, tBu), 66.97 (CH<sub>2</sub>-O), 59.06 (α-CH, Cys), 52.41 (α-CH, Ile), 43.96 (CH<sub>2</sub>-COO), 41.38 (β-CH<sub>2</sub>, Cys), 37.40 (β-CH, Ile), 31.93 (α-CH<sub>2</sub>, Pal), 30.33, 29.69, 29.43, 29.37, 29.22, 28.92, 25.52, 24.72 (CH<sub>2</sub>, Pal), 28.31 (CH<sub>3</sub>, tBu), 22.70 ( $\gamma$ -CH<sub>2</sub>, Ile), 15.35, 14.15, 11.58 (3 × CH<sub>3</sub>, Ile, Pal); MS (EI): m/z: calcd: 796.470; found: 796.475; elemental analysis calcd (%) for C45H67N2O8S (796.1): C 67.81, H 8.60, N 3.51; found: C 67.81, H 8.55, N 3.70.

**Boc-Arg(Aloc)**<sub>2</sub>-**Cys(Pal)-OPAOB (14b)**: DTT (0.109 g, 0.71 mmol) and triethylamine (48  $\mu$ L, 0.36 mmol) were added under argon to a solution of (Boc-Arg(Aloc)<sub>2</sub>-Cys-OPAOB)<sub>2</sub> (**13b**; 0.273 g, 0.18 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL)and the mixture was stirred at room temperature for 90 min. Then the solution was extracted with hydrochloric acid (1M, 3 × 20 mL) and the organic layer was dried with MgSO<sub>4</sub>, and filtered. At 0 °C to the solution was added triethylamine (48  $\mu$ L, 0.36 mmol) and palmitoyl chloride (0.244 g, 0.89 mmol) and the mixture was stirred at room temperature for 2 h. Then the solvent was removed under reduced pressure and the product

was isolated by flash chromatography on silica gel using n-hexane/ethyl acetate 2:1 to yield a colorless solid (0.294 g, 81 %). M.p. 44  $^{\circ}\mathrm{C}; [a]_{\mathrm{D}}^{20} = +\,8.3$  $(c=1.0 \text{ in CHCl}_3); R_f=0.43$  (n-hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 9.44$  (br, 1H, NH, guanidino), 9.26 (br, 1H, NH, guanidino), 7.38-7.26 (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.10 (d, <sup>3</sup>J = 7.2 Hz, 1 H, NH, amide), 7.05 (d,  ${}^{3}J = 8.5$  Hz, 2 H, OCCH, aromatic), 6.02 – 5.89 (m, 2H, CH=CH<sub>2</sub>), 5.59 (d,  ${}^{3}J$  = 8.3 Hz, 1H, NH, carbamate), 5.37 – 5.18 (m, 4H, CH=C $H_2$ ), 5.11 (d, <sup>2</sup>J = 12.7 Hz, 1H, CH<sub>2a</sub>-O, benzyl), 5.09 (d,  $^{2}J = 12.7$  Hz, 1 H, CH<sub>2b</sub>-O, benzyl), 4.72 – 4.70 (m, 1 H,  $\alpha$ -CH, Cys), 4.63 (d, <sup>3</sup>J = 5.6 Hz, 2 H, CH<sub>2</sub>-O, allyl), 4.64 – 4.55 (m, 2 H, CH<sub>2</sub>-O, allyl), 4.24 – 4.21 (m, 1H,  $\alpha$ -CH, Arg), 4.04–4.00 (m, 1H,  $\delta$ -CH<sub>2a</sub>, Arg), 3.90–3.87 (m, 1H,  $\delta$ -CH<sub>2b</sub>, Arg), 3.85 (s, 2 H, CH<sub>2</sub>-COO), 3.38 (dd, <sup>2</sup>J = 14.1 Hz, <sup>3</sup>J = 5.0 Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.30 (dd, <sup>2</sup>J = 14.1 Hz, <sup>3</sup>J = 6.4 Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.49 (t,  ${}^{3}J = 7.5$  Hz, 2H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.80–1.78 (m, 1H,  $\beta$ -CH<sub>2a</sub>, Arg), 1.71-1.67 (m, 3H, β-CH<sub>2b</sub>, γ-CH<sub>2</sub>, Arg), 1.61-1.58 (m, 2H, β-CH<sub>2</sub>, Pal), 1.44 (s, 9 H, *t*Bu), 1.25 (s, 24 H, CH<sub>2</sub>, Pal), 0.88 (t, <sup>3</sup>*J* = 6.9 Hz, 3 H, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta$  = 198.61 (C=O, thioester), 171.92 (C=O, amide), 169.78, 169.54 (2 × C=O, ester), 163.54 ( $C_q$ , guanidino), 160.70, 155.68, 155.53 (3 × C=O, carbamate), 150.78 (C<sub>q</sub>-O, aromatic), 133.31, 133.06  $(2 \times C_q, \text{ aromatic})$ , 132.74, 131.02  $(2 \times CH=CH_2)$ , 129.60, 129.28, 128.74, 127.39, 121.64 (CH, aromatic), 119.62, 117.83 (2 × CH=CH<sub>2</sub>), 79.81 (C<sub>a</sub>, tBu), 67.77, 66.85, 66.18 (CH<sub>2</sub>-O, 2 × allyl, 1 × benzyl), 53.87 (α-CH, Cys), 52.20 (α-CH, Arg), 44.10 (CH<sub>2</sub>-COO), 43.96 (δ-CH<sub>2</sub>, Arg), 41.38 (β-CH2, Cys), 31.92 (a-CH2, Pal), 30.31, 29.69, 29.65, 29.60, 29.44, 29.35, 29.23, 28.75, 25.48, 22.69 (CH<sub>2</sub>, Pal), 28.94 (β-CH<sub>2</sub>, Arg), 28.34 (CH<sub>3</sub>, tBu), 24.60 (y-CH<sub>2</sub>, Arg), 14.13 (CH<sub>3</sub>, Pal); MS (FAB, 3-NBA): m/z: calcd for [M+H]+: 1008.536; found: 1008.535; elemental analysis calcd (%) for C<sub>53</sub>H<sub>77</sub>N<sub>5</sub>O<sub>12</sub>S (1008.3): C 63.13, H 7.69, N 6.94; found: C 63.05, H 7.92, N 6.58.

H-Ile-Cys(Pal)-OPAOB · TFA (15a): TFA (0.72 mL, 9.41 mmol) was added at 0°C to a solution of Boc-Ile-Cys(Pal)-OPAOB (14a; 0.250 g, 0.31 mmol) in CH2Cl2 (30 mL) and the mixture was stirred at 0  $^\circ C$  for 45 min and at room temperature for 60 min. TFA and the solvent were coevaporated with toluene  $(2 \times 40 \text{ mL})$  under reduced pressure to yield a colorless solid (0.255 g, 100%). M.p.  $92^{\circ}$ C;  $[\alpha]_{D}^{20} = -2.7$  (c = 1.0 in CH<sub>3</sub>OH);  $R_f = 0.06$  (*n*-hexane/ethyl acetate 4:1); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD):  $\delta = 7.41$  (d,  ${}^{3}J = 8.6$  Hz, 2H, OCCHCH, aromatic), 7.38 – 7.26 (m, 5H, C<sub>6</sub>H<sub>5</sub>, aromatic), 7.06 (d, <sup>3</sup>J = 8.6 Hz, 2H, OCCH, aromatic), 5.17 (d,  $^{2}J = 12.2$  Hz, 1 H, CH<sub>2a</sub>-O), 5.13 (d,  $^{2}J = 12.2$  Hz, 1 H, CH<sub>2b</sub>-O), 4.68 (dd,  ${}^{3}J_{1} = 7.2$  Hz,  ${}^{3}J_{2} = 5.6$  Hz, 1 H,  $\alpha$ -CH, Cys), 3.88 (s, 2 H, CH<sub>2</sub>-COO), 3.72 (d,  ${}^{3}J = 5.2$  Hz, 1 H,  $\alpha$ -CH, Ile), 3.44 (dd,  ${}^{2}J = 14.0$  Hz,  ${}^{3}J = 5.6$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.25 (dd,  ${}^{2}J = 14.0$  Hz,  ${}^{3}J = 7.2$  Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.53 (t,  ${}^{3}J =$ 7.1 Hz, 2H, α-CH<sub>2</sub>, Pal), 1.91-1.83 (m, 1H, β-CH, Ile), 1.62-1.58 (m, 2H, β-CH<sub>2</sub>, Pal), 1.55-1.47 (m, 1 H, γ-CH<sub>2a</sub>, Ile), 1.27 (s, 24 H, CH<sub>2</sub>, Pal), 1.21-1.11 (m, 1H,  $\gamma$ -CH<sub>2b</sub>, Ile), 0.96 (d,  ${}^{3}J = 6.9$  Hz, 3H,  $\gamma$ -CH<sub>3</sub>, Ile), 0.92-0.87 (m, 6H,  $\delta$ -CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (100.6 MHz, CD<sub>3</sub>OD):  $\delta$  = 199.85 (C=O, thioester), 171.64 (C=O, amide), 170.68, 169.44 (2 × C=O, ester), 152.32 (C<sub>q</sub>-O, aromatic), 135.04, 134.45 ( $2 \times C_q$ , aromatic), 130.87, 130.40, 129.66, 128.28, 122.78 (CH, aromatic), 67.84 (CH<sub>2</sub>-O), 58.77 (α-CH, Cys), 53.78 (α-CH, Ile), 44.67 (CH<sub>2</sub>-COO), 41.85 (β-CH<sub>2</sub>, Cys), 38.07 (β-CH, Ile), 33.05 (a-CH<sub>2</sub>, Pal), 30.75, 30.52, 30.46, 30.34, 30.22, 29.92, 26.53, 26.06, 25.27 (CH<sub>2</sub>, Pal), 23.72 (γ-CH<sub>2</sub>, Ile), 14.94, 14.50, 11.77 (3 × CH<sub>3</sub>, Ile, Pal); MS (FAB, 3-NBA): m/z: calcd for  $[M - CF_3COO]^+$ : 697.4250; found: 697.4270;  $C_{40}H_{60}N_2O_6S \cdot C_2F_3O_2$  (810.0).

