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Synthesis and Conformational Analysis of Peptide Inhibitors of Farnesyltransferase

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Abstract—Farnesylation of the *ras* oncogene product by Farnesyl Transferase (FTase) is known to be a critical step in cell transformation leading to uncontrolled proliferation. The peptide CysValTicMet is a potent FTase inhibitor, but its degradation by amino-peptidases and its only weak internalization into cells make it a bad candidate for a future cancer drug. We have prepared improved CysValTicMet analogues using several approaches: (i) amino terminal modifications or introduction of pseudopeptides or non-natural amino acids to increase proteolytic stability, (ii) introduction of hydrophobic aliphatic chains to increase cell internalization and metabolic stability and (iii) transformation into prodrugs. Additionally, we have carried out comparative conformational analysis studies by molecular dynamics of some of the here presented peptides and of our recently described peptidomimetic inhibitors of FTase. Copyright © 1997 Elsevier Science Ltd

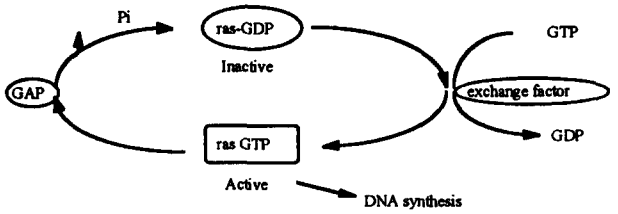


Figure 1. p21 Ras in the cellular transduction pathway.

Introduction

The p21 *Ras* protooncogene product plays a crucial role in cellular signal transduction pathways[1] (Fig. 1). This protein is strongly activated and/or overexpressed in about 30% of cancer desease cases.[2] During the last decade much effort has been invested to understand the mechanisms underlying *Ras*-induced cellular transformation. It has been shown that p21 Ras must be anchored to the inner side of the plasma membrane[3] by its farnesyl moiety linked to Cys_{186} to be activated.

This posttranslational modification is catalysed by the enzyme farnesyltransferase (FTase, Fig. 2). Several laboratories have focused on the working mechanism of this enzyme because of its key role in the control of *Ras* activity.[4] The development of the first synthetic peptides inhibiting FTase specifically, revealed this enzyme as a potential target for novel anti-cancer drug therapy.

Tetrapeptides derived from the C-terminus of p21 Ras were the first peptidic farnesyltransferase inhibitors described, the most potent being CVFM for the bovine FTase.[5] We initiated our research work on FTase inhibitors performing structure–activity relationship studies starting from this peptide. We showed that CVFM displayed a weaker inhibitory activity on human (1000 nM)[6] than on bovine FTase (60 nM).[7–8] We and others have also shown that locally constrained analogues of CVFM obtained by substitution of Phe by 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) and Val by (*N*-Me)Val led to potent inhibitors of FTase in isolated enzyme assays.[9–11] Based on these results, we designed and synthesized several novel peptide and pseudo-peptide analogues of CVFM. We demonstrated that introduction of a shifty amino acid related to Cys instead of Cys-Val and Tic instead of Phe resulted in a potent FTase inhibitor active not only in isolated enzyme, but also in cell based assays, at the condition that it was properly transformed into bridged disulfur prodrug. One of the pseudo-peptides, formu-

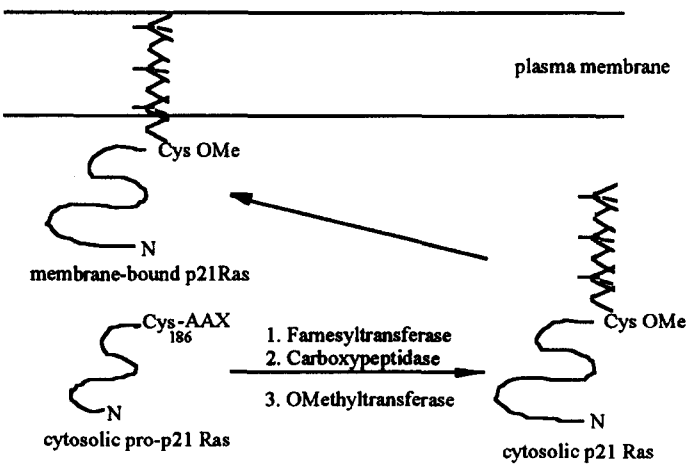


Figure 2. Mechanism of action of p21 Ras during signal transduction.

lated as a prodrug (product **31** below), suppressed specifically the ability of Ras transformed cells to form colonies in soft agar ($IC_{50}=2$ μM).[6]

Here we present the synthesis of additional analogues of Cys-ValTicMet by application of different modification strategies. Increased stability towards peptidases was inferred by (i) *N*-terminal modifications by introduction of hydrophobic aliphatic chains on the *N*-terminus or elimination of the terminal amine or (ii) transformation into prodrugs by introduction of lactones or thiolactones and (iii) inhibition of endopeptidases by introduction of aminomethylene or carboxy-retroinverso pseudopeptide backbone modifications.

Using these approaches, we attempted to obtain peptide analogues with increased FTase inhibitory activity not only in isolated enzyme but also in cell based assays due to their increased cell internalization and metabolic stability. Finally, we have carried out comparative conformational analysis studies by molecular dynamics of selected molecules emerging from the present work and from our recently described peptidomimetic inhibitors of FTase.[6,9-10]

Results and Discussion

Introduction of lipophilic chains on the *N*-terminus of CVTicM

In order to obtain more bioavailable peptides, lipophilic chains were introduced into the peptide inhibitors. Thus, a series of *N*-acylated peptides was synthesized in solution by introduction of different fatty acids (see Table 1).

In isolated enzyme assays, optimal activity was obtained for product **2** which contains a chain of 12 carbons. The stability of peptide **2** was monitored by protracted activity in isolated enzyme assay after incubation with THAC[12] cell extracts. This peptide showed a clearly higher stability than KCVFM or KCVTicM from our previous studies[10] (see Figure 3).

