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Thiocarbamate-linked peptides by chemoselective peptide ligation

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Abstract: Peptide chemical ligation chemistries, which allow the chemoselective coupling of unprotected peptide fragments, are useful tools for synthesizing native polypeptides or unnatural peptide-based macromolecules. We show here that the phenylthiocarbonyl group can be easily introduced into peptides on α or ε amino groups using phenylthiochloroformate and standard solid-phase method. It reacts chemoselectively with cysteinyl peptides to give an alkylthiocarbamate bond. *S*,*N*-shift of the alkylaminocarbonyl group from the Cys side chain to the α-amino group did not occur. The method was used for linking two peptide chains through their *N*-termini, for the synthesis of a cyclic peptide or for the synthesis of di- or tetravalent multiple antigenic peptides (MAPs). Thiocarbamate ligation is thus complementary to thioether, thioester or disulfide ligation methods.

Supporting information may be found in the online version of this article.

Keywords: ligation; phenylthiocarbamate; cysteine; alkylthiocarbamate; peptide; transthioesterification

INTRODUCTION

The peptide chemist toolbox contains a variety of chemoselective ligation methods that allow the convergent assembly of natural proteins or synthetic peptide scaffolds [1–3]. Native chemical ligation (NCL) [4,5], Staudinger ligation [6] and the decarboxylative condensation of *N*-alkylhydroxylamines and α-ketoacids recently reported by Bode *et al.* [7] lead to the formation of a native peptide bond at the ligation site. In particular, NCL is based on the reaction of a peptide thioester group with a cysteinyl peptide. Transthioesterification is followed by an intramolecular *S*,*N*-acyl shift that results in the formation of a peptide bond at a X-Cys junction. Application of NCL to selenocysteine was also described [8,9]. Methods based on the use of *N*-linked thiol-containing cleavable auxiliaries [10] were used to extend the principle of NCL to sites other than Cys residues. Ligation of peptide thioesters with *N*-terminal homocysteine [11] or homoselenocysteine [12] peptides followed by methylation permitted the formation of X-Met or X-selenoMet bonds.

Other methods result in the formation of unnatural covalent bonds such as disulfide [13], oxime [14], hydrazone [15–18], thioether [13], thiazolidine [18,19], thioester [20] and 1,2,3-triazole linkages [21]. These methods are of great interest when native peptide bonds are not absolutely required. In this context, we examined the potential use of the phenylthiocarbamate group in ligation chemistry. We first studied the modification of peptides by phenylthio or ethylthiocarbonyl moieties on either α–or ε-amino groups using standard solid-phase methods. We show that the reaction of phenylthiocarbonyl peptides with cysteinyl peptides leads to the formation of an alkylthiocarbamate moiety by transthioesterification. This alkylthiocarbamate was stable and did not rearrange through *S*,*N*-shift to the corresponding urea compound. The utility of the method for linking two peptide chains through their *N*-termini, for the synthesis of a cyclic peptide or for the synthesis of di- or tetravalent multiple antigenic peptides (MAPs), was examined.

RESULTS AND DISCUSSION

The chemistry of thiocarbamates **1** has been scarcely studied in the peptide field (Scheme 1) [22]. We have examined the reactivity between thiocarbamates **1** and *N*-terminal cysteinyl peptides **2**. Indeed, the thiocarbamate moiety of peptide **1** can potentially react with the thiol group of cysteine to give intermediate thiocarbamate **3**. The ability of thiocarbamate **3** to rearrange through an *S*,*N*-shift as in NCL to give urea **4** was questionable because of the reduced reactivity of the alkylthiocarbamate group compared to the thioester group. Whatever be the result of the ligation reaction, the formation of an alkylthiocarbamate or urea bond by the chemoselective reaction between thiocarbamates and *N*-terminal cysteinyl peptides was envisioned as a complementary ligation method to other thiol-based

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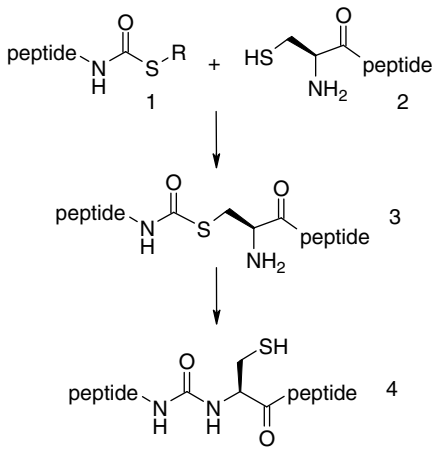


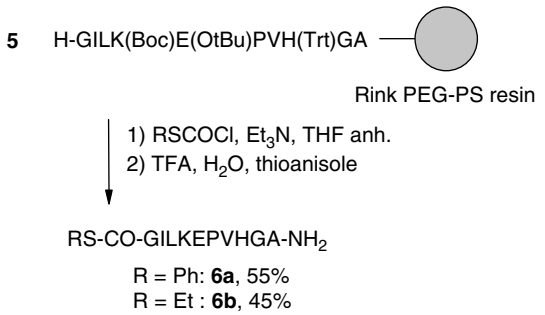
Scheme 1 Reaction between thiocarbamate **1** and cysteinyl peptide **2** can potentially lead to alkylthiocarbamate **3** and/or urea **4**.

chemistries and a way to obtain novel peptide scaffolds for structure–function studies.