H-Arg(Aloc)<sub>2</sub>-Cys(Pal)-OPAOB · TFA (15b): TFA (0.62 mL, 8.11 mmol) was added at 0 °C to a solution of Boc-Arg(Aloc)<sub>2</sub>-Cys(Pal)-OPAOB (14b; 0.272 g, 0.27 mmol) in CH2Cl2 (3 mL) and the mixture was stirred at 0 °C for 45 min and at room temperature for 60 min. TFA and the solvent were coevaporated with toluene  $(2 \times 10 \text{ mL})$  under reduced pressure to yield a colorless solid (0.288 g, quant.).  $[\alpha]_{D}^{20} = -13.7 (c = 1.0 \text{ in CH}_{3}\text{OH}); R_{f} = 0.03$ (*n*-hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta = 7.40$  (d, <sup>3</sup>J = 8.6 Hz, 2H, OCCHCH, aromatic), 7.37 – 7.28 (m, 5H, C<sub>6</sub>H<sub>5</sub>, aromatic), 7.06 (d,  ${}^{3}J = 8.6$  Hz, 2H, OCCH, aromatic), 5.98–5.94 (m, 2H, CH=CH<sub>2</sub>), 5.37 – 5.20 (m, 4H, CH=C $H_2$ ), 5.16 (d, <sup>2</sup>J = 12.7 Hz, 1H, CH<sub>2a</sub>-O, benzyl), 5.11 (d,  ${}^{2}J = 12.7$  Hz, 1 H, CH<sub>2b</sub>-O, benzyl), 4.71 (dt,  ${}^{3}J = 5.6$  Hz,  ${}^{4}J = 1.3$  Hz, 2 H, CH<sub>2</sub>-O, allyl), 4.67 (dd,  ${}^{3}J_{1} = 7.3$  Hz,  ${}^{3}J_{2} = 5.4$  Hz, 1 H,  $\alpha$ -CH, Cys), 4.60-4.58 (m, 2H, CH<sub>2</sub>-O, allyl), 3.98-3.95 (m, 1H, α-CH, Arg), 3.88 (s, 2H, CH<sub>2</sub>-COO), 3.87 - 3.84 (m, 2H,  $\delta$ -CH<sub>2</sub>, Arg), 3.45 (dd, <sup>2</sup>J = 14.0 Hz,  ${}^{3}J = 5.4$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.24 (dd,  ${}^{2}J = 14.0$  Hz,  ${}^{3}J = 7.4$  Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.53 (t,  ${}^{3}J$  = 7.0 Hz, 2 H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.90 – 1.82 (m, 1 H,  $\beta$ -CH<sub>2a</sub>, Arg), 1.81-1.73 (m, 3H, β-CH<sub>2b</sub>, γ-CH<sub>2</sub>, Arg), 1.62-1.57 (m, 2H, β-CH<sub>2</sub>,

Pal), 1.27 (s, 24 H, CH<sub>2</sub>, Pal), 0.89 (t,  ${}^{3}J = 6.9$  Hz, 3 H, CH<sub>3</sub>, Pal);  ${}^{13}C$  NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta = 199.58$  (C=O, thioester), 171.55 (C=O, amide), 170.62, 170.08 (2 × C=O, ester), 163.06 (C<sub>q</sub>, guanidino), 161.28, 156.08, (2 × C=O, carbamate), 152.19 (C<sub>q</sub>-O, aromatic), 134.93, 134.38 (2 × C<sub>q</sub>, aromatic), 134.01, 132.53 (2 × CH=CH<sub>2</sub>), 130.83, 130.62, 130.33, 129.60, 128.22, 122.73 (CH, aromatic), 119.75, 118.47 (2 × CH=CH<sub>2</sub>), 69.09, 67.77, 67.29 (CH<sub>2</sub>-CO, 2 × allyl, 1 × benzyl), 53.70 (*a*-CH, Cys), 53.42 (*a*-CH, Arg), 45.03 (CH<sub>2</sub>-COO), 44.62 ( $\delta$ -CH<sub>2</sub>, Arg), 41.80 ( $\beta$ -CH<sub>2</sub>, Cys), 32.96 (*a*-CH<sub>2</sub>, Pal), 30.67, 30.66, 30.60, 30.44, 30.37, 30.26, 30.15, 29.85, 26.43, 25.95, 23.63 (CH<sub>2</sub>, Pal), 28.92 ( $\beta$ -CH<sub>2</sub>, Arg), 24.47 ( $\gamma$ -CH<sub>2</sub>, Arg), 14.44 (CH<sub>3</sub>, Pal); MS (FAB, 3-NBA): *m*/*z*: calcd for [*M* – CF<sub>3</sub>COO]<sup>+</sup>: 909.4922; found: 909.4897; C<sub>44</sub>H<sub>70</sub>N<sub>5</sub>O<sub>10</sub>S + C<sub>2</sub>F<sub>3</sub>O<sub>2</sub> (1022.2).

Boc-Thr-Ile-Cys(Pal)-OPAOB (16a): EEDQ (53 mg, 0.22 mmol) was added at 0°C to a solution of Boc-Thr-OH (9f; 47 mg, 0.22 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL). Then H-Ile-Cys(Pal)-OPAOB • TFA (15a; 174 mg, 0.22 mmol) and triethylamine (31 µL, 0.22 mmol) were added and the mixture was stirred at room temperature for 18 h. Then the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using n-hexane/ethyl acetate 2:1 to yield a colorless solid (146 mg, 74%). M.p. 94°C;  $[\alpha]_{D}^{20} = -27.4$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{\rm f} = 0.19$  (*n*-hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta =$ 7.38–7.30 (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.06 (d,  ${}^{3}J = 8.5$  Hz, 2H, OCCH, aromatic), 6.95 (d,  ${}^{3}J = 8.3$  Hz, 1 H, NH, amide), 6.93 (d,  ${}^{3}J =$ 7.2 Hz, 1H, NH, amide), 5.51 (d,  ${}^{3}J = 7.9$  Hz, 1H, NH, carbamate), 5.11 (s, 2H, CH<sub>2</sub>-O), 4.76-4.72 (m, 1H, α-CH, Cys), 4.31-4.28 (m, 2H, α-CH, Thr,  $\beta$ -CH, Thr), 4.10 (d,  ${}^{3}J = 6.6$  Hz, 1H,  $\alpha$ -CH, Ile), 3.85 (s, 2H, CH<sub>2</sub>-COO), 3.55 (br, 1 H, OH, Thr), 3.32 (d,  ${}^{3}J = 5.6$  Hz, 2 H,  $\beta$ -CH<sub>2</sub>, Cys), 2.53 (t,  ${}^{3}J = 7.6$  Hz, 2H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.98–1.91 (m, 1H,  $\beta$ -CH, Ile), 1.63–1.58 (m, 2H, β-CH<sub>2</sub>, Pal), 1.45 (s, 9H, tBu), 1.31-1.19 (m, 1H, γ-CH<sub>2a</sub>, Ile), 1.25  $(s, 24 H, CH_2, Pal), 1.17 (d, {}^{3}J = 6.4 Hz, 3 H, \gamma - CH_3, Thr), 1.14 - 1.08 (m, 1 H,$ γ-CH<sub>2b</sub>, Ile), 0.90-0.86 (m, 9H, CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta = 199.75$  (C=O, thioester), 171.78, 170.83 (2 × C=O, amide), 169.81, 169.62 (2 × C=O, ester), 156.40 (C=O, carbamate), 150.86 (C<sub>q</sub>-O, aromatic), 133.30, 132.62 (2  $\times$  C $_{\rm q},$  aromatic), 129.70, 129.29, 128.76, 127.41, 121.70 (CH, aromatic), 80.32 (C<sub>a</sub>, tBu), 67.01 (CH<sub>2</sub>-O), 66.74 (β-CH, Thr), 57.98 (a-CH, Cys), 57.65 (a-CH, Thr), 52.70 (a-CH, Ile), 44.00 (CH<sub>2</sub>-COO), 41.40 (β-CH<sub>2</sub>, Cys), 36.71 (β-CH, Ile), 31.93 (α-CH<sub>2</sub>, Pal), 30.18, 29.69, 29.65, 29.61, 29.44, 29.36, 29.24, 28.96, 25.56 24.52 (CH22, Pal), 28.31 (CH33, tBu), 22.70 (γ-CH<sub>2</sub>, Ile), 18.23 (γ-CH<sub>3</sub>, Thr), 15.42, 14.13, 11.43 (3 × CH<sub>3</sub>, Ile, Pal); MS (FAB, 3-NBA): m/z: calcd for  $[M+H]^+$ : 898.5251; found: 898.5217; elemental analysis calcd (%) for C49H75N3O10S (898.2): C 65.52, H 8.41, N 4.67; found: C 65.05, H 8.74, N 4.20.