Table 1. Isolated enzyme and cell FTase inhibitory activity of hydrophobic peptides

	RCysValTicMet	IC_{50} [nM]	
Symbol	R	In vitro[a]	Cells[b]
1	CH_3—$(CH_2)_8$—CO	50	>10000
2	CH_3—$(CH_2)_{10}$—CO	23	>10000
3	CH_3—$(CH_2)_{12}$—CO	28	>10000
4	CH_3—$(CH_2)_{14}$—CO	85	>10000
5	CH_3—$(CH_2)_{16}$—CO	110	>10000
6	Boc	125	>10000
7	H	10	>10000

[a]FTtase inhibition was tested on human FTase as previously described.[6]
[b]Ras processing inhibition on intact THAC cells was carried out as previously described.[6]

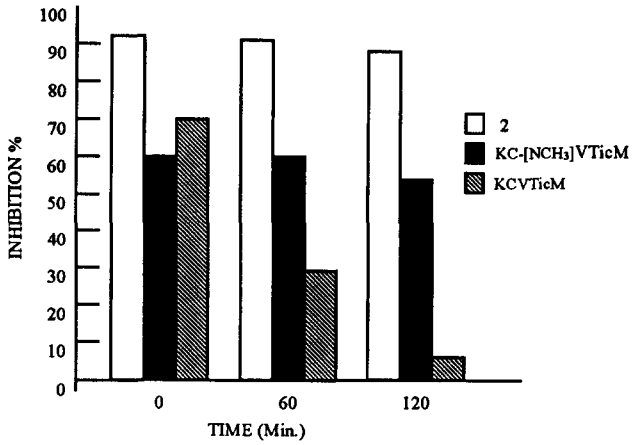


Figure 3. Protracted FTase inhibitory activity after incubation with THAC extracts.

In spite of its stability, peptide **2** had no FTase inhibitory activity in cell based assays when tested at a concentration of 10 μM.

Transformation of peptides into prodrugs

The introduction of certain amino acids into peptides can confer metabolic stability towards several proteases.[13] Thus, the deletion of the terminal amine by introduction of desamino acids has been shown to confer increased stability.[14] Moreover, transformation of the carboxyl terminal into lactones has been shown to increase inhibitory activity of the peptides in cell based assays.[15] We have introduced desamino-cysteine instead of Cys and Hser-lactone or Hcys-thiolactone instead of Met, as prodrug analogues of CVTicM. Lactone **8** (Table 2), displayed significant activity in isolated enzyme assays, but its thiolactone counterpart **9** showed no activity. It seems most likely that the ring of the thiolactone analogue, which has to be opened for restoration of the active free carboxylic acid form, remains closed inside the cell. In contrast, the Hser derivative **8** can be easily transformed into the free carboxylic acid. Due to its cytotoxicity, the FTase inhibitory activity of product **8** could not be measured in cell based assays. The biological activity analysis of these and other Hser active analogues previously described,[15] led us to the conclusion that the lactone prodrug **8** was absorbed by cells, resulting in an undesirable cytotoxic effect

Introduction of aminomethylene peptide isosters and (*N*-Me)Val

We and others[9,11] have previously shown that the introduction of aminomethylene peptide isoster ($\Psi[CH_2NH]$) instead of the peptide bonds Cys-Val into product **24** or Val-Tic into product **25** yielded analogues with enhanced activity as well in isolated enzyme as in cell-based assays. Here we have introduced Tic$\Psi[CH_2NH]$-Met or Tic$\Psi[CH_2N(CH_3)]$-Met instead of Tic-Met and (*N*-Me)Val instead of Val (Scheme 1).

Table 2. Isolated enzyme and cell FTase inhibitory activity of prodrug peptides

	R_1-AA_3-AA_2-AA_1				IC_{50} [nM]	
Symbol	R_1	AA_3	AA_2	AA_1	In vitro[a]	Cells[b]
8	HS—CH_2—CH_2—CO	*N*-Me Val	Tic	Homoserine lactone	350	cytotoxic
9	HS—CH_2—CH_2—CO	*N*-Me Val	Tic	L-Homocyslactone	56,000	>10,000
10	HS—CH_2—CH_2—CO	*N*-Me Val	Tic	D-Homocys lactone	83,000	>10,000
11	HS—CH_2—CH_2—CO	Val	Phe	L-HomoCys lactone	47,000	>10,000
12	HS—CH_2—CH_2—CO	Val	Phe	D-HomoCys lactone	49,000	>10,000

[a]FTtase inhibition was tested on human FTase as previously described.[6]
[b]*Ras* processing inhibition on intact THAC cells was carried out as previously described.[6]

These products and their intermediates were devoid of activity either in isolated enzyme or in cell based assays (see Table 3).

Products **20** and **24** from Table 3 were further studied by comparative conformational analysis (see below).

Introduction of a carboxy-retroinverso peptide isoster

In a preliminary report we have described a novel peptide isoster in which the C-α is interchanged with a carbonyl of the peptide bond. We designated this modification as 'carboxy retro-inverso' pseudo-peptide.[6] We have now synthesized a series of carboxy-retroinverso peptide isosters and analysed their inhibitory activity in isolated enzyme assays.

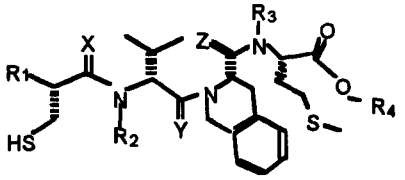
The carboxy retroinverso building block may be synthesized starting from the methyl ester of Tic as previously shown,[6] or as we report here, starting from a preformed peptide TicMetOEt (Scheme 2).

The resulting peptides (see Table 4) showed only low activity in isolated enzyme assays and no inhibitory activity in cell-based assays when tested at 100 μM. Peptide **30** is a carboxyretroinverso analogue of the recently published shifty pseudopeptide **31** (Scheme 2) with potent activity in cell based assays and which suppressed specifically the ability of *Ras* transformed cells to form colonies in soft agar.[6-9] The only difference between **30** and **31** is the reversal of the methylene and carbonyl groups. We have carried out conformational analysis by molecular dynamics to elucidate conformational requirements of product **31** for biological activity (see below).

Structure–activity relationships studies and conclusions

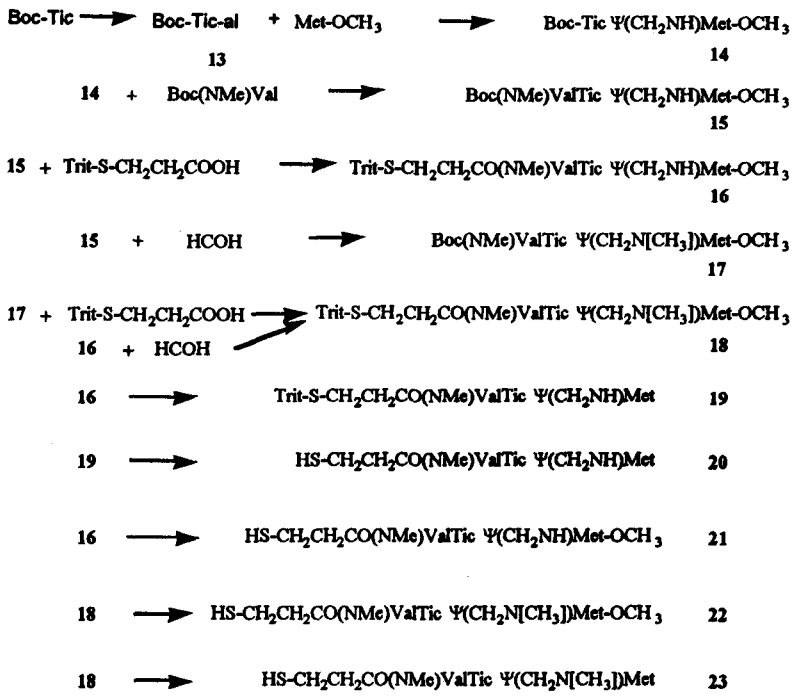
In order to perform structural–activity relationships analysis, some of the peptides were submitted to conformational analysis using a simulated annealing protocol. The peptides chosen for the structural