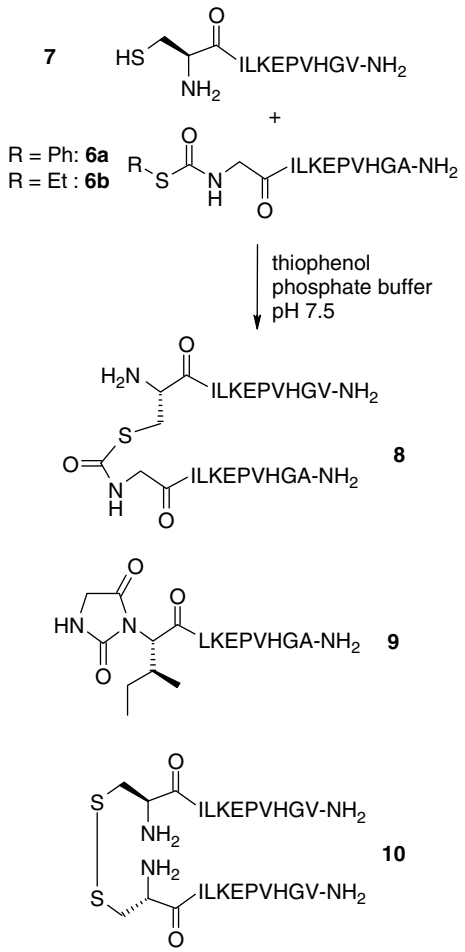
The incorporation of phenylthio or ethylthiocarbonyl groups at the *N*-terminus of peptides was performed using commercially available phenylthio- or ethylthiochloroformates, respectively (Scheme 2). The peptide was first assembled using standard Fmoc/*tert*-butyl solid-phase methods [23]. Phenylthio- or ethylthiochloroformates were reacted in anhydrous THF with the free α–amino group of peptidyl resin **5** in the presence of triethylamine. Deprotection and cleavage in TFA furnished cleanly thiocarbamates **6a** and **6b**, respectively.

Next, ligation of thiocarbamates **6a** and **6b** with cysteinyl peptide **7** [24] was examined (Scheme 3). The reaction was performed at pH 7.5 in the presence of thiophenol and benzylthiol under argon (2 mM peptide concentration), i.e. typical experimental conditions used for NCL. The reactions were monitored by RP-HPLC. The peaks were collected and analyzed by MALDI-TOF. Ethylthiocarbamate **6b** failed to react, whereas phenylthiocarbamate **6a** led to a mixture of three products. Besides the expected ligation product **8** (31% by RP-HPLC), we observed the formation of substantial amounts of hydantoin **9** (31%) and disulfide **10** (28%). Increasing the peptide *N*-phenylthiocarbamate concentration to 10 mM led to no significant improvement, whereas performing the reaction at 50 mM led to the clean formation of ligation product **8**, which could be isolated with 66% yield. No ligation product was detected when the same reaction was performed with the Ser analog of peptide **7** H-SILKEPVHGV-NH_2 [24], showing the requirement of a Cys residue for the ligation to proceed.

Several experiments were performed to ascertain the structure of the ligation product **8**. If the thiocarbamate transthioesterification product **3** (Scheme 1) rearranged spontaneously into urea **4**, ligation product between peptides **6a** and **7** should present a free thiol group, which should lead to the corresponding disulfide upon air oxidation in basic medium. We thus examined the stability of the RP-HPLC purified ligation product **8** in a pH 8.1 phosphate buffer at rt. The mixture was analyzed by LC-MS. The expected disulfide was not detected. Instead, we observed the formation of cysteinyl peptide **7** and its disulfide, and disulfides of peptide H-GILKEPVHGA-NH_2 and hydantoin **9**. After 2 h, the peak area corresponding to the



Scheme 2 Synthesis of thiocarbamates **6a** and **6b**.



Scheme 3 Ligation between cysteinyl peptide **7** and thiocarbamates **6a** and **6b**.

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ligation product **8** represented 38% of the total collected area. Moreover, reaction of the ligation product in the same buffer in the presence of iodoacetamide furnished the same degradation products together with peptide H-C(CH_2CONH_2)ILKEPVHGV-NH_2 resulting from the alkylation of peptide **7**. Both the lability in basic medium of the bond linking the two peptide molecules and the absence of a free thiol group in the ligation product are in favor of the thiocarbamate structure **8**.