Boc-Met-Arg(Aloc)<sub>2</sub>-Cys(Pal)-OPAOB (16b): HOBt (35 mg, 0.27 mmol) and DIC (33 µL, 0.22 mmol) were added at 0 °C to a solution of Boc-Met-OH (9i; 55 mg, 0.22 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (25 mL). Then H-Arg(Aloc)<sub>2</sub>-Cys(Pal)-OPAOB · TFA (15b; 225 mg, 0.22 mmol) and triethylamine (33 µL, 0.22 mmol) were added and the mixture was stirred at room temperature for 18 h. Then the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using n-hexane/ethyl acetate 2:1 to yield a colorless solid (163 mg, 65%).  $[\alpha]_{D}^{20} = -13.9$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{f} = 0.19$  (n-hexane/ethyl acetate 2:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 9.47$  (br, 1 H, NH, guanidino), 9.29 (br, 1H, NH, guanidino), 7.39-7.32 (m, 7H, C<sub>6</sub>H<sub>5</sub>, OCCHCH, aromatic), 7.31 (d,  ${}^{3}J = 6.1$  Hz, 1 H, NH, amide), 7.17 (d,  ${}^{3}J = 7.8$  Hz, 1 H, NH, amide), 7.05 (d, <sup>3</sup>*J* = 8.5 Hz, 2H, OCC*H*, aromatic), 6.02 - 5.89 (m, 2H, C*H*=CH<sub>2</sub>), 5.37 -5.19 (m, 4H, CH=CH<sub>2</sub>), 5.11 (d, <sup>2</sup>J = 12.4 Hz, 1H, CH<sub>2a</sub>-O, benzyl), 5.08 (d,  $^{2}J = 12.4$  Hz, 1 H, CH<sub>2b</sub>-O, benzyl), 4.69 (d,  $^{3}J = 5.7$  Hz, 2 H, CH<sub>2</sub>-O, allyl),  $4.68-4.64\ (m,1\,H,\alpha\text{-CH},Cys), 4.62-4.56\ (m,2\,H,CH_2\text{-}O,allyl), 4.53-4.50$ (m, 1H, α-CH, Arg), 4.27-4.25 (m, 1H, α-CH, Met), 3.99-3.95 (m, 2H, δ-CH<sub>2</sub>, Arg), 3.86 (s, 2 H, CH<sub>2</sub>-COO), 3.37 (dd, <sup>2</sup>J = 14.0 Hz, <sup>3</sup>J = 4.9 Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.29 (dd, <sup>2</sup>J = 14.0 Hz, <sup>3</sup>J = 6.6 Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.54 (t,  ${}^{3}J = 7.2$  Hz, 2H,  $\gamma$ -CH<sub>2</sub>, Met), 2.51 (t,  ${}^{3}J = 7.7$  Hz, 2H,  $\alpha$ -CH<sub>2</sub>, Pal), 2.12 – 2.05 (m, 1H, β-CH<sub>2a</sub>, Met), 2.09 (s, 3H, SCH<sub>3</sub>), 1.94-1.87 (m, 1H, β-CH<sub>2b</sub>, Met), 1.84-1.81 (m, 1 H, β-CH<sub>2a</sub>, Arg), 1.73-1.66 (m, 3 H, β-CH<sub>2b</sub>, γ-CH<sub>2</sub>, Arg), 1.61 – 1.55 (m, 2 H,  $\beta$ -CH<sub>2</sub>, Pal), 1.42 (s, 9 H, *t*Bu), 1.25 (s, 24 H, CH<sub>2</sub>, Pal), 0.88 (t, <sup>3</sup>*J* = 6.9 Hz, 3H, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta = 198.73$  (C=O, thioester), 171.58, 171.13 (2 × C=O, amide), 169.81, 169.45  $(2 \times C=0, \text{ ester})$ , 163.46 (C<sub>q</sub>, guanidino), 160.91, 155.67 (3 × C=O, carbamate), 150.79 (C<sub>a</sub>-O, aromatic), 133.30, 132.72 (2 × C<sub>a</sub>, aromatic), 133.03, 131.00 (2 × CH=CH<sub>2</sub>), 129.65, 129.29, 128.75, 127.40, 121.65 (CH, aromatic), 119.63, 118.03 (2 × CH=CH<sub>2</sub>), 80.05 (C<sub>q</sub>, tBu), 67.82, 66.89, 66.22 (CH<sub>2</sub>-O, 2 × allyl, 1 × benzyl), 53.52 (*a*-CH, Cys), 52.67 (*a*-CH, Arg), 52.36 (*a*-CH, Met), 44.05, 44.00 (CH<sub>2</sub>-COO,  $\delta$ -CH<sub>2</sub>, Arg), 41.39 ( $\beta$ -CH<sub>2</sub>, Cys), 31.92, 31.73 (*a*-CH<sub>2</sub>, Pal,  $\gamma$ -CH<sub>2</sub>, Met), 30.17 ( $\beta$ -CH<sub>2</sub>, Met), 29.69, 29.66, 29.62, 29.45, 29.36, 29.25, 28.50, 25.50, 22.69 (CH<sub>2</sub>, Pal), 28.96 ( $\beta$ -CH<sub>2</sub>, Arg), 28.28 (CH<sub>3</sub>, *t*Bu), 24.55 ( $\gamma$ -CH<sub>2</sub>, Arg), 15.32 (SCH<sub>3</sub>, Met), 14.13 (CH<sub>3</sub>, Pal); MS (FAB, 3-NBA): *m*/*z*: calcd for C<sub>38</sub>H<sub>86</sub>N<sub>6</sub>O<sub>13</sub>S<sub>2</sub>: 1139.6; found: 1139.5 [*M*+H]<sup>+</sup>.

Boc-Thr-Ile-Cys(Pal)-OH (17a): Boc-Thr-Ile-Cys(Pal)-OPAOB (16a; 0.050 g, 0.06 mmol) was added to a solution of dimethyl- $\beta$ -cyclodextrin (2.226 g, 1.67 mmol) in phosphate buffer (100 mL, 0.05 м, pH 7) and the mixture was sonicated until a clear solution resulted. Then immobilized penicillin G acylase (500 U) was added and the reaction mixture was shaken at room temperature for 24 h. After filtering off the enzyme the pH of the aqueous phase was adjusted to pH 2, the aqueous solution was treated with benzyltriethylammonium bromide (0.909 g, 3.34 mmol) and extracted with diethyl ether (5  $\times$  30 mL). The combined organic layers were dried with MgSO<sub>4</sub> and the solvent was removed under reduced pressure. The resulting crude product was triturated with *n*-hexane  $(3 \times 5 \text{ mL})$  to separate the desired compound from side products to yield a colorless solid (31 mg, 81 %). M.p. 128 °C;  $[\alpha]_{\rm D}^{20} = -13.4$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{\rm f} = 0.03$  (nhexane/ethyl acetate 1:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 7.38$  (d, <sup>3</sup>J = 6.8 Hz, 1 H, NH, amide), 7.30 (d,  ${}^{3}J = 8.7$  Hz, 1 H, NH, amide), 5.66 (d,  ${}^{3}J =$ 8.2 Hz, 1 H, NH, carbamate), 4.66 – 4.62 (m, 1 H,  $\alpha$ -CH, Cys), 4.35 (dd,  ${}^{3}J_{1} =$ 8.7 Hz,  ${}^{3}J_{2} = 7.6$  Hz, 1 H,  $\alpha$ -CH, Thr), 4.31–4.27 (m, 1 H,  $\beta$ -CH, Thr), 4.18 (d,  ${}^{3}J = 6.2$  Hz, 1 H,  $\alpha$ -CH, Ile), 3.48 (dd,  ${}^{2}J = 14.1$  Hz,  ${}^{3}J = 4.4$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.30 (dd,  ${}^{2}J = 14.1$  Hz,  ${}^{3}J = 7.4$  Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.57 (t,  ${}^{3}J = 7.5$  Hz, 2H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.98–1.94 (m, 1H,  $\beta$ -CH, Ile), 1.67–1.61 (m, 2H, β-CH<sub>2</sub>, Pal), 1.45 (s, 9H, tBu), 1.31-1.27 (m, 1H, γ-CH<sub>2a</sub>, Ile), 1.25 (s, 24 H, CH<sub>2</sub>, Pal), 1.17 (d,  ${}^{3}J = 6.3$  Hz, 3 H,  $\gamma$ -CH<sub>3</sub>, Thr), 1.15 – 1.09 (m, 1 H,  $\gamma$ -CH<sub>2b</sub>, Ile), 0.92 (d,  ${}^{3}J = 6.8$  Hz, 3H,  $\gamma$ -CH<sub>3</sub>, Ile), 0.90–0.87 (m, 6H,  $\delta$ -CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta = 200.27$  (C=O, thioester),  $171.91, 171.76 (2 \times C=O, amide), 156.45 (C=O, carbamate), 80.55 (C_a, tBu),$ 66.14 (β-CH, Thr), 58.08 (α-CH, Cys), 57.96 (α-CH, Thr), 53.06 (α-CH, Ile), 44.05 (β-CH<sub>2</sub>, Cys), 36.67 (β-CH, Ile), 31.93 (α-CH<sub>2</sub>, Pal), 29.84, 29.70, 29.66, 29.63, 29.47, 29.36, 29.27, 29.02, 25.58, 24.52 (CH<sub>2</sub>, Pal), 28.31 (CH<sub>3</sub>, tBu), 22.69 (γ-CH<sub>2</sub>, Ile), 18.29 (γ-CH<sub>3</sub>, Thr), 15.40, 14.12, 11.30 (3 × CH<sub>3</sub>, Ile, Pal); MS (FAB, 3-NBA): m/z: calcd for  $[M+Na]^+$ : 696.4234; found: 698.4227; elemental analysis calcd (%) for C34H63N3O8S (674.0): C 60.59, H 9.42, N 6.23; found: C 60.44, H 9.10, N 6.13.

Boc-Arg(Aloc)<sub>2</sub>-Cys(Pal)-OH (18): Boc-Arg(Aloc)<sub>2</sub>-Cys(Pal)-OPAOB (14b; 5 mg, 5  $\mu$ mol) was added to a solution of dimethyl- $\beta$ -cyclodextrin (200 mg, 150 µmol) in phosphate buffer (2 mL, 0.05 M, pH 7) and the mixture was sonicated until a clear solution resulted. Then immobilized penicillin G acylase (50 U) was added and the reaction mixture was shaken at room temperature for 24 h. After the enzyme was filtered off, the pH of the aqueous phase was adjusted to 2, and the aqueous solution was treated with benzyltriethylammonium bromide (81 mg, 300 µmol) and extracted with diethyl ether (5  $\times$  10 mL). The combined organic layers were dried with  $MgSO_4$  and the solvent was removed under reduced pressure. The resulting crude product was triturated with *n*-hexane  $(3 \times 1 \text{ mL})$  to separate the desired compound from side products to yield a colorless solid (3 mg. 77%). M.p. 83°C;  $[\alpha]_{D}^{20} = +11.7$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{f} = 0.10$  (n-hexane/ ethyl acetate 1:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 5:1):  $\delta = 6.02 - 5.94$  $(m, 2H, CH=CH_2), 5.40-5.22 (m, 4H, CH=CH_2), 4.74 (dt, {}^{3}J=5.8 Hz, {}^{4}J=$ 1.1 Hz, 2H, CH<sub>2</sub>-O, allyl), 4.64 (dd,  ${}^{3}J_{1} = 7.2$  Hz,  ${}^{3}J_{2} = 4.6$  Hz, 1H,  $\alpha$ -CH, Cys), 4.62 (dt,  ${}^{3}J = 4.5$  Hz,  ${}^{4}J = 1.3$  Hz, 2H, CH<sub>2</sub>-O, allyl), 4.12-4.08 (m, 1H,  $\alpha$ -CH, Arg), 4.01–3.96 (m, 1H,  $\delta$ -CH<sub>2a</sub>, Arg), 3.93–3.89 (m, 1H,  $\delta$ -CH<sub>2b</sub>, Arg), 3.49 (dd,  ${}^{2}J = 13.9$  Hz,  ${}^{3}J = 4.7$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.24 (dd,  $^{2}J = 13.9$  Hz,  $^{3}J = 7.2$  Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.55 (t,  $^{3}J = 7.6$  Hz, 2 H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.80-1.75 (m, 1H, β-CH<sub>2a</sub>, Arg), 1.72-1.68 (m, 1H, β-CH<sub>2b</sub>, Arg),  $1.66 - 1.60 \text{ (m, 4H, } \beta\text{-CH}_2, \text{Pal, } \gamma\text{-CH}_2, \text{Arg}), 1.45 \text{ (s, 9H, } t\text{Bu}), 1.26 \text{ (s, 24H, } t$ CH<sub>2</sub>, Pal), 0.88 (t,  ${}^{3}J = 6.9$  Hz, 3H, CH<sub>3</sub>, Pal);  ${}^{13}C$  NMR (125.7 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 5:1): δ = 199.10 (C=O, thioester), 172.72 (C=O, carboxylic acid), 171.36 (C=O, amide), 163.38 (Cq, guanidino), 160.53, 156.09, 155.60  $(3 \times C=0, \text{ carbamate}), 132.80, 130.99 (2 \times CH=CH_2), 119.53, 117.74 (2 \times CH=CH_2), 119.53, 117.74 (2 \times CH=CH_2))$ CH=CH<sub>2</sub>), 79.91 (C<sub>q</sub>, tBu), 67.85, 66.28 (2 × CH<sub>2</sub>-O, allyl), 54.28 ( $\alpha$ -CH, Cys), 51.89 (α-CH, Arg), 44.42 (δ-CH<sub>2</sub>, Arg), 43.90 (β-CH<sub>2</sub>, Cys), 31.84 (α-CH<sub>2</sub>, Pal), 30.21, 30.13, 29.59, 29.56, 29.51, 29.34, 29.26, 29.15, 29.02, 28.13, 25.46, 23.76, 22.58 (CH<sub>2</sub>, Pal), 28.88 (β-CH<sub>2</sub>, Arg), 28.13 (CH<sub>3</sub>, tBu), 24.82 (y-CH<sub>2</sub>, Arg), 13.88 (CH<sub>3</sub>, Pal); MS (FAB, 3-NBA): *m*/*z*: calcd: 784.4530; found: 784.4556  $[M+H]^+$ ; elemental analysis calcd (%) for  $C_{38}H_{65}N_5O_{10}S$ (784.0): C 58.22, H 8.36, N 8.93; found C 58.09, H 8.43, N 8.72.