Table 3. Isolated enzyme and cell FTase inhibitory activity of aminomethylene peptide isosters derived from CVTicM



								FTase inhibition			
								In vitro[a]	Cells[b]		
Symbol	R_1	R_2	R_3	R_4	X	Y	Z	IC_{50}c	Dose[d]	Score[e]	Ref.
20	H	CH_3	H	H	O	O	H, H	63000	1000	–	–
21	H	CH_3	H	CH_3	O	O	H, H	95000	1000	–	–
22	H	CH_3	CH_3	CH_3	O	O	H, H	55000	1000	–	–
23	H	CH_3	CH_3	H	O	O	H, H	50000	1000	–	–
24	NH_2	H	H	H	H, H	O	O	0.6	100	+	9, 11
25	NH_2	H	H	H	H, H	H, H	O	1.2	15	+	9, 11

[a]FTtase inhibition was tested on human FTase as previously described.[6]
[b]*Ras* processing inhibition on intact THAC cells was carried out as previously described.[6]
[c][nM].
[d][μM].
[e]Score = +, 30–60%; –, 0%.



Scheme 1. Synthesis of $\Psi(CH_2NH)$ and $\Psi(CH_2NCH_3)$ peptide isosters analogues of CVTicM (for details, see experimental procedures).

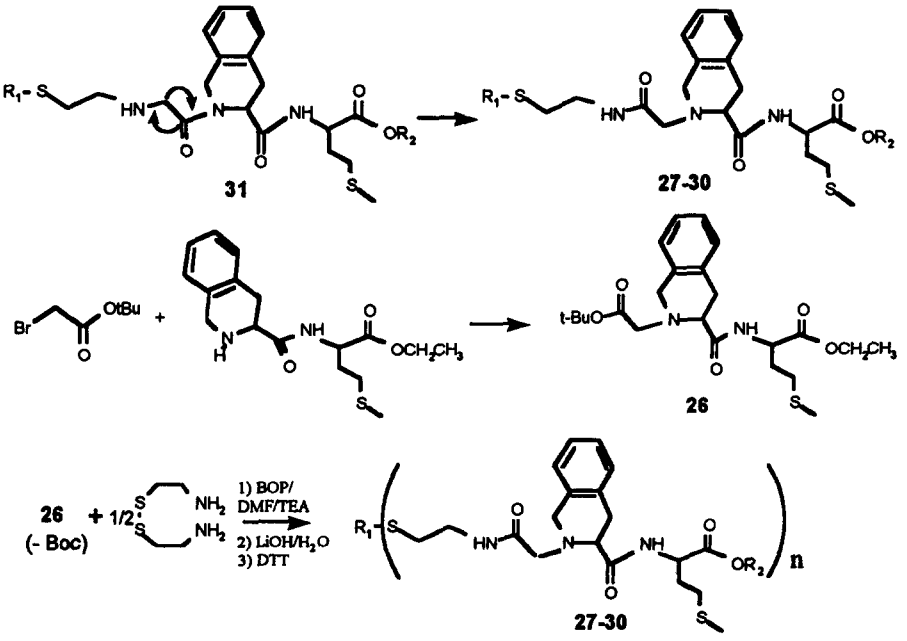
Table 4. Isolated enzyme inhibitory activity of carboxy-retroinverso peptide isosters derived from CVTicM

Symbol	R_1	R_2	n	IC_{50} [μM][a]
T27	—	CH_2CH_3	2	1
28	—	H	2	1.1
29	H	CH_2CH_3	1	2.15
30	H	H	1	2.1
31	H	H	1	0.1

[a]FTtase inhibition was tested on human FTase as previously described.[6]

analysis were **20** compared with **24** and **30** compared with **31**.

The analysis revealed that out of the 100 structures generated for our most active peptide (**24**), only 51 are in an extended conformation [see Fig. 4(b)]. In a recent publication, we have shown that there exists a strong correlation between the propensity of a peptide derived from CVFM to adopt an extended conformation and its activity on FTase.[10] As an example, the peptide CNMeVTicM (IC_{50} = 5nM) is always found in an extended form. As peptide **24**, which is derived from



Scheme 2. Synthesis of 'carboxy-retroinverso' pseudo-peptides (for details see experimental procedures).

CNMeVTicM, is more active, we could postulate that although the conformational analysis showed that peptide **24** could adopt some pseudo-turn conformation, the extended form should be the active one and that the difference in activity is essentially due to the reduction of the amide bond. It should be pointed out that the distance observed between the carboxylate *C*-terminus and the aromatic ring is closely the same (ca 8 Å) for all the 100 structures and the same value is also obtained for CNMeVTicM. (We have chosen an aromatic ring as anchorage point because many recent publications[16-17] have shown the importance of this hydrophobic site in the binding of inhibitors to FTase.)

The analysis of the conformations obtained for peptide **20** ($IC_{50} = 63000$ nM) shows that the percentage of extended structures obtained is about 50% [see Fig. 4(a)], but the mean distance between the carboxylate *C*-terminus and the aromatic ring is ~5 Å. In a recent publication Sebti et al. have shown the critical incidence of the COOH position on the biological activity using a biphenyl-derivates series.[17] Another critical difference between products **24** and **20** is the absence of a *N*-terminal amine on the latter. This amine is postulated to play, together with the thiol, an important role in the chelation of zinc.

Structural features obtained for peptide **31** are closely related to those obtained for peptide **24**. Nevertheless, two differences may explain the difference in activity ($IC_{50} = 100$ nM versus 0.6 nM): first, the N-terminal amine is absent in **31** and second, peptide **31** is shorter than **24**, leading to a shorter distance between the aromatic ring and the thiol than in peptide **24** [Fig. 4(b)].