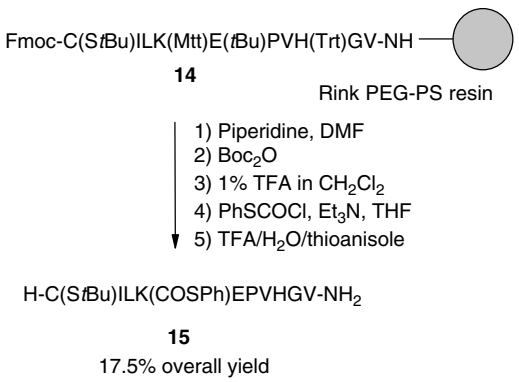
The high molecular weight of peptide **8** complicated its NMR analysis. A detailed ^1H, ^{13}C, COSY, TOSCY, HSQC and HMBC NMR study of the bond formed during the ligation process was performed using peptide **13** H-C(CO-IKA-NH_2)YG-NH_2 (71% yield), obtained by ligating cysteinyl tripeptide **11** H-CYG-NH_2 with phenylthiocarbamate **12** PhSCO-IKA-NH_2 (see Supporting information). The CO–NH proton of Ile residue appeared as a doublet at 8.65 ppm (DMF-d_7). The protons on the α-amino group of Cys residue were fast exchanging as expected for thiocarbamate structure **3**. The α and β protons of Cys for peptide **13** were found at 4.32 (triplet) and 3.47 (multiplet) ppm, whereas the α and β protons of Cys for peptide **11** were found at 4.41 and 3.18–3.27 ppm, respectively. The ~0.2–0.3 ppm downfield shift of β Cys protons in ligation product **13** compared to Cys peptide **11** is again in favor of the thiocarbamate structure **3**.

The stability of the alkylthiocarbamate structure **3** (Scheme 1) is in accordance with literature data. The synthesis of *S*-alkylcarbamoyl cysteines by reacting cysteine hydrochloride with alkylisocyanates was reported by Ross *et al.* [25]. The ethylcarbamoyl group was later used by Storey *et al.* for the temporary protection of the thiol of cysteine in convergent protein synthesis [26]. This group was stable toward strong acids such as TFA. *S*-ethylcarbamoyl cysteine showed only 10% deblocking in pH 8.5 0.25 M ammonium bicarbonate buffer after 66 h, showing that the *S*,*N*-shift of the ethylcarbamoyl group was not a favored process. The reactivity of thiocarbamate **3** (Scheme 1) should be close to those of *S*-ethylcarbamoyl cysteine, and indeed no rearrangement leading to urea **4** could be detected on our model ligation experiments. Drozd reported an *S*,*N*-shift for a dimethylcarbamoyl group but using *o*-mercaptoaniline derivatives and harsh experimental conditions [27].

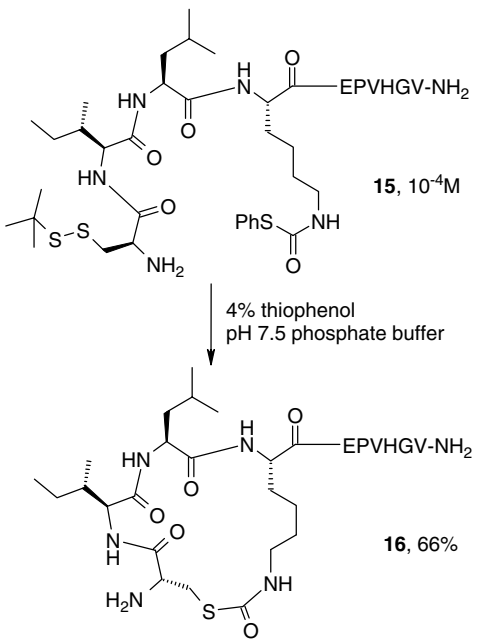
We next examined the potential utility of the ligation method for the synthesis of cyclic peptides, by reaction of a phenylthiocarbamate group with an internal cysteine residue. For this, peptide **15** featuring a phenylcarbamate group on the lysine side chain and an *N*-terminal *S*-(*tert*-butylsulfenyl)cysteine was synthesized as described in Scheme 4. The peptide chain was assembled on a Rink-type resin [28] using *tert*-butylsulfenyl and Mtt protections for Cys^1 and Lys^4 side chains, respectively. The Fmoc group on the α-amino group of Cys^1 was first replaced by a Boc group. Then, the Mtt group on Lys^4 was removed selectively using 1% TFA in CH_2Cl_2 [29]. The phenylthiocarbamate group was introduced in the Lys^4 side chain using PhSCOCl/Et_3N in dry THF. Finally, deprotection and cleavage with TFA furnished peptide **15** (17.5%).

