Preparation of the β^3-Homoselenocysteine Derivatives Fmoc-β^3hSec(PMB)-OH and Boc-β^3hSec(PMB)-OH for Solution and Solid-Phase-Peptide Synthesis and Selenoligation

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Dedicated, in admiration, to Professor *Teruaki Mukaiyama* on the occasion of his 80th birthday

The title compounds, **4** and **7**, have been prepared from the corresponding α-amino acid derivative selenocystine (**1**) by the following sequence of steps: cleavage of the Se–Se bond with $NaBH_4$, *p*-methoxybenzyl (PMB) protection of the SeH group, Fmoc or Boc protection at the N-atom and *Arndt–Eistert* homologation (*Schemes 1* and *2*). A β^3-heptapeptide **8** with an N-terminal β^3-hSec(PMB) residue was synthesized on *Rink amide AM* resin and deprotected ('in air') to give the corresponding diselenide **9**, which, in turn, was coupled with a β^3-tetrapeptide thiol ester **10** by a seleno-ligation. The product β^3-undecapeptide was identified as its diselenide and its mixed selenosulfide with thiophenol (*Scheme 3*). The differences between α- and β-Sec derivatives are discussed.

1. Introduction. – Selenium is an essential trace element in human nutrition [1]. Recent studies have also reported beneficial effects of selenium compounds for cancer prevention [2]. Among selenium derivatives, selenocysteine (Sec or U), the 21st proteinogenic amino acid [3], has been the focus of considerable attention[3]). This moiety is found in a variety of naturally occuring enzymes (selenoproteins) such as glutathione peroxidase, iodothyronine deiodinase, and thioredoxin reductase [4]. The basis for the incorporation of Sec into proteins has been established by *Böck*'s pioneering work on *Escherichia coli* mutants [5]. Selenocysteine is incorporated into selenoproteins by a process that decodes the UGA codon as selenocysteine. For most human selenoproteins, 'homologues' exist, in which selenocysteine is replaced by cysteine. These enzymes are normally weaker catalysts when compared to the corresponding selenoproteins [6]. The selenol group ($pK_a \approx 5.7$) is more acidic than the thiol group ($pK_a \approx 8.5$) [7]. Thus, in contrast to cysteine, selenocysteine is ionized at physiological pH. Furthermore, the Se-atom has a higher polarizability, which makes the selenol group a 'softer' S_N2-type nucleophile.

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[2]) Part of the projected Ph.D. thesis of *G.C.*, ETH-Zürich.

[3]) For a comprehensive review '*From Selenium to Selenoproteins: Synthesis, Identity, and Their Role in Human Health*' and for a discussion on '*The Importance of Selenium to Human Health*' see [2b,c].

Diselenides have lower reduction potential as compared to disulfides, and selenocysteine-containing peptides form diselenides more rapidly than the corresponding cysteine-containing peptides form disulfides [8]. The presence of selenium in an amino acid sequence provides a spectroscopic as well as a mechanistic probe of peptide properties[4]).

Furthermore, the selenol group of selenocysteine can be easily eliminated under mild oxidative conditions to form dehydroalanines, which are convenient precursors for the preparation of peptide conjugates [10]. For these reasons, interest in selenocysteine and its derivatives has dramatically increased in recent years.

Many different syntheses of selenocysteine derivatives have been reported [11]. In a common approach, a selenolate acts as nucleophile on an activated OH group of a serine analog. For solid-phase peptide synthesis (SPPS) *N*-Boc- or *N*-Fmoc-protected derivatives are the most convenient. The currently most useful protective group for a selenol is the *para*-methoxybenzyl-group (PMB or Mob group) [11b,c]. The PMB group can be removed under various conditions. For instance, *Fujii* and co-workers used oxidative conditions (I_2 in AcOH or DMSO in CF_3COOH (TFA)) or strong *Lewis* acids (Me_3SiBr (TMSBr) or TMSOTf (Tf = trifluoromethylsulfonyl)) [11b]. An alternative method utilizes 2-nitrophenylsulfenyl chloride [12] in analogy to protocols for deprotection of related cysteine derivatives [13][14]. A milder method for deprotection of Sec(PMB) has been recently reported by *Hondal* and co-workers [15]: best results were obtained using 2,2′-dithiobis(5-nitropyridine) (DTNP).