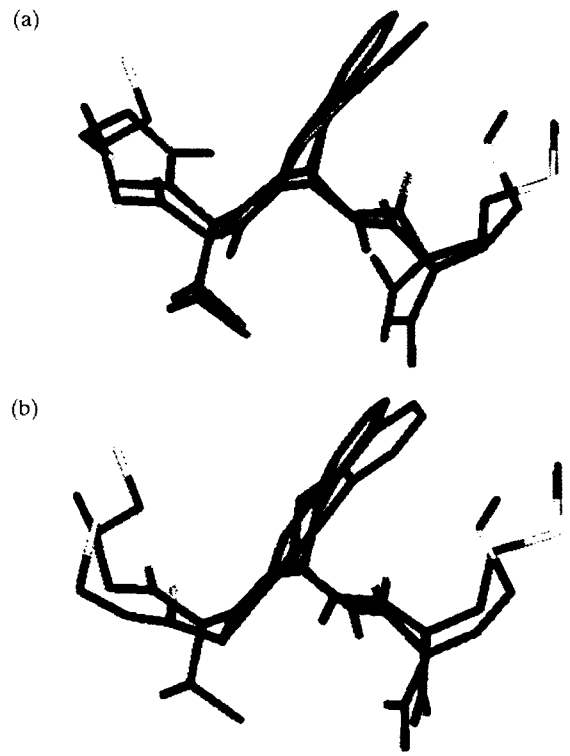


Figure 4. Superposition of representative conformation of: 4(a) = **20** (orange) and **24** (magenta) and, 4(b) = **24** (magenta) and **31** (blue), obtained by simulated annealing protocol.

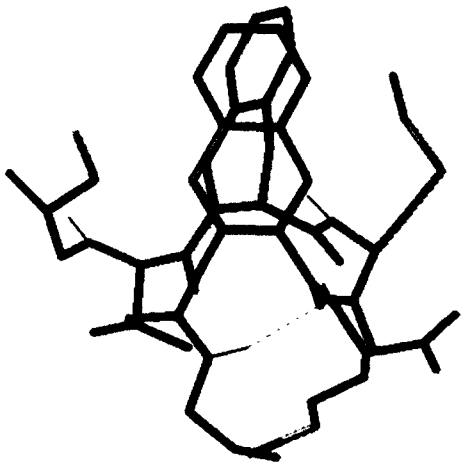


Figure 5. Superposition of representative conformation of **30** (green) and **24** (magenta), obtained by simulated annealing protocol.

The last peptide analysed was peptide **30** (see Fig. 5). This peptide is less active than peptide **31** ($IC_{50} = 2100$ nM). Structural analysis showed that peptide **30** preferentially adopts a turn-like conformation due to the displacement of the amide bond between the Tic and the thiol function. This turn-like conformation is presumably stabilized by the formation of an internal hydrogen bond (see Fig. 5).

To sum up our structural analysis, it seems that different points are critical for inhibitory activity. The previously shown requirement for an extended structure is not the only feature important for activity. The position of the *C*-terminal carboxylate, as well as the position of the thiol function in relation to the hydrophobic anchorage point seem to be another critical point. The last requirement is the presence of a *N*-terminal amine, which is postulated to interact with the zinc in association with the thiol group.

We are currently investigating by additional modifications on the peptide backbone, whether the about mentioned conformational differences between the active and inactive products are relevant for biological activity.

Experimental

Materials and methods

Triethyl amine was purchased from Aldrich–France and used without further purification (negative ninhydrin test), Boc-amino acids and BOP reagent were purchased from Neosystem, France. THF, analytical grade, was purchased from Merck–Clevenot, France, and was kept over molecular sieves (3 Å) and used without further purification. All solvents were analytically pure grade and were used without further purification. HPLC was performed on a Merck–Hitachi gradient pump equipped with a AS-2000A Auto

sampler, a L-6200A Intelligent Pump and a UV–vis detector L-4000 with tuneable wavelength set at 220 nm for analytical separations and at 235 nm for semi-preparative separations. The flow was fixed at 1 mL/min for analytical separations and the conditions are defined for each product. The mobile phases were water (0.05% TFA) and acetonitrile HPLC grade Merck (0.05% TFA). The columns were Lichrocart RP-18 250 × 4 mm i.d. (analytical) from Merck and Bio-Sil C8 or C18 HL 90–10 250 × 10 mm i.d. (semi-preparative) from Bio-Rad. NMR and MS were carried out at the Analysis Department of Rhône Poulenc-Rorer, Vitry-sur-Seine. ^{1}H NMR spectra were recorded on Bruker AC-300 and Bruker AM-400 spectrometers. Samples were dissolved in $CDCl_3$ or DMSO. Chemical shifts are in ppm relative to TMS internal standard. MS were carried out on a VG Autospec by LSIMS technique equipped with a caesium cannon, the matrix was a mixture of glycerol and thioglycerol or nitro-benzyl alcohol (FABMS) and/or a Perkin–Elmer Sciex API (III) MS.

Protracted isolated FTase inhibitory activity of peptides

The inhibitory activity of peptides on isolated FTase was measured in the absence (control) or the presence of THAC lysates. THAC lysates were prepared as previously described.[6] 100 µL of cell lysates were incubated with the peptides at 20 µM, during 90 min at 37 °C, with withdrawals at 0, 15, 30, 60 and 90 min and immediate freezing at −80 °C.

Structural evaluation by molecular modelling studies

Molecular modelling studies were performed using the Insight II/Discover package (Biosym Technologies Inc.) on a Silicon Graphics Indigo II workstation. Simulated annealing protocol (900 K [2 ps], 300 K [2 ps], minimization) was used to generate 100 structures for each modelling molecule. The simulations were carried out in vacuo with *N*- and *C*-termini uncharged to avoid artefactual turn formation. The obtained structures were analysed using Insight II tools.

General synthetic methods

Method A. Coupling with BOP for peptide assembling. To a stirred solution of the amino component (1 mmol) in MeCN or DMF (10 mL) were added BOP reagent (1.1 mmol) the acid component (1.1 mmol) and DIEA (3 mmol) at room temperature. After 15 min the pH was checked for basicity (in cases were the pH was lower than pH = 9, more DIEA was added) and the reaction mixture left for 1 h at room temperature. The reaction was followed by fluorescamine[18] test. When the fluorescamine test was negative, the solvent was evaporated and the crude product dissolved in EtOAc (100 ml) and washed as usually. Final products were purified by semipreparative HPLC and identified by MS.

Method B. Ester hydrolysis after final assembling of peptides. The hydrolysis of the esters for the synthesis of **1–6** were carried out prior to thiol deprotection for preventing thiol oxidation. Briefly, 0.1 mmol of the ester was dissolved in 1 ml THF at 0 °C and LiOH (1 ml of a 0.5 N solution) was added, the mixture was stirred and after 30 min the solution was concentrated to remove THF. A solution of $KHSO_4$ (10 mL) was added and the precipitated product was extracted with ethyl acetate (3 × 10 mL). The organic phase was washed with brine (3 × 10 mL) and water (3 × 10 mL). The solution was dried on $MgSO_4$ and evaporated to dryness. The absence of ester was monitored by TLC. The products were deprotected from their thiol protecting group without further purification.