Boc-Thr-Ile-Cys(Pal)-Ile-OAll (19): Triethylamine (20 µL, 0.14 mmol) and EEDQ (35 mg, 0.14 mmol) were added at 0 °C to a solution of Boc-Thr-Ile-Cys(Pal)-OH (17a; 80 mg, 0.12 mmol) and L-isoleucine-allylester tosylate (12 f; 42 mg, 0.12 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and the mixture was stirred for 18 h at room temperature. The solvent was removed under reduced pressure and the product was purified by flash chromatography on silica gel using *n*-hexane/acetone 7:1 to yield a colorless solid (70 mg, 71 %).  $[\alpha]_{\rm D}^{20} =$ -39.9 (c = 1.0 in CH<sub>3</sub>OH);  $R_{\rm f} = 0.13$  (n-hexane/acetone 7:1); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta = 5.96$  (ddt,  ${}^{3}J_{\text{trans}} = 17.2$  Hz,  ${}^{3}J_{\text{cis}} = 10.4$  Hz,  ${}^{3}J_{\text{vic}} = 10.4$  5.7 Hz, 1 H, CH=CH<sub>2</sub>), 5.36 (d,  ${}^{3}J_{\text{trans}} = 17.2$  Hz, 1 H, CH=CH<sub>2a</sub>), 5.24 (d,  ${}^{3}J_{cis} = 10.4 \text{ Hz}, 1 \text{ H}, \text{ CH}=CH_{2b}$ , 4.65 (dd,  ${}^{2}J = 13.2 \text{ Hz}, {}^{3}J = 5.8 \text{ Hz}, 1 \text{ H}$ ,  $CH_{2a}$ -O), 4.62 (dd,  ${}^{2}J = 13.2$  Hz,  ${}^{3}J = 5.7$  Hz, 1 H,  $CH_{2b}$ -O), 4.56 (dd,  ${}^{3}J_{1} =$ 8.4 Hz,  ${}^{3}J_{2} = 5.6$  Hz, 1 H,  $\alpha$ -CH, Cys), 4.38 (d,  ${}^{3}J = 6.0$  Hz, 1 H,  $\alpha$ -CH, Ile<sub>1</sub>), 4.26 (d,  ${}^{3}J = 6.7$  Hz, 1 H,  $\alpha$ -CH, Ile<sub>2</sub>), 4.16 – 4.13 (m, 1 H,  $\beta$ -CH, Thr), 4.08 (d,  ${}^{3}J = 4.0$  Hz, 1 H,  $\alpha$ -CH, Thr), 3.33 (dd,  ${}^{2}J = 13.9$  Hz,  ${}^{3}J = 5.6$  Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.13 (dd,  ${}^{2}J = 13.9$  Hz,  ${}^{3}J = 8.4$  Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.56 (t,  $^{3}J = 7.4$  Hz, 2 H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.95 – 1.88 (m, 1 H,  $\beta$ -CH, Ile<sub>1</sub>), 1.88 – 1.81 (m, 1 H,  $\beta$ -CH, Ile<sub>2</sub>), 1.65 – 1.61 (m, 2H,  $\beta$ -CH<sub>2</sub>, Pal), 1.58 – 1.42 (m, 2H,  $2 \times \gamma$ -CH<sub>2a</sub>, Ile), 1.46 (s, 9 H, *t*Bu), 1.28 (s, 24 H, CH<sub>2</sub>, Pal), 1.26 – 1.15 (m, 2 H, 2 ×  $\gamma$ -CH<sub>2b</sub>, Ile), 1.18 (d,  ${}^{3}J = 6.2$  Hz, 3H,  $\gamma$ -CH<sub>3</sub>, Thr), 0.93-0.88 (m, 15 H, CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta = 200.22$  (C=O, thioester), 173.27, 172.12, 171.92 (3 × C=O, amide), 171.78 (C=O, ester), 157.96 (C=O, carbamate), 133.25 (CH=CH<sub>2</sub>), 118.97 (CH=CH<sub>2</sub>), 80.79 (C<sub>q</sub>, tBu), 68.48 (β-CH, Thr), 66.63 (CH2-O), 61.05 (α-CH, Cys), 59.23 (α-CH, Ile), 58.34 (α-CH, Ile), 53.97 (α-CH, Thr), 44.74 (β-CH<sub>2</sub>, Cys), 38.32, 38.20  $(2 \times \beta$ -CH, Ile), 33.02 ( $\alpha$ -CH<sub>2</sub>, Pal), 31.09, 30.71, 30.67, 30.50, 30.42, 30.35, 29.97, 26.53, 26.23 (CH<sub>2</sub>, Pal), 28.71 (CH<sub>3</sub>, tBu), 25.73, 23.69 (2 × γ-CH<sub>2</sub>, Ile), 20.01 ( $\gamma$ -CH<sub>3</sub>, Thr), 15.95, 14.46, 11.75, 11.69 (5 × CH<sub>3</sub>, Ile, Pal); MS (EI): *m/z*: calcd for C<sub>43</sub>H<sub>78</sub>N<sub>4</sub>O<sub>9</sub>S: 826.5490; found: 826.5487.

H-Thr-Ile-Cys(Pal)-Ile-OAll · TFA (20): TFA (0.12 mL, 1.60 mmol) was added at 0°C to a solution of Boc-Thr-Ile-Cys(Pal)-Ile-OAll (19; 44 mg, 0.05 mmol) in  $CH_2Cl_2$  (0.50 mL) and the mixture was stirred at 0 °C for 45 min and at room temperature for 60 min. TFA and the solvent were coevaporated with toluene  $(2 \times 10 \text{ mL})$  under reduced pressure to yield a colorless solid (45 mg, quant.).  $[\alpha]_{D}^{20} = -32.7$  (c = 1.0 in CH<sub>3</sub>OH);  $R_{f} = 0.06$ (*n*-hexane/acetone 7:1); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta = 5.96$  (ddt,  ${}^{3}J_{\text{trans}} = 17.1 \text{ Hz}, \; {}^{3}J_{\text{cis}} = 10.6 \text{ Hz}, \; {}^{3}J_{\text{vic}} = 5.9 \text{ Hz}, \; 1 \text{ H}, \; \text{CH=CH}_{2}$ ), 5.36 (d,  ${}^{3}J_{\text{trans}} = 17.1 \text{ Hz}, 1 \text{ H}, \text{ CH}=CH_{2a}), 5.25 \text{ (d, } {}^{3}J_{\text{cis}} = 10.6 \text{ Hz}, 1 \text{ H}, \text{ CH}=CH_{2b}),$ 4.65 (dd,  ${}^{2}J = 13.3$  Hz,  ${}^{3}J = 5.8$  Hz, 1 H, CH<sub>2a</sub>-O), 4.63 (dd,  ${}^{2}J = 13.3$  Hz,  ${}^{3}J = 13.3$ 5.8 Hz, 1 H,  $CH_{2b}$ -O), 4.57 (dd,  ${}^{3}J_{1} = 7.9$  Hz,  ${}^{3}J_{2} = 6.2$  Hz, 1 H,  $\alpha$ -CH, Cys), 4.39 (d,  ${}^{3}J = 6.0$  Hz, 1 H,  $\alpha$ -CH, Ile<sub>1</sub>), 4.29 (d,  ${}^{3}J = 7.4$  Hz, 1 H,  $\alpha$ -CH, Ile<sub>2</sub>), 4.07 – 4.04 (m, 1 H,  $\beta$ -CH, Thr), 3.75 (d,  ${}^{3}J$  = 6.6 Hz, 1 H,  $\alpha$ -CH, Thr), 3.30  $(dd, {}^{2}J = 13.8 Hz, {}^{3}J = 6.2 Hz, 1 H, \beta$ -CH<sub>2a</sub>, Cys), 3.14  $(dd, {}^{2}J = 13.8 Hz, {}^{3}J =$ 7.9 Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.57 (t,  ${}^{3}J = 8.0$  Hz, 2 H,  $\alpha$ -CH<sub>2</sub>, Pal), 1.93 – 1.87 (m, 1H,  $\beta\text{-CH},\,{\rm Ile_1}),\,1.87-1.80$  (m, 1H,  $\beta\text{-CH},\,{\rm Ile_2}),\,1.65-1.63$  (m, 2H,  $\beta\text{-}$ CH<sub>2</sub>, Pal), 1.59–1.42 (m, 2H,  $2 \times \gamma$ -CH<sub>2a</sub>, Ile), 1.31 (d,  ${}^{3}J = 3.8$  Hz, 3H,  $\gamma$ -CH<sub>3</sub>, Thr), 1.29 (s, 24 H, CH<sub>2</sub>, Pal), 1.27-1.14 (m, 2H, 2×γ-CH<sub>2b</sub>, Ile), 0.95-0.89 (m, 15H, CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta = 200.23$  (C=O, thioester), 172.77, 172.16, 171.68 (3 × C=O, amide), 168.44 (C=O, ester), 133.19 (CH=CH<sub>2</sub>), 119.03 (CH=CH<sub>2</sub>), 67.56 ( $\beta$ -CH, Thr), 66.66 (CH2-O), 59.88 (a-CH, Cys), 59.35 (a-CH, Ile), 58.28 (a-CH, Ile), 53.81 ( $\alpha$ -CH, Thr), 44.73 ( $\beta$ -CH<sub>2</sub>, Cys), 38.35, 38.12 ( $2 \times \beta$ -CH, Ile), 33.00 (a-CH<sub>2</sub>, Pal), 31.14, 30.70, 30.65, 30.49, 30.40, 30.34, 29.95, 26.53, 26.19 (CH<sub>2</sub>, Pal), 25.76, 23.67 (2 × γ-CH<sub>2</sub>, Ile), 20.19 (γ-CH<sub>3</sub>, Thr), 15.96, 15.84, 14.44, 11.70, 11.52 (5 × CH<sub>3</sub>, Ile, Pal); MS (FAB, 3-NBA): m/z: calcd for  $[M - CF_3COO]^+$ : 728.5122; found: 728.5154;  $C_{38}H_{71}N_4O_7S \cdot C_2F_3O_2$  (841.1).