Cyclization of peptide **15** was performed at 0.1 mM peptide concentration to avoid the formation of oligomers (Scheme 5). Thiophenol allowed the *in situ* removal of the *tert*-butylsulfenyl group and the clean conversion of peptide **15** into cyclic peptide **16** (66% isolated yield).

We finally examined the utility of the ligation method for the synthesis of MAPs [30]. MAPs are peptide dendrimers with branched structure, which have found a variety of applications in the development of vaccines, diagnostic products and artificial enzymes. These structures present multiple copies of a particular peptide on a lysine-based core. The synthesis of unambiguous and homogeneous MAPs is usually carried out by ligating chemoselectively purified peptide



Scheme 4 Synthesis of thiocarbamate **15**.



Scheme 5 Cyclization of peptide **15**.

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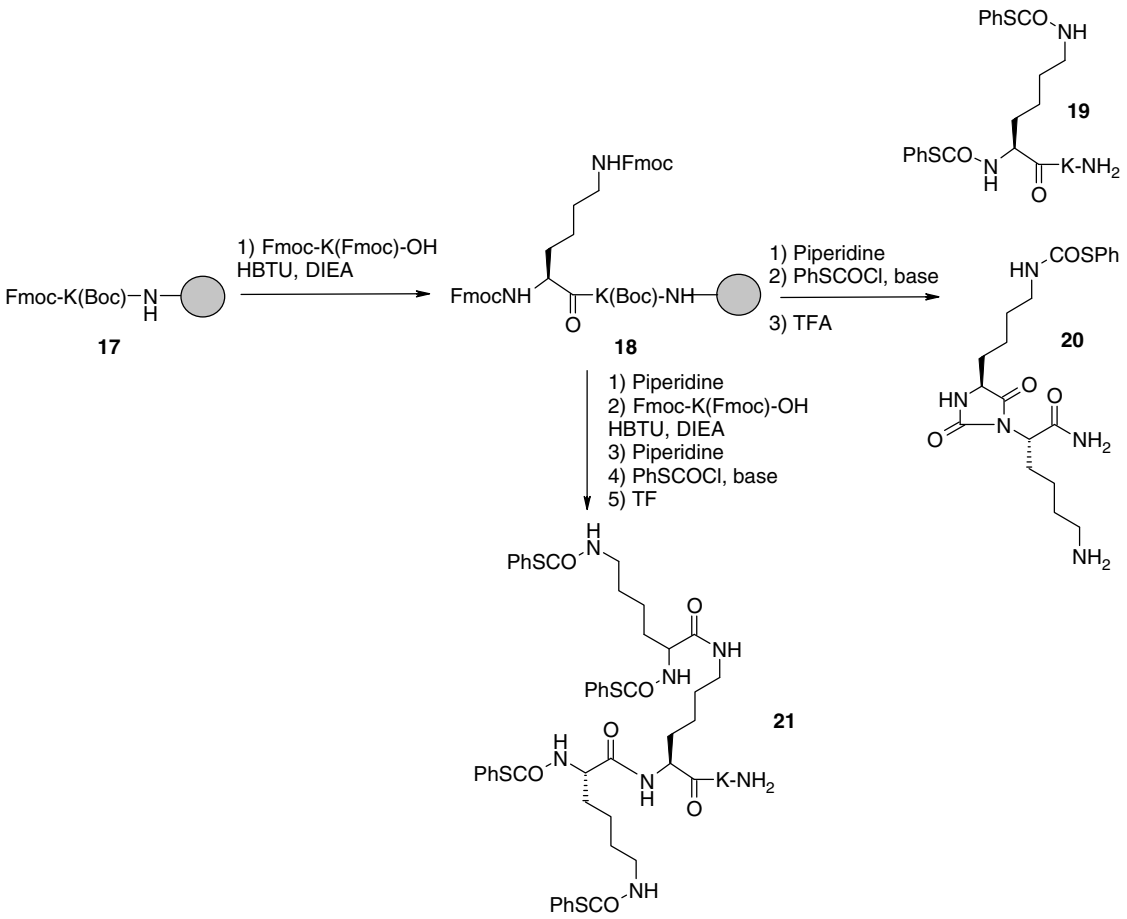

Scheme 6 Synthesis of phenylthiocarbamate functionalized lysinyl cores **19** and **21**.

segments to the lysinyl core peptide presenting the appropriate functional groups at its periphery [18].