Decanoyl-CysValTicMet (1). The peptide was assembled by method A using Boc chemistry starting from $MetOCH_3$, after *N*-deprotection of Cys the peptide was coupled with decanoic acid by method A. The ester was hydrolysed by method B and finally, the Acm protective group of Cys was cleaved as previously described.[19] The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on C-18 semipreparative column with a flow of 6 mL/min: 0–1 min: 30% MeCN; 1–15 min: 30–80% MeCN, 15–20 min: 80–100% MeCN; 20–45 min: 100% MeCN, R_f = 28 min. APIMS m/e 665 MH^+.

Lauroyl-CysValTicMet (2). The peptide was assembled and deprotected as **1**, except that lauric acid was used as the *N*-terminal acyl group. The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on a C-8 semipreparative column with a flow of 6 mL/min: 0–1 min: 30% MeCN; 1–15 min: 30–80% MeCN, 15–20 min: 80–100% MeCN; 20–45 min: 100% MeCN, R_f = 31 min. APIMS m/e 693 MH^+, 715 MNa^+.

Myristoyl-CysValTicMet (3). The peptide was assembled and deprotected as **1**, except that myristic acid was used as the *N*-terminal acyl group. The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on a C-8 semipreparative column with a flow of 6 mL/min: 0–1 min: 30% MeCN; 1–15 min: 30–80% MeCN, 15–20 min: 80–100% MeCN; 20–45 min: 100% MeCN, R_f = 35 min. APIMS m/e 721 MH^+.

Palmitoyl-CysValTicMet (4). The peptide was assembled and deprotected as **1**, except that palmitic acid was used as the *N*-terminal acyl group. The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on a C-8 semipreparative column with a flow of 6 mL/min: 0–1 min: 30% MeCN; 1–15 min: 30–80% MeCN, 15–20 min: 80–100% MeCN; 20–45 min: 100% MeCN, R_f = 38 min. APIMS m/e 749 MH^+.

Stearoyl-CysValTicMet (5). The peptide was assembled and deprotected as **1**, except that stearic acid was used as the *N*-terminal acyl group. The peptide was purified by semipreparative HPLC with a linear gradient of MeCN/H_2O on a C-8 semiprepara-

tive column with a flow of 6 mL/min: 0–1 min: 30% MeCN; 1–15 min: 30–80% MeCN, 15–20 min: 80–100% MeCN; 20–45 min: 100% MeCN, R_f= 39 min. APIMS m/e 777 MH^+.

Boc-CysValTicMet (6). The peptide was assembled and deprotected as **1**, except that the Boc protecting group was left in the *N*-terminal position. The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on a C-8 semipreparative column with a flow of 6 mL/min: 0–1 min: 30% MeCN; 1–15 min: 30–80% MeCN, 15–20 min: 80–100% MeCN; 20–45 min: 100% MeCN, R_f= 32 min. APIMS m/e 610 MH^+.

$HSCH_2CH_2CO$—[NCH_3]ValTicHser-lactone (8). The peptide was assembled by method A using Boc chemistry by sequential coupling of Boc-Tic, Boc-[NCH3]Val and (Acm)mercaptopropionic acid to D,L-Hser-Lactone. The final Acm protected peptide was deprotected as previously described.[19] The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on a C-8 semipreparative column with a flow of 6 mL/min: 0–20 min: 0–25% MeCN; 20–30 min: 22–100% MeCN, 30–40 min: 100% MeCN, R_f= 28 min. APIMS m/e 477 MH^+.

$HSCH_2CH_2CO$—[NCH_3]ValTicHcys-lactone (9 and 10). The peptide was assembled by method A using Boc chemistry by sequential coupling of Boc-Tic, Boc-[NCH3]Val and (Acm)mercaptopropionic acid to D,L-Hcys-lactone. The final Acm protected peptide was deprotected as previously described. The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on a C-8 semipreparative column with a flow of 6 mL/min: 0–20 min: 20% MeCN; 20–120 min: 20–100% MeCN, 120–140 min: 100% MeCN, R_f=82 min **(9)**, R_f=105 min **(10)**. Two products with the same molecular weight were separated, we assumed that each product corresponds to D or L isomers of Hcys (no data). APIMS m/e (for **9** and **10**) 478 MH^+.

$HSCH_2CH_2CO$—[NCH_3]ValPheHcys-lactone (11 and 12). The peptide was synthesized as **9–10** by sequential coupling of Boc-Phe, Boc-[NCH3]Val and (Acm)mercaptopropionic acid to D,L-Hcys-lactone. The final Acm-protected peptide was deprotected as previously described. The peptide was purified by HPLC with a linear gradient of MeCN/H_2O on a C-8 semipreparative column with a flow of 6 mL/min: 0–20 min: 20% MeCN; 20–120 min: 20–100% MeCN, 120–140 min: 100% MeCN, R_t=72 min **(11)**, R_f=95 min **(12)**. Two products with the same molecular weight were separated, we assumed that each product corresponds to D or L isomers of Hcys (no data). APIMS m/e 452 MH^+.

2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde (13). (Benzotriazol-1-yloxy)tris (dimethylamino)phosphonium hexafluorophosphate (BOP) (4.42 g, 10 mmol) was added to a stirred solution of Boc-TicOH (2.77 g, 10 mmol) and triethylamine (TEA; 1.33 mL, 10 mmol) in CH_2Cl_2 (30 mL). After 5 min, *N*,*N*-dimethylhydroxylamine hydrochloride (1.2 g, 12 mmol) and triethylamine (1.7 mL, 12 mmol) were added to the solution. The reaction was left overnight and then the mixture was diluted with CH_2Cl_2 (90 mL) and washed with 0.5 M $KHSO_4$ (3 × 50 mL), satd $NaHCO_3$ (3 × 50 mL) and brine (3 × 50 mL). The organic solution was dried on $MgSO_4$ and evaporated, the product (3 g, 93% yield) was analysed by NMR and used without further purification.

To a stirred solution of the *N*-methoxy-*N*-methylamide derivative (3 g, 9.3 mmol) in 30 mL Et_2O under argon atmosphere, 380 mg (10 mmol) of $LiAlH_4$ was added. The reduction was carried out for 1 h at 0 °C and the reaction mixture was then hydrolysed with a solution of $KHSO_4$ (2 g, 15 mmol) in 40 mL of H_2O. The aqueous phase was separated and extracted with ether (3 × 50 mL). The four organic phases were combined, washed with 3 N HCl, a satd soln of $NaHCO_3$ and brine, and finally dried over $MgSO_4$. The solvent was evaporated and the pure aldehyde was obtained as an oil (1 g, 3.8 mmol, yield 40%): ^1H NMR (400 MHz, $CDCl_3$, at a temperature of 333 K, δ in ppm): 1.50 [s, 9H: $C(CH_3)_3$]; 3.10 and 3.18 (respectively, dd and bd, J=17 and 7 Hz and J=17 Hz, 1H each: CH_2Ar); from 4.40 to 4.65 (vbm, 1H: NCH); 4.58 and 4.72 (2 d, J=17 Hz, 1H each: NCH_2Ar); from 7.05 to 7.25 (m, 4H: H aromatics); 9.50 (s, 1H: HC=O). The aldehyde was immediately used for the synthesis of **14**.