**NBDAca-Thr-Ile-Cys(Pal)-Ile-OAll (21):** HOBt (7 mg, 0.04 mmol), triethylamine (5 µL, 0.04 mmol) and EDC (7 mg, 0.04 mmol) were added at 0 °C to a solution of H-Thr-Ile-Cys(Pal)-Ile-OAll •TFA (**20**; 30 mg, 0.04 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and the mixture was stirred for 18 h at room temperature. The solvent was removed under reduced pressure and the product was purified by flash chromatography on silica gel using chloroform/methanol 50:1 to yield an orange solid (31 mg, 86%). M.p. 144 °C;  $[a]_D^{20} = -91.6$  (c = 1.0 in CHCl<sub>3</sub>);  $R_r = 0.80$  (chloroform/methanol 5:1); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta = 8.48$  (d, <sup>3</sup>J = 8.4 Hz, CH, NBD), 7.29 (br, 1H, NH, amide), 7.17 (d, <sup>3</sup>J = 7.1, 2H, NH, amide), 6.86 (br, 1H, NH, amide), 6.65 (br, 1H, NH, amide), 6.17 (d, <sup>3</sup>J = 8.4 Hz, CH, NBD), 5.94– 5.85 (m, 1H, CH=CH<sub>2</sub>), 5.33 (d, <sup>3</sup> $J_{trans} = 17.2$  Hz, 1H, CH=CH<sub>2a</sub>), 5.25 (d, <sup>3</sup> $J_{cis} = 10.6$  Hz, 1H, CH=CH<sub>2b</sub>), 4.67–4.58 (m, 4H, CH<sub>2</sub>-O,  $\alpha$ -CH, Ile<sub>2</sub>,  $\beta$ -CH, Ile<sub>1</sub>), 4.55–4.52 (m, 1H,  $\alpha$ -CH<sub>2</sub>, aminocaproyl), 3.31–3.24 (m, 2H,  $\beta$ -

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CH<sub>2</sub>, Cys), 2.56 (t,  ${}^{3}J = 6.9$  Hz, 2H,  $\alpha$ -CH<sub>2</sub>, Pal), 2.38 – 2.34 (m, 2H,  $\epsilon$ -CH<sub>2</sub>, aminocaproyl), 1.99-1.95 (m, 1 H, β-CH, Ile<sub>1</sub>), 1.91-1.88 (m, 1 H, β-CH, Ile<sub>2</sub>), 1.86-1.82 (m, 2H, β-CH<sub>2</sub>, aminocaproyl), 1.80-1.74 (m, 2H, δ-CH<sub>2</sub>, aminocaproyl), 1.63–1.60 (m, 2H,  $\beta$ -CH<sub>2</sub>, Pal), 1.55–1.51 (m, 2H, 2× $\gamma$ -CH<sub>2a</sub>, Ile), 1.43-1.41 (m, 2H, γ-CH<sub>2</sub>, aminocaproyl), 1.24 (s, 24H, CH<sub>2</sub>, Pal), 1.16 (d,  ${}^{3}J = 4.9$  Hz, 3H,  $\gamma$ -CH<sub>3</sub>, Thr), 1.12–1.07 (m, 2H, 2× $\gamma$ -CH<sub>2b</sub>, Ile), 0.91-0.86 (m, 15H, CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (100.6 MHz, CDCl<sub>3</sub>):  $\delta = 201.39$  (C=O, thioester), 171.32, 171.06, 169.50 (5 × C=O, amide, ester), 144.33, 144.01 (4 × Cq, NBD), 136.51 (CH=CH<sub>2</sub>), 131.57 (2 × CH, NBD), 118.97 (CH=CH<sub>2</sub>), 66.98 (β-CH, Thr), 65.90 (CH<sub>2</sub>-O, allyl), 58.49 (a-CH, Cys), 56.86 (a-CH, Ile), 53.83 (a-CH, Ile), 53.43 (a-CH, Thr), 44.07 (α-CH<sub>2</sub>, aminocaproyl), 43.64 (β-CH<sub>2</sub>, Cys), 37.73, 36.71 (2×β-CH, Ile), 35.83 (α-CH<sub>2</sub>, Pal), 31.94 (ε-CH<sub>2</sub>, aminocaproyl), 30.60, 29.71, 29.46, 29.37, 29.27, 29.03, 27.90, 26.32, 26.19, 25.60, 25.09, 24.81, 24.64, 22.70 (CH<sub>2</sub>, Pal,  $\beta$ -CH<sub>2</sub>,  $\delta$ -CH<sub>2</sub>,  $\gamma$ -CH<sub>2</sub>, aminocaproyl,  $\gamma$ -CH<sub>2</sub>, Ile), 18.46 ( $\gamma$ -CH<sub>3</sub>, Thr), 15.63, 15.50, 14.14, 11.58 (5 × CH<sub>3</sub>, Ile, Pal); MS (FAB, 3-NBA): m/z: calcd for C<sub>50</sub>H<sub>83</sub>N<sub>8</sub>O<sub>11</sub>S: 1003.590; found: 1003.586 [M+H]+

NBDAca-Thr-Ile-Cys(Pal)-Ile-OH (22): Dimethylbarbituric acid (2 mg, 0.02 mmol) and a catalytic amount of  $[Pd(PPh_{3})_{4}]$  were added under argon to a solution of NBDAca-Thr-Ile-Cys(Pal)-Ile-OAll (21; 31 mg, 0.03 mmol) in dry THF (10 mL). The mixture was stirred for 15 min at room temperature and then the solvent was removed under reduced pressure. The resulting residue was triturated with diethyl ether  $(3 \times 2 \text{ mL})$  to yield the desired compound as an orange solid (25 mg, 86%). M.p. 162°C;  $[\alpha]_{\rm D}^{20} = -12.7$  (c = 1.0 in CHCl<sub>3</sub>/CH<sub>3</sub>OH 1:);  $R_{\rm f} = 0.44$  (chloroform/methanol 5:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 1:1):  $\delta = 8.52$  (d, <sup>3</sup>J = 8.6 Hz, CH, NBD), 6.30 (d,  ${}^{3}J = 8.7$  Hz, CH, NBD), 4.59 – 4.56 (m, 1 H,  $\alpha$ -CH, Cys), 4.45 (d,  ${}^{3}J = 3.9$  Hz, 1 H,  $\alpha$ -CH, Thr), 4.41 – 4.38 (m, 1 H,  $\alpha$ -CH, Ile<sub>1</sub>), 4.30 – 4.27 (m, 1H,  $\alpha$ -CH, Ile<sub>2</sub>), 4.21 (dq,  ${}^{3}J_{1} = 3.9$  Hz,  ${}^{3}J_{2} = 6.4$  Hz, 1H,  $\beta$ -CH, Thr), 3.57 - 3.50 (m, 2H,  $\alpha$ -CH<sub>2</sub>, aminocaproyl), 3.35 (dd, <sup>2</sup>J = 13.1 Hz, <sup>3</sup>J = 5.4 Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys), 3.16 (dd, <sup>2</sup>J = 13.1 Hz, <sup>3</sup>J = 8.6 Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys), 2.57 (t,  ${}^{3}J = 7.5$  Hz, 2H,  $\alpha$ -CH<sub>2</sub>, Pal), 2.36 (t,  ${}^{3}J = 7.3$  Hz, 2H,  $\epsilon$ -CH<sub>2</sub>, aminocaproyl), 1.92-1.88 (m, 2H,  $2 \times \beta$ -CH, Ile), 1.86-1.80 (m, 2H,  $\beta$ -CH<sub>2</sub>, aminocaproyl), 1.77-1.71 (m, 2H, δ-CH<sub>2</sub>, aminocaproyl), 1.65-1.61 (m, 2H,  $\beta$ -CH<sub>2</sub>, Pal), 1.55–1.48 (m, 4H, 2× $\gamma$ -CH<sub>2a</sub>, Ile,  $\gamma$ -CH<sub>2</sub>, aminocaproyl), 1.27 (s, 24 H, CH<sub>2</sub>, Pal), 1.22-1.14 (m, 2 H, 2 × γ-CH<sub>2b</sub>, Ile), 1.18 (d,  ${}^{3}J = 6.4$  Hz, 3 H,  $\gamma$ -CH<sub>3</sub>, Thr), 0.95–0.87 (m, 15 H, CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 1:1):  $\delta = 200.61$  (C=O, thioester), 175.37 (C=O, carboxylic acid), 173.69, 172.76, 171.90, 170.86 (4×C=O, amide), 146.06, 145.17, 144.89 (4  $\times$  Cq, NBD), 132.53, 129.46 (2  $\times$  CH, NBD), 67.78 (β-CH, Thr), 58.85, 58.70, 57.61, 53.62 (α-CH), 44.46 (α-CH<sub>2</sub>, aminocaproyl), 44.19 (β-CH<sub>2</sub>, Cys), 37.97, 37.55 (2×β-CH, Ile), 36.25 (α-CH2, Pal), 32.50 (ε-CH2, aminocaproyl), 30.74, 30.23, 30.20, 30.18, 30.00, 29.91, 29.84, 29.54, 28.47, 27.03, 26.10, 25.80, 25.62, 25.20, 23.21 (CH<sub>2</sub>, Pal, β-CH<sub>2</sub>, δ-CH<sub>2</sub>, γ-CH<sub>2</sub>, aminocaproyl, γ-CH<sub>2</sub>, Ile), 19.47 (γ-CH<sub>3</sub>, Thr), 15.77, 15.74, 14.28, 11.78, 11.60 (5 × CH<sub>3</sub>, Ile, Pal); MS (FAB, 3-NBA): m/z: calcd for C<sub>47</sub>H<sub>79</sub>N<sub>8</sub>O<sub>11</sub>S: 963.5589; found: 963.5562 [*M*+H]<sup>+</sup>.