Di- and tetravalent lysinyl cores modified by phenylthiocarbonyl groups were synthesized on the solid phase as described in Scheme 6. First, Fmoc-L-Lys(Boc)-OH was coupled to a Rink-type resin. Removal of the Fmoc group with piperidine was followed by reaction of α and ε amines with phenylthiochloroformate in the presence of triethylamine as before. However, deprotection and cleavage from the resin with TFA failed to give the expected divalent core **19**. Instead, MALDI-TOF analysis of the product suggested the exclusive formation of hydantoin **20**. To minimize hydantoin formation, we examined the potential utility of *N*-methylmorpholine or pyridine as alternative bases. The duration of the reaction was also reduced to 30 min instead of 2 h to minimize the contact between the phenylthiocarbamate groups and the base. Pyridine permitted the isolation of the target divalent lysinyl core **19** with 24% yield. However, hydantoin formation was still observed, together with products arising from incomplete phenylthiocarbonylation. Interestingly, hydantoin formation was almost suppressed with *N*-methylmorpholine as base. Using these experimental conditions, divalent lysinyl core **19** was isolated with 42% yield following RP-HPLC purification. Likewise, the tetravalent tree **21** was isolated with 13% yield.

Ligation of lysinyl cores **19** and **21** to cysteinyl peptide **7** was performed as described in Scheme 7. The concentration of peptide **7** was 50 mM to minimize hydantoin formation as before. Using stoichiometric conditions, divalent MAP **22** was isolated with 30% yield after purification. The same procedure failed to give any tetravalent MAP **23** probably because of the low solubility of core **21** in water. Alternately, ligation in DMF in the presence of thiophenol and DIEA furnished the tetravalent MAP **23** with 23% yield after purification.

CONCLUSION

In conclusion, peptide phenylthiocarbamates can be easily synthesized using standard Fmoc/*tert*-butyl solid-phase methods. The phenylthiocarbonyl group could be introduced on α or ε-amino groups. Ligation with cysteinyl peptides proceeded efficiently, provided that the peptide concentration was 50 mM to avoid hydantoin formation. The ligation product features an alkylthiocarbamate bond formed by transthioesterification of the phenylthiocarbamate by the thiol of the Cys

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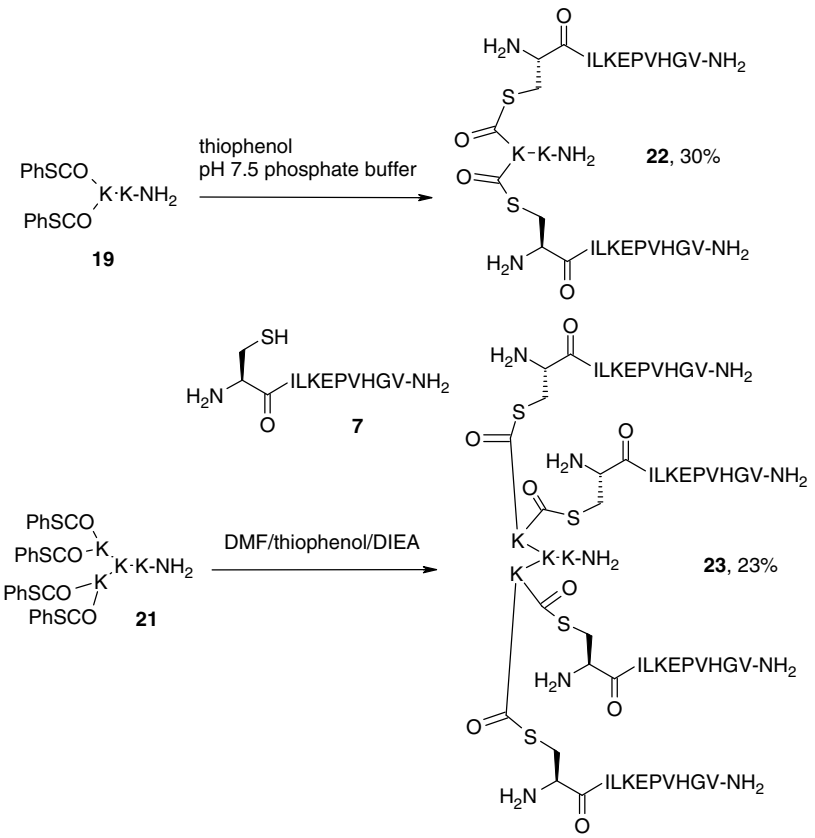

Scheme 7 Ligation of cysteinyl peptide **7** with di- or tetravalent cores **19** and **21**.

residue. S,*N*-shift to give a urea bond did not occur. The use of thiophenol during the ligation is recommended to minimize the formation of disulfide bonds between cysteines. Introduction of phenylthiocarbonyl on a ε-amino group and ligation with an internal cysteine led to the formation of a cyclic peptide. Finally, di-and tetravalent lysinyl cores modified on their periphery by phenylthiocarbonyl moieties were synthesized successfully and ligated to give MAPs. Thus, the phenylthiocarbamate/cysteine ligation method complements the ligation techniques currently available to the peptide chemist and should open novel opportunities for the synthesis of complex peptide scaffolds. Rapid degradation of the thiocarbamate linkage was observed at pH 8.1. Interestingly, the thiocarbamate linkage is stable at or below pH 7.5 and thus is compatible with most experimental procedures used in biochemistry or biology.