Boc-TicΨ(CH_2NH)MetOCH$_3$ (14). 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde **13** (1 g, 3.8 mmol) and Met-OCH_3·HCl (0,99 g, 5 mmol) were dissolved in methanol (5 mL). To the stirred solution were added 0.62 g of $NaCNBH_3$ and the solution was stirred for 1 h at room temperature. The methanol was evaporated, the oil was dissolved in CH_2Cl_2 (50 mL) and washed with $KHSO_4$ (3 × 50 mL), $NaHCO_3$ (3 × 50 mL), brine (3 × 50 mL) and finally dried over $MgSO_4$. After solvent evaporation, Boc-TicΨ(CH_2NH)MetOCH$_3$ (1.3 g, 87% yield) was obtained as an oil: HPLC, NMR and MS demonstrate the high purity of the product which was used without further purification: HPLC, B 50–100% in 50 min; R_f=5.2 min. ^1H NMR (400 MHz, $(CD_3)_2SO$, at a temperature of 373 K, δ in ppm): 1.48 [s, 9H: $C(CH_3)_3$]; 1.72 and 1.86 (2 m, 1H each: CH_2 of Met); 2.07 (s, 3H: SCH_3 of Met); from 2.40 to 2.60 (m: SCH_2 of Met); from 2.80 to 3.10 (m: CH_2Ar and CH_2N); 3.35 (m, 1H: NCH); 3.63 (s, 3H: $COOCH_3$); 4.20 and 4.68 (2 d, J=17 Hz, 1H each: NCH_2Ar); 4.35 (m, 1H: NCH); from 7.10 to 7.25 (m, 4H: H aromatics). FABMS m/e 408 M^+.

Boc-(*N*-Me)Val-TicΨ(CH_2NH)MetOCH$_3$ (15). Product **14** (1.3 g, 3.19 mmol) was deprotected by treatment with TFA (20 mL) during 1 h at room temperature. The TFA was the evaporated and coevaporated with CH_2Cl_2 (3 × 20 mL), the solid deprotected TicΨ(CH_2NH)MetOCH$_3$ was washed with ether (2 × 10 mL). To the same flask was added DMF (10 mL), the solution was stirred and the pH was brought to 7 (atmosphere over the solution) by addition of triethyl-

amine. Boc-(NMe)Val (0,737 g, 3,19 mmol) was added and additional TEA (2 mL, 2 mmol) was added followed by BOP (1.42 g, 3,2 mmol). The reaction was monitored by HPLC, completion was observed after 2 h, then 100 ml of 0.5 M $KHSO_4$ was added and the product was extracted with ethyl acetate (3 × 30 mL). The organic phases were combined and washed with $NaHCO_3$ (3 × 50 mL), brine (3 × 50 mL) and finally dried over $MgSO_4$. After solvent evaporation Boc-(NMe)Val-TicΨ(CH_2NH)MetOCH_3 (1.5 g, 90% yield) was obtained as an oil. HPLC, NMR and MS demonstrate the high purity of the product which was used without further purification. HPLC: 3 min B 30%, B 30–100% in 22 min; R_f=22.82. ^{1}H NMR (400 MHz, $(CD_3)_2SO$ at a temperature of 423 K, δ in ppm): 0.90 (d, J=6.5 Hz, 6H: CH_3 of Val); 1.48 [s, 9H: C$(CH_3)_3$]; 1.75 and 1.88 (2 m, 1H each: CH_2 of Met); 2.07 (s, 3H: SCH_3 of Met); from 2.00 to 2.20 (m, 1H: CH of Val); 2.35 (m, 2H: SCH_2 of Met); from 2.50 to 3.10 (m: CH_2Ar–CH_2N and NCH_3); 3.32 (m, 1H: NCH); 3.65 (s, 3H: $COOCH_3$); 4.32 (m, 1H: NCH); from 4.60 to 4.80 and 5.00 (respectively, m and bd, J=16 Hz, 2H and 1H: NCH_2Ar and NCH); from 7.10 to 7.30 (m, 4H: H aromatics). FABMS m/e 521 M^+.

Trityl-SCH_2CH_2CO(NCH_3)Val-TicΨ(CH_2NH)MetOCH_3 (16). Product **15** (0.24 mg, 0.46 mmol) was deprotected by treatment with TFA (10 mL) during 1 h at room temperature. The TFA was the evaporated and coevaporated with CH_2Cl_2 (3 × 10 mL) the solid deprotected (NCH_3)Val-TicΨ(CH_2NH)MetOCH_3 was washed with ether (2 × 10 mL). To the same flask DMF (5 mL) was added, the solution was stirred and the pH was brought to 7 (atmosphere over the solution) by addition of triethylamine. Trityl-S-CH_2CH_2COOH (0.162 g, 0.46 mmol) was added and additional TEA (0.135 mL, 1 mmol) was added followed by BOP (0.22 g, 0.5 mmol). The reaction was monitored by HPLC, completion was observed after 1 h, then 50 mL of 0.5 M $KHSO_4$ were added and the product was extracted with ethyl acetate (3 × 10 ml). The organic phases were combined and washed with $NaHCO_3$ (3 × 10 mL), brine (3 × 10 mL) and finally dried over $MgSO_4$. After solvent evaporation trityl-SCH_2CH_2CO(NCH_3)Val-TicΨ(CH_2NH)MetOCH_3 (0.34 g, 95% approximate yield by HPLC) was obtained as an oil and purified by HPLC using a 55 min linear gradient of 0–100% MeCN and a flow of 4 mL/min with a semipreparative C18 column and monitored by analytical HPLC: 3 min B 30%, B 30–100% in 22 min, 10 min B 100%, R_f=32 min. ^{1}H NMR [400 MHz, $(CD_3)_2SO$, at a temperature of 423 K, δ in ppm]: 0.90 (d, J=6.5 Hz, 6H: CH_3 of Val); 1.73 and 1.88 (2 m, 1H each: CH_2 of Met); 2.07 (s, 3H: SCH_3 of Met); from 2.10 to 2.40 (m, 7H: CH of Val—SCH_2CH_2CON and SCH_2 of Met); from 2.50 to 2.80 (m: CH_2Ar and NCH_3); 2.88 and 2.94 (2 dd, respectively, J=12 and 2 Hz and J=12 and 6 Hz, 1H each: CH_2N); 3.30 (m, 1H: NCH); 3.62 (s, 3H: $COOCH_3$); 4.22 and 4.93 (2 bd, J=16 Hz, 1H each: NCH_2Ar); 4.70 (m, 1H: NCH); 5.08 (m, 1H: NCH); from 7.00 to 7.50 (m, 19H: H aromatics). FABMS m/e 751 M^+.