Consequently, typical building blocks for SPPS of Sec-containing peptides are Fmoc-Sec(PMB)-OH and Boc-Sec(PMB)-OH. However, special care must be taken while performing SPPS with these derivatives, since they are prone to racemization and elimination under basic conditions. In particular, iterative piperidine deprotection steps can lead to the formation of dehydroalanine moieties within the peptide and subsequently to the corresponding piperidine adducts [16][17] (replacement of CH_2SeR by $CH_2-N(CH_2)_5$). To minimize these problems the incorporation of selenium residues is usually performed with preformed pentafluorophenyl esters (OPfp esters) in the presence of 1-hydroxy-1*H*-benzotriazole (HOBt) [16][17], which obviates the need for tertiary amines.

For the synthesis of larger peptides, *Kent*'s native chemical ligation, the coupling of two unprotected peptides, has proven to be a useful tool [18]. In this convergent synthetic strategy, a C-terminal thioester group of one peptide is coupled with an N-terminal cysteine residue of another peptide. This technique has also been extended to the coupling of a C-terminal thioester with an N-terminal Sec moiety[5]). In contrast to the conventional native chemical (thio)ligation, where disulfide bonds are cleaved with thiophenol, a phosphine (usually tris(2-carboxyethyl)phosphine (TCEP)) is often added for reductive cleavage of diselenide bonds in the reaction mixture of a (seleno)ligation [19][20]. In some cases, though, thiophenol is sufficient for this purpose [11c][21][22]. Expressed protein ligation [23] has been used to prepare

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[4]) A general overview on the replacement of sulfur by other chalcogens in peptides and proteins is given in [9].

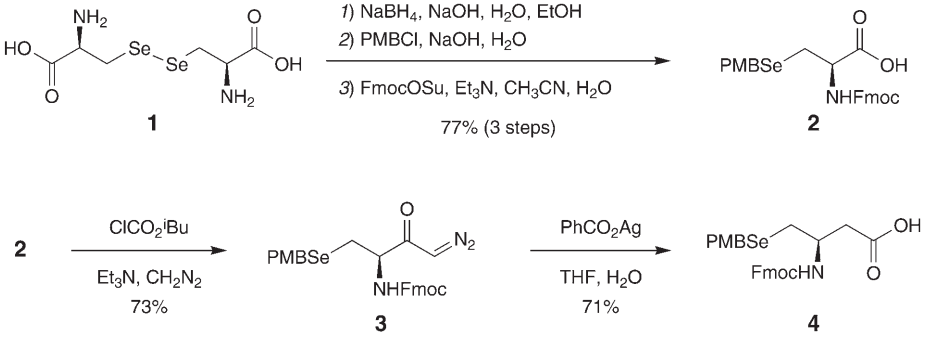
[5]) An overview of the use of Sec derivatives for chemoselective ligations is given in [19].

selenoproteins [20][24]; here, the thioesters are generated by using recombinant DNA technology and condensed with a synthetic Sec-containing segment.

β-Peptides represent a biomedically promising class of peptidomimetics. Like their α-counterparts, β-peptides fold to secondary structures such as turns, helices, or sheets [25]. Furthermore, they can mimic α-peptidic hormones, as well as peptide–protein and protein–protein interactions [25a]. They may be orally bioavailable [26], and, most importantly, they are enzymatically and metabolically stable [25a]. The β^2- and β^3-amino acid homologues of the 20 proteinogenic α-amino acids have all been prepared and incorporated in β-peptides, the properties of which have been studied extensively [25a]. Since the 21st proteinogenic amino acid, selenocysteine (Sec), confers interesting features to α-peptides and proteins, we decided to prepare its β^3-homologue and to explore its use in β-peptide synthesis. We here describe the preparation of Fmoc-β^3Sec(PMB)-OH and of Boc-β^3Sec(PMB)-OH, as well as their use in solid phase synthesis and in native chemical ligation.