EXPERIMENTAL SECTION

Synthesis of Peptides 6a and 6b

Peptide H-GILK(Boc)E(*t*Bu)PVH(Trt)GA was assembled on a Novasyn TGR resin (Novabiochem, 0.3 mmol, 0.23 mmol/g) using a Pioneer peptide synthesizer (Applied Biosystems) and standard Fmoc/*tert*-butyl protocols [23]. The resin was washed thoroughly with anhydrous THF and then reacted with phenylthiochloroformate (Sigma-Aldrich, ref 561 231, 1.20 mmol, 173 µl) and Et_3N (1.2 mmol, 154 µl) in THF (12 ml) during 1 h 30 min at rt. The resin was then washed twice with CH_2Cl_2 and Et_2O and dried *in vacuo*. Deprotection and cleavage from the resin was performed in TFA/H_2O/thioanisole (96.5/2.5/1 v/v/v, 30 ml) during 1 h at rt. The peptide was precipitated in Et_2O/*n*-heptane (1/1 by vol, 300 ml) and centrifugated. The solid was solubilized in water and lyophilized. The crude product was purified by RP-HPLC to give 201 mg (48%) of peptide **6a**.

Peptide **6a**: $C_{53}H_{82}N_{14}O_{13}S$; MW, 1155.37; RP-HPLC and capillary zone electrophoresis (CZE) purities (pH 3.0 20 mM sodium citrate or 50 mM pH 4.5 sodium acetate buffers) were >98%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 1155.6, found 1155.6.

Peptide **6b** was obtained similarly. Starting from 0.05 mmol of Novasyn TGR resin, 30.1 mg (45%) of peptide **6b** were obtained after RP-HPLC purification.

Peptide **6b**: $C_{49}H_{82}N_{14}O_{13}S$; MW, 1107.33; RP-HPLC and CZE purities (pH 3.0 sodium citrate or pH 4.5 sodium acetate buffers) were >98%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 1107.6, found 1107.3.

Synthesis of Peptide 12

Peptide **12** was assembled on a Novasyn TGR resin (Novabiochem, 0.25 mmol, using a CEM microwave peptide synthesizer (Orsay, France) and standard Fmoc/*tert*-butyl protocols [23].

The resin was washed thoroughly with anhydrous THF and then reacted with phenylthiochloroformate (0.25 mmol, 36 µl) and *N*-methylmorpholine (0.25 mmol, 27 µl) in THF (3 ml)

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during 30 min at rt. The resin was then washed twice with CH_2Cl_2 and Et_2O and dried *in vacuo*. Deprotection and cleavage from the resin was performed in TFA/H_2O/thioanisole (96.5/2.5/1 v/v/v, 8 ml) during 1 h at rt. The peptide was precipitated in Et_2O/*n*-heptane (1/1 by vol, 250 ml) and centrifugated. The solid was solubilized in water and lyophilized. 111.8 mg of peptide **12** was obtained ($R = 77\%$).

Peptide **12**: $C_{22}H_{35}N_5O_4S$; MW, 465.34; RP-HPLC purity, 97%; CZE purity (pH 3.0 sodium citrate buffer), 96%; CZE purity (pH 4.5 sodium acetate buffer), 93%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic), 466.2, found 466.1.

^{1}H NMR (300 MHz, (DMF, δ_H): 0.88 (3H, t, CH_3 δ Ile), 0.95 (3H, d, CH_3 γ Ile), 1.32 (3H, d, CH_3 Ala), 1.34 (1H, m, CH_2 γ Ile), 1.5 (3H, m, CH_2 ε Lys, CH_2 γ' Ile), 1.83 (5H, m, CH_2 δ Lys, CH_2 γ Lys, CH_2 β Ile), 3.03 (2H, m, CH_2 β Lys), 4.35 (3H, m, CH α Lys, CH α Ile, CH α Ala), 7.09 (1H, s, $CONH_2$) 7.54 (6H, m, ArH, $CONH_2$), 7.97 (1H, d, NH Ala), 8.28 (1H, d, NH Lys), 8.41 (1H, d, NH Ile). ^{13}C NMR (75.4 MHz, DMF, δ_C) 13.0, 13.2, 16.6, 17.6, 20.3, 20.4, 24.7, 26.8, 27.2, 29.1, 29.2, 33.5, 33.7, 39.1, 39.3, 41.7, 41.8, 51.0, 55.3, 59.7, 62.4, 72.7, 131.3, 131.4, 131.6, 137.5, 167.4, 170.6, 173.2, 173.4, 173.5, 177.0.