Boc-(*N*-Me)Val-TicΨ(CH_2NCH_3)MetOCH_3 (17). Product **15** (0.15 g, 0.2 mmol) was dissolved in acetonitrile (0.73 mL), a solution of 37% aqueous formaldehyde (97 mL, 0.8 mmol) was added followed by 0.0242 mmol $NaCNBH_3$. The reaction was monitored by HPLC, completion was observed after 1 h, then the solvent was evaporated and the product dissolved in ethyl acetate (10 mL), washed with 0.5 M $KHSO_4$ (3 × 5 ml), $NaHCO_3$ (3 × 5 mL), brine (3 × 5 mL), and finally dried over $MgSO_4$. After solvent evaporation Boc-(*N*-Me)Val-TicΨ(CH_2NCH_3)MetOCH_3 (107 g, 100% yield) was obtained as an oil. HPLC, NMR and MS demonstrate the high purity of the product which was used without further purification: HPLC: 3 min B 30%, B 30–100% in 22 min. R_f=25 min. FABMS m/e 535 M^+.

Trityl-SCH_2CH_2CO(NCH_3)Val-TicΨ(CH_2NCH_3)MetOCH_3 (18). Method A: Product **17** (0.16 mg, 0.3 mmol) was deprotected by treatment with TFA (10 mL) during 1 h at room temperature. The TFA was then evaporated and coevaporated with CH_2Cl_2 (3 × 10 mL), the solid deprotected (NCH_3)Val-TicΨ(CH_2NCH_3)MetOCH_3 was washed with ether (2 × 10 mL). To the same flask DMF (5 mL) was added, the solution was stirred and the pH was adjusted to 7 (atmosphere over the solution) by addition of triethylamine. Trityl-S-CH_2CH_2COOH (0.106 g, 0.31 mmol) was added followed by additional TEA (0.17 ml, 1.2 mmol) and BOP (0.137 g, 0.31 mmol). The reaction was monitored by HPLC, completion was observed after 1.5 h, subsequently, 50 mL of 0.5 M $KHSO_4$ were added and the product was extracted with ethyl acetate (3 × 10 mL). The organic phases were combined and washed with $NaHCO_3$ (3 × 10 mL), brine (3 × 10 mL), and finally dried over $MgSO_4$. After solvent evaporation trityl-SCH_2CH_2CO(NCH_3)Val-TicΨ(CH_2NCH_3)MetOCH_3 (0.203 g, 90% approximate yield by HPLC) was obtained as an oil and purified by HPLC using a 55 min linear gradient of 0–100% MeCN and a flow of 4 mL/min with a semipreparative C18 column, R_f=45 min. ^{1}H NMR [400 MHz, $(CD_3)_2SO$, at a temperature of 423 K, δ in ppm]: 0.90 (d, J=6.5 Hz, 6H: CH_3 of Val); 1.73 and 1.88 (2 m, 1H each: CH_2 of Met); 2.07 (s, 3H: SCH_3 of Met); from 2.10 to 2.40 (m, 10H: CH of Val—NCH_3—SCH_2CH_2CON and SCH_2 of Met); from 2.50 to 2.80 (m: CH_2Ar and NCH_3); 2.87 and 2.93 (2 dd, respectively, J=12 and 2 Hz and J=12 and 6 Hz, 1H each: CH_2N); 3.30 (m, 1H: NCH); 3.62 (s, 3H: $COOCH_3$); 4.22 and 4.93 (2 bd, J=16 Hz, 1H each: NCH_2Ar); 4.70 (m, 1H: NCH); 5.08 (m, 1H: NCH); from 7.00 to 7.50 (m, 19H: H aromatics). FABMS m/e 765 M^+.

Method B: Product **16** (0.180 g, 0.24 mmol) was dissolved in 1 mL acetonitrile and a solution of 37% aq formaldehyde (117 mL, 1,2 mmol) was added followed by 0.029 mg $NaCNBH_3$. The reaction was monitored by HPLC, completion was observed after 1 h, then the solvent was evaporated and the product dissolved in ethyl acetate (10 mL), washed with 0.5 M $KHSO_4$ (3 × 5 mL), $NaHCO_3$ (3 × 5 mL), brine (3 × 5 mL), and finally dried over $MgSO_4$. After solvent evaporation

trityl-$SCH_2CH_2CO(NCH_3)$Val-Tic$\Psi(CH_2NCH_3)$Met-OCH_3 (0.183 g, 95% approximate yield by HPLC) was obtained as an oil. The product was similar to the purified product obtained by Method A above, as verified by NMR and MS. The product was used without further purification.

Trityl-$SCH_2CH_2CO(NCH_3)$Val-Tic$\Psi(CH_2NH)$MetOH (19). Product **16** (0.155 mg, 0.22 mmol) was dissolved in 2 mL THF at 0 °C and then LiOH, 0.5 M, 2 mL, was added. The saponification was monitored by HPLC. After 30 the reaction was completed, the solution was neutralized with Phosphate tampon (pH = 5.5) and the solvent was evaporated. The crude salted trityl-$SCH_2CH_2CO(NCH_3)$Val-Tic$\Psi(CH_2NH)$MetOH was purified by HPLC using a 55 min linear gradient of 0–100% MeCN and a flow of 4 mL/min with a semi-preparative C18 column, R_f = 38 min; 0.1 g of the pure final product were obtained (50% yield). FABMS m/e 737 M^+. APIMS m/e 738 MH^+.

HS—$CH_2CH_2CO(NCH_3)$Val-Tic$\Psi(CH_2NH)$MetOH (20). Product **19** (0.06 g, 0.09 mmol) was treated with a mixture of 2 mL CH_2Cl_2, 1 mL TFA and 120 ml triethylsilane at 0 °C during 1 h. The solvent was then evaporated and the solid product was washed with ether (3 × 1 mL). The crude product was purified by HPLC using a 55 min linear gradient of 0–100% MeCN and a flow of 4 mL/min with a semi-preparative C18 column, R_f = 27.2 min. FABMS m/e 495 M^+. APIMS m/e 496 MH^+.