Boc-Arg(Aloc)<sub>2</sub>-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OAll (23): Triethylamine (5  $\mu L,$  38  $\mu mol)$  and EEDQ (12 mg, 46  $\mu mol)$  were added at 0  $^{\circ}C$  to a solution of Boc-Arg(Aloc)2-Cys(Pal)-OH (18; 30 mg, 38 µmol) and H-Thr-Ile-Cys(Pal)-Ile-OAll • TFA (20; 32 mg, 38 µmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and the mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure and the product was purified by sizeexclusion chromatography on Sephadex LH 20 using chloroform/methanol 1:1 to yield a colorless solid (26 mg, 46 %).  $[\alpha]_{D}^{20} = -33.4$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{\rm f} = 0.31$  (chloroform/methanol 10:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta =$ 9.46 (br, 2H, NH, guanidino), 7.56 (br, 1H, NH, amide), 7.37 (br, 1H, NH, amide), 7.24 (br, 1 H, NH, amide), 7.09 (d, <sup>3</sup>J = 8.3 Hz, 1 H, NH, amide), 6.99 (d,  ${}^{3}J = 6.2$  Hz, 1 H, NH, amide), 6.02 - 5.87 (m, 3 H, CH=CH<sub>2</sub>), 5.40 (br, 1 H, NH, carbamate), 5.37 - 5.23 (m, 6 H, CH=CH<sub>2</sub>), 4.73 (d,  ${}^{3}J = 5.8$  Hz, 2 H, CH<sub>2</sub>-O, allyl), 4.66 - 4.57 (m, 4 H,  $2 \times$  CH<sub>2</sub>-O, allyl), 4.52 - 4.49 (m, 2 H,  $2 \times \alpha$ -CH, Cys), 4.33–4.26 (m, 3H,  $\beta$ -CH, Thr,  $2 \times \alpha$ -CH), 4.08–4.02 (m,  $2H, 2 \times \alpha$ -CH), 3.97 - 3.93 (m, 2H,  $\delta$ -CH<sub>2</sub>, Arg), 3.40 (dd,  $^{2}J = 14.3$  Hz,  $^{3}J =$ 4.9 Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys<sub>1</sub>), 3.33 (dd, <sup>2</sup>J = 14.4 Hz, <sup>3</sup>J = 4.3 Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys<sub>1</sub>), 3.30 - 3.22 (m, 2H,  $\beta$ -CH<sub>2</sub>, Cys<sub>2</sub>), 2.59 - 2.53 (m, 4H,  $2 \times \alpha$ -CH<sub>2</sub>, Pal), 2.06-2.02 (m, 1 H, β-CH, Ile<sub>1</sub>), 1.92-1.89 (m, 1 H, β-CH<sub>2a</sub>, Arg), 1.88-1.85 (m, 1H, β-CH, Ile<sub>2</sub>), 1.75-1.70 (m, 3H, β-CH<sub>2b</sub>, γ-CH<sub>2</sub> Arg), 1.68-1.48 (m, 6 H,  $2 \times \beta$ -CH<sub>2</sub>, Pal,  $2 \times \gamma$ -CH<sub>2a</sub>, Ile), 1.46 (s, 9 H, *t*Bu), 1.44 (d, <sup>3</sup>*J* = 4.8 Hz, 3H,  $\gamma$ -CH<sub>3</sub>, Thr), 1.31–1.14 (m, 2H,  $2 \times \gamma$ -CH<sub>2b</sub>, Ile), 1.25 (s, 48H, CH<sub>2</sub>, Pal), 0.94-0.75 (m, 18 H,  $4 \times CH_3$ , Ile,  $2 \times CH_3$ , Pal); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta = 200.07$  (2 × C=O, thioester), 173.15, 171.55,

170.98, 170.84, 170.39, 169.70 (C=O, 5 × amide, 1 × ester), 156.24, 155.51 (3 × C=O, carbamate), 132.90, 131.80, 130.78 (3 × CH=CH<sub>2</sub>), 118.61 (3 × CH=CH<sub>2</sub>), 80.81 (C<sub>q</sub>, *t*Bu), 68.02, 65.62 (3 × CH<sub>2</sub>-O, allyl), 66.75 ( $\beta$ -CH, Thr), 59.27, 58.91, 56.93, 55.64, 55.14, 53.51 (6 ×  $\alpha$ -CH), 50.81 ( $\delta$ -CH<sub>2</sub>, Arg), 44.02 (2 ×  $\beta$ -CH<sub>2</sub>, Cys), 37.54, 36.07 (2 ×  $\beta$ -CH, Ile), 31.91 (2 ×  $\alpha$ -CH<sub>2</sub>, Pal), 30.32, 29.95, 29.69, 29.65, 29.46, 29.35, 29.29, 29.24, 29.03, 28.62, 25.64, 25.54, 25.10, 24.92, 24.68, 22.68 (CH<sub>2</sub>, Pal,  $\beta$ -CH<sub>2</sub>,  $\gamma$ -CH<sub>2</sub>, Arg,  $\gamma$ -CH<sub>2</sub>, Ile), 28.33 (CH<sub>3</sub>, *t*Bu), 19.62 ( $\gamma$ -CH<sub>3</sub>, Thr), 15.62, 15.46, 14.11, 11.67, 11.59 (6 × CH<sub>3</sub>, Ile, Pal); MS (FAB, 3-NBA): *m*/*z*: calcd for C<sub>76</sub>H<sub>134</sub>N<sub>9</sub>O<sub>16</sub>S<sub>2</sub>: 1492.9; found: 1492.5 [*M*+H]<sup>+</sup>.

H-Arg(Aloc)<sub>2</sub>-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OAll · TFA (24): TFA (54 µL, 0.67 mmol) was added at 0°C to a solution of Boc-Arg(Aloc)2-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OAll (2; 20 mg, 13 µmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.12 mL) and the mixture was stirred at 0 °C for 45 min and at room temperature for 60 min. TFA and the solvent were coevaporated with toluene  $(2 \times 10 \text{ mL})$  under reduced pressure to yield a colorless solid (20 mg, quant.).  $[\alpha]_{D}^{20} = -14.3$  $(c=0.5 \text{ in CHCl}_3); R_f=0.12$  (chloroform/methanol 10:1); <sup>1</sup>H NMR  $(500 \text{ MHz}, \text{ CDCl}_3/\text{CD}_3\text{OD} 5:1): \delta = 6.00 - 5.89 \text{ (m, 3H, CH=CH}_2), 5.41 - 6.00 - 5.89 \text{ (m, 3H, CH=CH}_2)$ 5.23 (m, 6 H, CH=CH<sub>2</sub>), 4.75 (dt, <sup>3</sup>J = 5.9 Hz, <sup>4</sup>J = 1.2 Hz, 2 H, CH<sub>2</sub>-O, allyl),  $4.65-4.59 \text{ (m, 4H, } 2 \times \text{CH}_2\text{-O, allyl}\text{), } 4.56-4.54 \text{ (m, 1H, } \alpha\text{-CH), } 4.48-4.44$ (m, 2H,  $\beta$ -CH, Thr,  $\alpha$ -CH), 4.35 (d,  ${}^{3}J$  = 4.0 Hz, 1H,  $\alpha$ -CH), 4.26 (d,  ${}^{3}J$  = 6.3 Hz, 1 H, α-CH), 4.22-4.20 (m, 1 H, α-CH), 4.16-4.12 (m, 1 H, α-CH),  $3.56 - 3.48 (m, 2 H, \delta$ -CH<sub>2</sub>, Arg),  $3.45 - 3.40 (m, 1 H, \beta$ -CH<sub>2a</sub>, Cys<sub>1</sub>),  $3.32 (dd, \beta)$  ${}^{2}J = 14.1 \text{ Hz}, {}^{3}J = 5.4 \text{ Hz}, 1 \text{ H}, \beta \text{-CH}_{2b}, \text{Cys}_{1}), 3.21 \text{ (dd, } {}^{2}J = 14.0 \text{ Hz}, {}^{3}J =$ 7.7 Hz, 1 H,  $\beta$ -CH<sub>2a</sub>, Cys<sub>2</sub>), 3.14 (dd, <sup>2</sup>J = 14.0 Hz, <sup>3</sup>J = 8.4 Hz, 1 H,  $\beta$ -CH<sub>2b</sub>, Cys<sub>2</sub>), 2.56 (t,  ${}^{3}J = 7.6$  Hz, 4H, 2 ×  $\alpha$ -CH<sub>2</sub>, Pal), 2.05 – 1.98 (m, 1H,  $\beta$ -CH, Ile<sub>1</sub>), 1.93-1.75 (m, 5H, β-CH, Ile<sub>2</sub>, β-CH<sub>2</sub>, γ-CH<sub>2</sub>, Arg), 1.64-1.62 (m, 4H,  $\gamma$ -CH<sub>2b</sub>, Ile), 1.26 (s, 48 H, CH<sub>2</sub>, Pal), 1.15 (d, <sup>3</sup>J = 5.6 Hz, 3 H,  $\gamma$ -CH<sub>3</sub>, Thr), 0.93–0.87 (m, 18H,  $4\times CH_3,$  Ile,  $2\times CH_3,$  Pal);  $^{13}C$  NMR (125.7 MHz,  $CDCl_3/CD_3OD 5:1$ ):  $\delta = 200.34$ , 199.85 (2 × C=O, thioester), 172.06, 171.29, 170.60, 170.35, 170.21, 169.64 (6 × C=O, 5 × amide, 1 × ester), 162.89 (C<sub>a</sub>, guanidino), 160.62, 155.38 (2 × C=O, carbamate), 132.74, 131.76, 130.99  $(3 \times CH=CH_2)$ , 120.05, 119.00, 118.56  $(3 \times CH=CH_2)$ , 68.52, 66.57, 66.00  $(3 \times CH_2$ -O, allyl), 67.23 ( $\beta$ -CH, Thr), 58.62, 58.47, 57.17, 54.26, 53.13, 52.57 (6 × α-CH), 44.16 (δ-CH<sub>2</sub>, Arg), 43.64 (2 × β-CH<sub>2</sub>, Cys), 37.61, 36.83 (2 × β-CH, Ile), 32.08 (2  $\times \alpha$  -CH<sub>2</sub>, Pal), 30.36, 29.84, 29.80, 29.77, 29.60, 29.51, 29.43, 29.15, 27.10, 25.69, 25.63, 25.24, 24.79, 23.54 (CH<sub>2</sub>, Pal, β-CH<sub>2</sub>, γ-CH<sub>2</sub>, Arg, γ-CH<sub>2</sub>, Ile), 22.83 (γ-CH<sub>3</sub>, Thr), 18.85, 17.30, 17.11, 15.56, 15.51, 14.16, 11.54, 11.41 ( $6 \times CH_3$ , Ile, Pal); MS (FAB, 3-NBA): m/z: calcd for C<sub>71</sub>H<sub>126</sub>N<sub>9</sub>O<sub>14</sub>S<sub>2</sub>: 1393.9; found: 1393.7 [M]<sup>+</sup>.