Synthesis of Peptide 15

Peptide H-C(S*t*Bu)ILK(Mtt)E(*t*Bu)PVH(Trt)GV was assembled on a Novasyn TGR resin (Novabiochem, 0.19 mmol, 0.23 mmol/g) using a Pioneer peptide synthesizer (Applied Biosystems) and standard Fmoc/*tert*-butyl protocols [23]. The peptidyl resin was reacted with Boc_2O (415 mg, 1.90 mmol) in DMF during 1 h. The resin was then washed with DMF (4×2 min) and CH_2Cl_2 (4×2 min). The absence of unreacted amino groups was verified using the 2,4,6-trinitrobenzene-1-sulfonic acid test [31]. Selective deprotection of Mtt group was performed using 1% TFA in CH_2Cl_2 (17×11.4 ml) [29]. The resin was neutralized with 5% DIEA in CH_2Cl_2 (5×2 min) and washed with CH_2Cl_2 (6×2 min). The resin was then reacted during 2 h with PhSCOCl (110 µl, 0.76 mmol) and Et_3N (107 µl, 0.76 mmol) in anhydrous THF (7.6 ml). The resin was then washed with THF (4×2 min), CH_2Cl_2 (6×2 min), Et_2O (2×2 min) and dried. Deprotection and cleavage from the resin was performed in TFA/water/thioanisole (96.5/2.5/1 v/v/v) during 1 h. The peptide was precipitated in cold Et_2O/*n*-heptane (1/1 by vol), redissolved in water and lyophilized. RP-HPLC purification furnished 33.9 mg (17.5%) of peptide **15**.

Peptide **15**: $C_{60}H_{96}N_{14}O_{13}S_3$; MW, 1316.64; RP-HPLC purity, 91%; CZE purity (pH 3.0 sodium citrate buffer), >98%; CZE purity (pH 4.5 sodium acetate buffer), >98%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 1317.7, found 1317.9.

Synthesis of Lysinyl Cores 19 and 21

Lysinyl cores **19** was prepared starting from NovaSyn TGR resin (0.025 mmol, 0.23 mmol/g). Fmoc-L-Lys(Boc)-OH and Fmoc-L-Lys(Fmoc)-OH were coupled successively using standard Fmoc/*tert*-butyl protocols. The terminal Fmoc groups were removed using 20% piperidine in DMF (5 and 15 min). The resin was washed with DMF (5×2 min) and then with anhydrous THF (8×2 min) under argon. The resin was reacted for 30 min with phenylthiochloroformate (0,05 mmol, 7.2 µl) and *N*-methylmorpholine (0.05 mmol, 5.6 µl) in 500 µl of anhydrous THF under argon. The resin was then washed with THF (3×2 min), CH_2Cl_2 (3×2 min), Et_2O (3×2 min) and dried. Deprotection and cleavage from the resin was performed during 1 h at rt in a mixture of TFA/H_2O/thioanisole (96.5/2.5/1 v/v/v). The crude product was precipitated in 50 ml of Et_2O/*n*-heptane (1/1 v/v), solubilized in water and lyophilized. RP-HPLC purification yielded 7.0 mg (42%) of peptide **19** as a white hygroscopic powder.

Peptide **19**: $C_{26}H_{35}N_5O_4S_2$; MW, 545.21; RP-HPLC purity, >98%; CZE purity (pH 3.0 sodium citrate buffer), 91%; CZE purity (pH 4.5 sodium acetate buffer), 88%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 546.2, found 546.2.

Tetravalent lysinyl core **21** was obtained similarly. Starting from 0.1 mmol of Novasyn TGR resin; 15.8 mg (13.3%) of peptide **21** was obtained after RP-HPLC purification.

Peptide **21**: $C_{52}H_{67}N_9O_8S_4$, MW, 1073.39; RP-HPLC purity, 93%; CZE purity (pH 3.0 sodium citrate buffer), 93%; CZE purity (pH 4.5 sodium acetate buffer), 84%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 1074.4, found 1074.5.

Ligations

Synthesis of peptide **8**. Ligation between peptide **6a** (9.60 mg, 7.0 µmol) and peptide **7** (10.0 mg, 7.0 µmol) was performed in a pH 7.48 0.2 M sodium phosphate buffer containing 4% by vol of thiophenol under argon at 20 °C. Final peptide concentration was 50 mM. After 24 h, the crude reaction mixture was purified by RP-HPLC to give 11.9 mg (66%) of peptide **8**.