HS—$CH_2CH_2CO(NCH_3)$Val-Tic$\Psi(CH_2NH)$MetOCH$_3$ (21). Product **16** (0.05 g, 0.07 mmol) was treated with a mixture of 2 mL CH_2Cl_2, 1 mL TFA and 120 mL triethylsilane at 0 °C during 1 h. The solvent was then evaporated and the solid product was washed with ether (3 × 1 mL). The crude product was purified by HPLC using a 55 min linear gradient of 0–100% MeCN and a flow of 4 mL/min with a semi-preparative C18 column, R_f = 35.3 min. FABMS m/e 509 M^+. APIMS m/e 510 MH^+.

HS—$CH_2CH_2CO(NCH_3)$Val-Tic$\Psi(CH_2NCH_3)$MetOCH$_3$ (22). Product **18** (0.06 g, 0.08 mmol) was treated with a mixture of 2 mL CH_2Cl_2, 1 mL TFA and 120 mL triethylsilane at 0 °C during 1 h. The solvent was then evaporated and the solid product was washed with ether (3 × 1 mL). The crude product was purified by HPLC using a 55 min linear gradient of 0–100% MeCN and a flow of 4 mL/min with a semi-preparative C18 column, R_f = 37 min. FABMS m/e 523 M^+. APIMS m/e 524 MH^+.

HS—$CH_2CH_2CO(NCH_3)$Val-Tic$\Psi(CH_2NCH_3)$MetOH (23). Product **18** (0.1 g, 0.1 mmol) was dissolved in 2 ml THF at 0 °C then LiOH, 0.5 M, 2 mL, was added. The saponification was monitored by HPLC. After 30 min the reaction was completed, the solution was evaporated and the crude product was treated with a mixture of 2 mL CH_2Cl_2, 1 mL TFA and 120 mL triethylsilane at 0 °C during 1 h. The solvent was then evaporated and the solid product was washed with ether (3 × 1 mL). The crude product was dissolved in degassed H_2O/MeOH (2 mL) and purified by HPLC using a 55 min linear gradient of 0–100% MeCN and a flow of 4 mL/min with a semi-preparative C18 column, R_f = 28 min. FABMS m/e 509 M^+. APIMS m/e 510 MH^+.

***N*-[2-*tert*-Butoxycarbonylmethyl-1,2,3,4-tetrahydro-isoquinoline-3-carbonyl]-methionine ethyl ester (26).** BocTicMetOCH_2CH_3 (436 mg, 1 mmol) was treated with TFA for 1 h. The TFA was then evaporated and the deprotected product was washed with ether (2 × 5 mL). The product was dissolved in DMF (1 mL) and TEA was added (0.35 mL, 5 mmol), the pH = 10 (of a wet pH paper). *tert*-Butyl bromoacetate (0.161 mL, 1 mmol) was then added. The solution was stirred overnight. Water was added (10 mL) and the precipitated product was extracted with ethyl acetate (3 × 10 mL), the organic combined phases were washed with $KHSO_4$, $NaHCO_3$ and brine, dried on $MgSO_4$ and the solvent evaporated to dryness. Purification of the crude *tert*-butyl ester (0.41 g, 90% yield) was not necessary as HPLC and NMR analysis revealed no impurities. HPLC: linear gradient of MeCN/H_2O on a C-18 analytical column with a flow of 1 mL/min: 0–3 min: 50% MeCN; 3–20 min: 50–100% MeCN, 20–35 min: 100% MeCN, R_f = 7.6 min. ^{1}H NMR [400 MHz, $(CD_3)_2SO$, δ in ppm]: 1.19 (t, J = 7 Hz, 3H: CH_3 of ethyl); 1.47 [s, 9H: $C(CH_3)_3$]; 1.80 and 1.92 (2 m, 1H each: CH_2 of Met); 2.00 (s, 3H: SCH_3 of Met); 2.22 (m, 2H: SCH_2 of Met); 2.98 (limit AB, J = 16.5 and 6.5 Hz, 2H: CH_2Ar); 3.42 and 3.48 (2 d, J = 16.5 Hz; 1H each: NCH_2Ar); 3.76 (t, J = 6.5 Hz, 1H: NCH); 3.90 and 3.95 (2 d, J = 15 Hz, 1H each: NCH_2COO); 4.05 (q, J = 7 Hz, 2H: OCH_2 of ethyl); 4.35 (m, 1H: NCH of Met); from 7.05 to 7.25 (m, 4H: H aromatics); 8.23 (d, J = 8 Hz, 1H: NHCO of Met). FABMS m/e 450 M^+.

[$SCH_2CH_2NHCOCH_2$-TicMetOCH_2CH_3]$_2$ (27). Product **26** (450 mg, 1 mmol) was treated with TFA for 1 h. The TFA was then evaporated and the deprotected product was washed with ether (2 × 5 mL). The product was then coupled with Cystamine ([$SCH_2CH_2NH_2$]$_2$, 0.5 mmol) in DMF by method A.

The product was purified by semi-preparative HPLC with a linear gradient of MeCN/H_2O; 0–50 min: 20–100% MeCN; 50–60 min: 100% MeCN on a C-18 semipreparative column with a flow of 4 mL/min, R_f = 22 min. APIMS m/e 905 MH^+.

[$SCH_2CH_2NHCOCH_2$-TicMet]$_2$ (28). Product 27 was hydrolysed by method B. The crude product was purified by semi-preparative HPLC with a linear gradient of MeCN/H_2O; 0–3 min: 30% MeCN; 3–25 min: 30–100% , 25–35 min: 100% MeCN on a semi-preparative C-18 column with a flow of 6 mL/min, R_f = 12 min. APIMS m/e 849 MH^+.

H$SCH_2CH_2NHCOCH_2$-TicMetOCH_2CH_3 (29). Product **27** (0.1 mmol) was dissolved into 30 mL of tris 50 mM pH = 8 containing 0.2 M dithiothreitol (6 mL DTT, 1

M). The reaction was followed by HPLC (completion after 1 h). The product was purified by semi-preparative HPLC with a linear gradient of MeCN/H_2O; 0–50 min: 0% MeCN; 50–120 min: 0–100% MeCN on a semi-preparative C-18 column with a flow of 4 mL/min, R_f=91 min. APIMS m/e 454 MH^+.

$HSCH_2CH_2NHCOCH_2$-TicMet (30). Product **28** (0.1 mmol) was dissolved into 30 mL of tris 50 mM pH=8, containing 0.2 M dithiothreitol (6 mL DTT, 1 M). The reaction was followed by HPLC (completion after 1 h). The product was purified by semi-preparative HPLC with a linear gradient of MeCN/H_2O; 0–50 min: 0% MeCN; 50–120 min: 0–100% MeCN on a C-18 semipreparative column with a flow of 4 mL/min, R_f=81 min. APIMS m/e 426 MH^+.

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