NBDAca-Met-OAll (25): HOBt (14 mg, 0.09 mmol), triethylamine (10 µL, 0.08 mmol) and EDC (14 mg, 0.08 mmol) were added at 0 °C to a solution of N-(7-nitrobenz-2-oxa-1,3-diazole-4-yl)-aminocaproic acid (20 mg, 0.08 mmol) and L-methionine-allyl ester tosylate (12g; 29 mg, 0.08 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and the mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure and the product was purified by flash chromatography on silica gel using chloroform/methanol 50:1 to yield an orange oil (26 mg, 69 %).  $[\alpha]_{D}^{20} = +16.3$  (c = 1.0 in CHCl<sub>3</sub>);  $R_{\rm f} = 0.74$  (chloroform/methanol 5:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 8.48$ (d,  ${}^{3}J = 8.6$  Hz, 1 H, NBD), 6.68 (br, 1 H, NH, amine), 6.27 (d,  ${}^{3}J = 7.7$  Hz, 1H, NH, amide), 6.17 (d, <sup>3</sup>J = 8.6 Hz, 1H, NBD), 5.95-5.87 (m, 1H, CH=CH<sub>2</sub>), 5.35 (d,  ${}^{3}J_{trans} = 17.2$  Hz, 1 H, CH=CH<sub>2a</sub>), 5.27 (d,  ${}^{3}J_{cis} = 10.3$  Hz, 1 H, CH=CH<sub>2b</sub>), 4.79-4.75 (m, 1 H,  $\alpha$ -CH, Met), 4.66 (d,  $^{3}J = 5.3$  Hz, 2 H, CH2-O, allyl), 3.55-3.51 (m, 2H, α-CH2, aminocaproyl), 2.61-2.55 (m, 2 H, γ-CH<sub>2</sub>, Met), 2.31 (t,  ${}^{3}J$  = 7.0 Hz, 2 H, ε-CH<sub>2</sub>, aminocaproyl), 2.23 – 2.17 (m, 1 H, β-CH<sub>2a</sub>, Met), 2.09-1.99 (m, 1 H, β-CH<sub>2b</sub>, Met), 2.05 (s, 3 H, SCH<sub>3</sub>, Met), 1.87 - 1.82 (m, 2H,  $\beta$ -CH<sub>2</sub>, aminocaproyl), 1.80 - 1.73 (m, 2H,  $\delta$ -CH<sub>2</sub>, aminocaproyl), 1.57-1.51 (m, 2H, γ-CH<sub>2</sub>, aminocaproyl); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>):  $\delta = 172.56$ , 171.83 (2 × C=O, amide, ester), 144.28, 144.07, 143.95, 123.68 (4 × C<sub>a</sub>, NBD), 136.58, 98.57 (2 × CH, NBD), 131.33 (CH=CH<sub>2</sub>), 119.16 (CH=CH<sub>2</sub>), 66.27 (CH<sub>2</sub>-O, allyl), 51.64 (a-CH, Met), 43.68 (α-CH<sub>2</sub>, aminocaproyl), 35.87 (γ-CH<sub>2</sub>, Met), 31.50 (ε-CH<sub>2</sub>, aminocaproyl), 29.68 (β-CH<sub>2</sub>, Met), 27.91 (β-CH<sub>2</sub>, aminocaproyl), 26.22 (δ-CH<sub>2</sub>, aminocaproyl), 24.54 (y-CH2, aminocaproyl), 15.46 (SCH3, Met); MS (EI): m/z: calcd for C<sub>20</sub>H<sub>27</sub>N<sub>5</sub>O<sub>6</sub>S: 465.2; found: 465.2.

**NBDAca-Met-OH (26)**: Dimethylbarbituric acid (5 mg, 0.03 mmol) and a catalytic amount of  $[Pd(PPh_3)_4]$  were added under argon to a solution of NBDAca-Met-OAll (**25**, 26 mg, 0.06 mmol) in THF (10 mL). The mixture was stirred for 15 min at room temperature and then the solvent was removed under reduced pressure. The resulting residue was triturated with

diethyl ether  $(3 \times 2 \text{ mL})$  to yield the desired compound an an orange oil (24 mg, 94%).  $[\alpha]_{D}^{20} = +10.3$  (c = 1.0 in DMF);  $R_{f} = 0.39$  (chloroform/ methanol 5:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 1:1):  $\delta = 8.51$  (d, <sup>3</sup>J = 8.2 Hz, 1 H, NBD), 6.26 (d,  ${}^{3}J = 8.0$  Hz, 1 H, NBD), 4.33 (dd,  ${}^{3}J_{1} = 7.3$  Hz,  ${}^{3}J_{2} = 5.2$  Hz, 1 H,  $\alpha$ -CH, Met), 3.55 – 3.49 (m, 2 H,  $\alpha$ -CH<sub>2</sub>, aminocaproyl), 2.51–2.49 (m, 2H,  $\gamma$ -CH<sub>2</sub>, Met), 2.29 (t,  ${}^{3}J$  = 7.2 Hz, 2H,  $\epsilon$ -CH<sub>2</sub>, aminocaproyl), 2.15-2.09 (m, 1 H, β-CH<sub>2a</sub>, Met), 2.07 (s, 3 H, SCH<sub>3</sub>, Met), 2.06-1.92 (m, 1H, β-CH<sub>2b</sub>, Met), 1.84-1.78 (m, 2H, β-CH<sub>2</sub>, aminocaproyl), 1.74-1.68 (m, 2H,  $\delta$ -CH<sub>2</sub>, aminocaproyl), 1.52-1.48 (m, 2H,  $\gamma$ -CH<sub>2</sub>, aminocaproyl); <sup>13</sup>C NMR (125.7 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 1:1):  $\delta = 180.34$ (C=O, carboxylic acid), 174.12 (C=O, amide), 145.23, 144.34, 127.80 ( $4 \times C_q$ , NBD), 136.89 (2 × CH, NBD), 54.62 (a-CH, Met), 48.34 (a-CH<sub>2</sub>, aminocaproyl), 36.25 (γ-CH<sub>2</sub>, Met), 32.67 (ε-CH<sub>2</sub>, aminocaproyl), 30.59 (β-CH<sub>2</sub>, Met), 26.73 (β-CH<sub>2</sub>, aminocaproyl), 25.44 (δ-CH<sub>2</sub>, aminocaproyl), 23.63 (γ-CH2, aminocaproyl), 17.94 (SCH3, Met); MS (FAB, 3-NBA): m/z: calcd for C<sub>17</sub>H<sub>24</sub>N<sub>5</sub>O<sub>6</sub>S: 426.1466, found 426.1437 [M+H]<sup>+</sup>.