Peptide **8**: $C_{96}H_{160}N_{28}O_{25}S$; MW 2137.18; RP-HPLC purity, 94%; CZE purity (pH 3.0 sodium citrate buffer), >98%; CZE purity (pH 4.5 sodium acetate buffer), 96%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 2138.2, found 2138.1

Peptide **13**. Ligation between peptide **11** (29.91 mg, 66.0 µmol) and peptide **12** (38.11 mg, 66.0 µmol) was performed in a pH 7.45 0.2 M sodium phosphate buffer containing 4% by vol of thiophenol under argon at 20 °C. Final peptide concentration was 50 mM. After 24 h, the crude reaction mixture was purified by RP-HPLC to give 37.9 mg (71%) of peptide **13.**

Peptide **13**: $C_{30}H_{49}N_9O_8S$; MW 695.34; RP-HPLC purity, 98.5%; CZE purity (pH 3.0 sodium citrate buffer); 98.6%; CZE purity (pH 4.5 sodium acetate buffer), 98.8%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 696.3, found 696.2.

^{1}H NMR (300 MHz, DMF, δ_H): 0.85 (3H, t, CH_3 δ Ile), 0.92 (3H, d, CH_3 γ Ile), 1.22 (1H, m, CH_2 γ Ile), 1.30 (3H, d, CH_3 β Ala), 1.53 (3H, m, CH_2 ε Lys, CH_2 γ' Ile), 1.80 (5H, m, CH_2 δ Lys, CH_2 γ Lys, CH β Ile), 3.08 (4H, m, H β, β' Tyr, CH_2 β Lys), 3.40 (2H, m, CH_2 Cys), 3.85 (2H, t, CH_2 Gly), 4.25 (1H, t, CH α Ile), 4.37 (3H, m, CH α Ala, CH α Lys, CH α Cys), 4.64 (1H, dd, CH α Tyr), 6.73 (2H, d, J 8.5, $H_{3,5}$ Tyr), 7.10 (2H, d, J 8.4, $H_{2,6}$ Tyr), 7.14 (1H, s, $CONH_2$), 7.19 (1H, s, $CONH_2$) 7.45 (1H, s, $CONH_2$), 7.59 (1H, s, $CONH_2$) 8.07 (1H, d, NH Ala), 8.20 (1H, d, NH Lys), 8.33 (1H, t, NH Gly), 8.63 (1H, d, NH Ile), 8.92 (1H, d, NH Tyr). ^{13}C NMR (75.4 MHz, DMF δ_C): 13.2, 17.6, 20.5, 24.8, 27.3, 29.3, 39.2, 41.9, 44.7, 51.1, 55.2, 55.8, 58.0, 62.9, 117.5, 130.1, 132.8, 159.0, 168.3, 169.9, 173.3, 173.5, 173.7, 173.9, 177.2.

Peptide **16**. Peptide **15** (17.9 mg, 11.6 µmol) was dissolved under argon in 116 ml of pH 7.5 0.1 M sodium phosphate buffer and 4.7 ml of thiophenol. After 26 h, thiophenol was extracted with cyclohexane (6×120 ml). The crude product was purified by RP-HPLC to give 9.5 mg (66%) of peptide **16**.

Peptide **16**: $C_{50}H_{82}N_{14}O_{13}S$; MW, 1118.59; RP-HPLC and CZE purities (pH 3.0 sodium citrate or pH 4.5 sodium acetate

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buffers), >98%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 1119.6, found 1119.8.

Peptide **22**. Peptide **7** (20.8 mg, 14.5 μmol) and divalent lysinyl core **19** (4.78 mg, 7.25 μmol) were reacted under argon in a pH 7.4 sodium phosphate buffer containing 20% by vol of *tert*-butanol and 4% by vol of thiophenol. After 5 days at 20 °C, the crude reaction mixture was purified by RP-HPLC to give 6.6 mg (30%) of peptide **22**.

Peptide **22**: $C_{112}H_{191}N_{33}O_{28}S$; MW, 2510.39; RP-HPLC purity, 97%; CZE purity (pH 3.0 sodium citrate buffer), 87%; CZE purity (pH 4.5 sodium acetate buffer), 81%; MALDI-TOF $[M + H]^+$ calcd (monoisotopic) 2511.4, found 2511.5.

Peptide **23**. Peptide **7** (20.02 mg, 14.0 μmol) and tetravalent lysinyl core **21** (2.76 mg, 2.33 μmol) were reacted under argon in a mixture of DMF/thiophénol/DIEA (253/11/16 μl). After 43 h at rt, the crude reaction mixture was purified by RP-HPLC to give 3.2 mg (22.8%) of peptide **23**.

Peptide **23**: $C_{224}H_{379}N_{65}O_{56}S_4$; MW, 5003.76; RP-HPLC purity, >99%; CZE purity (pH 3.0 sodium citrate buffer), 93%; CZE purity (pH sodium acetate buffer), 95%. ESI (30 V, 80 °C source temperature, 120 °C desolvatation temperature, N_2 flow 350 l/h): $[M + 4H]^{4+}$ m/z calcd 1252.1, found 1252.97; $[M + 5H]^{5+}$ calcd 1001.9, found 1002.5.

Supporting Information

Supporting information may be found in the online version of this article.

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