NBDAca-Met-Arg(Aloc)2-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OAll (27): HOBt (3 mg, 5.0 µmol), triethylamine (1 µL, 7 mmol) and EDC (3 mg, 4.6 µmol) were added at 0 °C to a solution of NBDAca-Met-OH (26; 2 mg, 4.6 µmol) and H-Arg(Aloc)<sub>2</sub>-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OAll • TFA (24; 7 mg, 4.6  $\mu mol)$  in  $CH_2Cl_2$  (20 mL) and the mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure and the product was purified by size-exclusion chromatography on Sephadex LH 20 using chloroform/methanol 1:1 to yield a yellow solid (6 mg, 72%).  $[a]_{D}^{20} = -8.0$  (c = 0.2 in CHCl<sub>3</sub>/CH<sub>3</sub>OH 1:1);  $R_{f} = 0.80$ (chloroform/methanol 5:1); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 5:1):  $\delta = 8.52$  (d,  ${}^{3}J = 5.2$  Hz, 1H, CH, NBD), 6.28 (d,  ${}^{3}J = 5.3$  Hz, 1H, CH, NBD), 6.02-5.90 (m, 3H, CH=CH2), 5.48-5.27 (m, 6H, CH=CH2), 4.85-4.74 (m, 2H, CH<sub>2</sub>-O, allyl), 4.69-4.61 (m, 4H, 2 × CH<sub>2</sub>-O, allyl), 4.48-4.42 (m, 2H,  $2 \times a$ -CH, Cys), 4.34-4.29 (m, 2H,  $2 \times a$ -CH), 4.27-4.19 (m, 3H,  $\beta$ -CH, Thr, 2 ×  $\alpha$ -CH), 4.11–4.05 (m, 1H,  $\alpha$ -CH), 3.98–3.90 (m, 2H,  $\delta$ -CH<sub>2</sub>, Arg), 3.64–3.59 (m, 1H,  $\beta$ -CH<sub>2a</sub>, Cys<sub>1</sub>), 3.56–3.47 (m, 3H,  $\alpha$ -CH<sub>2</sub>, aminocaproyl, β-CH<sub>2b</sub>, Cys<sub>1</sub>), 3.33-3.28 (m, 1H, β-CH<sub>2a</sub>, Cys<sub>2</sub>), 3.22-3.16  $(m, 1H, \beta$ -CH<sub>2b</sub>, Cys<sub>2</sub>), 2.93 – 2.84  $(m, 2H, \gamma$ -CH<sub>2</sub>, Met), 2.60 – 2.53  $(m, 4H, \gamma$ -CH<sub>2</sub>), 2.51 – 2.51  $(m, 4H, \gamma)$ , 2.51  $(m, 4H, \gamma$  $2 \times \alpha$ -CH<sub>2</sub>, Pal), 2.34–2.29 (m, 2H,  $\epsilon$ -CH<sub>2</sub>, aminocaproyl), 2.09 (s, 3H, SCH<sub>3</sub>, Met), 2.08 – 2.01 (m, 2H,  $\beta$ -CH<sub>2</sub>, Met), 2.01 – 1.90 (m, 3H, 2 ×  $\beta$ -CH, Ile,  $\beta$ -CH<sub>2a</sub>, Arg), 1.86–1.80 (m, 3H,  $\beta$ -CH<sub>2b</sub>,  $\gamma$ -CH<sub>2</sub> Arg), 1.72–1.60 (m, 10 H,  $2 \times \beta$ -CH<sub>2</sub>, Pal,  $2 \times \gamma$ -CH<sub>2a</sub>, Ile,  $\beta$ -CH<sub>2</sub>,  $\delta$ -CH<sub>2</sub>, aminocaproyl), 1.54 – 1.48 (m, 2 H, γ-CH<sub>2</sub>, aminocaproyl), 1.44 (d, <sup>3</sup>J = 5.0 Hz, 3 H, γ-CH<sub>3</sub>, Thr), 1.26 (s, 48 H, CH<sub>2</sub>, Pal), 1.19-1.10 (m, 2 H,  $2 \times \gamma$ -CH<sub>2b</sub>, Ile), 1.00-0.81 (m, 18H,  $4 \times CH_3$ , Ile,  $2 \times CH_3$ , Pal); MS (FAB, 3-NBA): m/z: calcd for  $C_{91}H_{152}N_{14}O_{19}S_3$ : 1842.1; found: 1842.0  $[M+H]^+$ .

NBDAca-Met-Arg-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OH (28): Dimethylbarbituric acid (1.5 mg, 6.6 µmol) and a catalytic amount of [Pd(PPh<sub>3</sub>)<sub>4</sub>] were added under argon to a solution of NBDAca-Met-Arg(Aloc)2-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OAll (27; 6 mg, 3.3 µmol) in THF (10 mL). The mixture was stirred for 30 min at room temperature and then the solvent was removed under reduced pressure. The resulting residue was purified by size exclusion chromatography on Sephadex LH 20 using chloroform/ methanol 1:1 to yield yellow solid (5 mg, 96%).  $[\alpha]_{D}^{20} = -22.5$  (c = 0.2 in CHCl<sub>3</sub>/CH<sub>3</sub>OH 1:1);  $R_f = 0.10$  (chloroform/methanol 5:1); <sup>1</sup>H NMR  $(500 \text{ MHz}, \text{CDCl}_3/\text{CD}_3\text{OD} 1:1): \delta = 8.55 - 8.51 \text{ (m, 1 H, CH, NBD)}, 6.40 - 8.51 \text{ (m, 1 H, CH, NBD)}$ 6.37 (m, 1 H, CH, NBD), 4.41 - 4.36 (m, 3 H,  $3 \times \alpha$ -CH), 4.34 - 4.31 (m, 1 H,  $\alpha$ -CH), 4.28–4.21 (m, 3H, 2× $\alpha$ -CH,  $\beta$ -CH, Thr), 4.18–4.12 (m, 1H,  $\alpha$ -CH), 3.70-3.63 (m, 2H, δ-CH<sub>2</sub>, Arg), 3.47-3.41 (m, 2H, α-CH<sub>2</sub>, aminocaproyl), 3.31-3.11 (m, 4H,  $2 \times \beta$ -CH<sub>2</sub>, Cys), 2.93-2.88 (m, 2H,  $\gamma$ -CH<sub>2</sub>, Met), 2.62–2.55 (m, 4H,  $2 \times \alpha$ -CH<sub>2</sub>, Pal), 2.38–2.30 (m, 3H,  $\epsilon$ -CH<sub>2</sub>, aminocaproyl,  $\beta$ -CH<sub>2a</sub>, Met), 2.09–2.03 (m, 1H,  $\beta$ -CH<sub>2b</sub>, Met), 2.06 (s, 3H, SCH<sub>3</sub>, Met), 1.95 - 1.88 (m, 3 H,  $2 \times \beta$ -CH, Ile,  $\beta$ -CH<sub>2a</sub>, Arg), 1.75 - 1.62 (m, 11 H,  $\beta$ -CH<sub>2</sub>,  $\delta$ -CH<sub>2</sub>, aminocaproyl,  $\beta$ -CH<sub>2b</sub>,  $\gamma$ -CH<sub>2</sub>, Arg, 2 ×  $\beta$ -CH<sub>2</sub>, Pal), 1.55–1.47 (m, 4H,  $2 \times \gamma$ -CH<sub>2a</sub>, Ile,  $\gamma$ -CH<sub>2</sub>, aminocaproyl), 1.27 (s, 48H, CH<sub>2</sub>, Pal), 1.21 (d,  ${}^{3}J = 6.0$  Hz, 3 H,  $\gamma$ -CH<sub>3</sub>, Thr), 1.17 – 1.12 (m, 2 H, 2 ×  $\gamma$ -CH<sub>2b</sub>, Ile), 0.96-0.88 (m, 18H, CH<sub>3</sub>, Ile, CH<sub>3</sub>, Pal); MS (FAB, 3-NBA): m/z: calcd for C<sub>77</sub>H<sub>133</sub>N<sub>14</sub>O<sub>15</sub>S<sub>3</sub>: 1589.9; found: 1589.7 [M+H]<sup>+</sup>.

General procedure for the preparation of loaded lipid vesicles:<sup>[40]</sup> A solution of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC;  $7.5 \,\mu$ L, 20 mM) in methanol was mixed with the desired volumes of lipopeptide and/or quencher stock solutions. Subsequently 150  $\mu$ L of aqueous buffer (10 mM (*N*-2-hydroxylethyl)piperazine-*N*'-2-ethansulfonic acid, 150 mM KCl, 8 mM NaCl, 1 mM MgCl<sub>2</sub>; pH 7) was added. The mixtures were freeze-thawed three times before extruding through 0.1  $\mu$ m pore size

polycarbonate filters ( $20 \times$ ). The remaining organic solvents were removed in a speedvac apparatus (Concentrator 5301, Eppendorf).

### Vesicles generated according to the general procedure:

### Lipid vesicles loaded with fluorescence quencher and lipopeptides:

a) A solution of NBDac-Thr-Ile-Cys(Pal)-Ile-OH (**22**; 15  $\mu$ L, 100  $\mu$ M) in chloroform/methanol 1:4 and a solution of *N*-(lissamine-rhodamine-sulfonyl)phosphatidylethanolamine (15  $\mu$ L, 200  $\mu$ M) in methanol.

b) A solution of NBDac-Met-Arg-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OH (**28**; 15  $\mu$ L, 100  $\mu$ M) in chloroform/methanol 1:4 and a solution of *N*-(lissamine-rhodamine-sulfonyl)phosphatidylethanolamine (15  $\mu$ L, 200  $\mu$ M) in methanol.

#### Lipid vesicles loaded with lipopeptides:

а) A solution of NBDac-Thr-Ile-Cys(Pal)-Ile-OH (22; 15  $\mu L,$  100  $\mu m)$  in chloroform/methanol 1:4.

b) A solution of NBDac-Met-Arg-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OH (28; 15  $\mu$ L, 100  $\mu$ M) in chloroform/methanol 1:4.

General procedure for the preparation of acceptor vesicles: A solution of POPC in methanol ( $30 \ \mu$ L,  $20 \ m$ M) was mixed with 150  $\mu$ L buffer ( $10 \ m$ M (N-2-hydroxylethyl)piperazine-N'-2-ethansulfonic acid, 150 mM KCl, 8 mM NaCl, 1 mM MgCl<sub>2</sub>; pH 7). The mixtures were freeze-thawed three times before extruding through 0.1  $\mu$ m pore size polycarbonate filters ( $20 \times$ ). The remaining organic solvents were removed under reduced pressure.

Monitoring of flip-flop kinetics by dithionite reduction: A mixture of loaded vesicles (with NBDac-Thr-Ile-Cys(Pal)-Ile-OH **22** or NBDac-Met-Arg-Cys(Pal)-Thr-Ile-Cys(Pal)-Ile-OH (**28**; 6.1  $\mu$ L) was added at 20 °C to a buffer solution (1200  $\mu$ L, pH 7). After 15 min a solution of sodium dithionite in the buffer (12  $\mu$ L, 1M) was added and the time-dependent fluorescence decrease was detected at the emission wavelength of NBD (535 nm).<sup>[27]</sup> Finally a solution of Triton X-100 (60  $\mu$ L, 20%) was added after 50 min.

Intervesicle transfer of the single palmitoylated tetrapeptide 22: A mixture of loaded vesicles (with NBDac-Thr-Ile-Cys(Pal)-Ile-OH (22) and the fluorescence quencher (6.1  $\mu$ L) was added at 20 °C to a buffer solution (1200  $\mu$ L, pH 7). Then the desired amounts of acceptor vesicle mixture were added and the time-dependent fluorescence increase was detected at the emission wavelength of NBD (535 nm).

Comparison of intervesicle transfer of the double palmitoylated heptapeptide 28 with the single palmitoylated tetrapeptide 22: A mixture of vesicles loaded with lipopeptides 22 or 28 and the fluorescence quencher was added to 1200  $\mu$ L buffer. After 30 min an access of acceptor vesicles was added and the time-dependent fluorescence increase was detected at the emission wavelength of NBD (535 nm). All experiments were performed at 20 °C.

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