2. Preparation of Fmoc-β^3hSec(PMB)-OH and Boc-β^3hSec(PMB)-OH. – Fmoc-β^3hSec(PMB)-OH (**4**) was synthesized starting from commercial selenocystine (**1**). The Se–Se bond of **1** was cleaved with $NaBH_4$, and the selenol was protected with a PMB group, according to the procedure published by *Fujii* and co-workers [11b], and *Vermeulen* and co-workers [11e]. Subsequent treatment with *N*-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}oxy)succinimide (Fmoc-OSu) furnished Fmoc-Sec(PMB)-OH (**2**) [11b] (*Scheme 1*) in 77% yield over three steps.

Scheme 1. *Preparation of Fmoc-β^3hSec(PMB)-OH* (**4**). For abbreviations, see accompanying text.

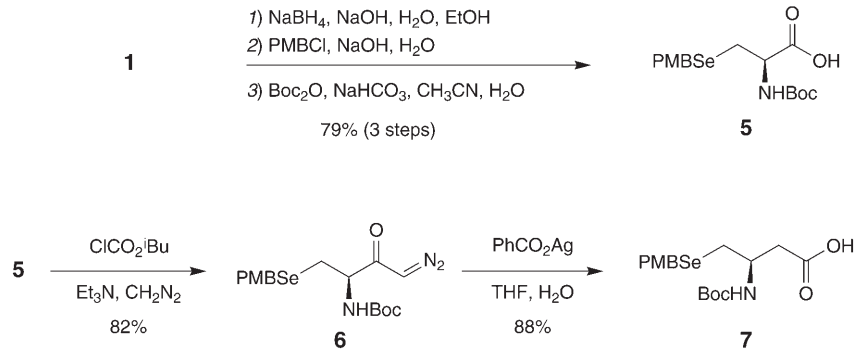


Homologation to the β-amino-acid derivative **4** was accomplished by the *Arndt–Eistert* protocol [25d][27][28], wherein the diazo ketone **3** was converted to **4** by an ultrasound-promoted [29] *Wolff* rearrangement [30] with PhCOOAg.

Boc-β^3hSec(PMB)-OH (**7**) was obtained in the same way as the Fmoc derivative (*Scheme 2*). Again, the corresponding α-amino acid (α-Boc-Sec(PMB)-OH, (**5**)) was first prepared, and homologation through the diazo ketone **6** led to the desired *N*- and *Se*-protected carboxylic acid **7**.

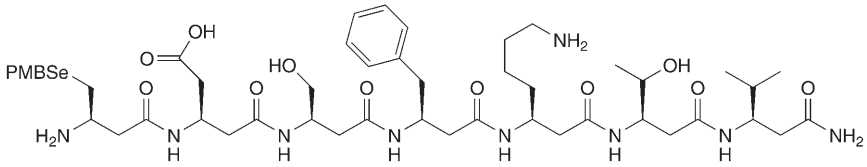
According to these simple protocols, Fmoc-β^3hSec(PMB)-OH **4** and Boc-β^3hSec(PMB)-OH **7** can be prepared in five steps starting from commercial precursors in 40 and 57% overall yield, respectively.

Scheme 2. *Preparation of Boc-β³hSec(PMB)-OH* (**7**)



Due to the high nucleophilicity of selenium compounds of the general formula R–Se–R, we had expected difficulties in the reaction with CH_2N_2 (**2** → **3** and **5** → **6**) and in the *Wolff*-rearrangement step of the intermediate diazo ketone (**3** → **4** and **6** → **7**)[6][7]). However, the overall yield of diazo-ketone formation and *Wolff* rearrangement (**2** → **4**, **5** → **7**) was comparable to that of the corresponding sulfur derivative (Fmoc-Cys(PMB)-OH or Fmoc-Cys(Acm)-OH → Fmoc-β³hCys(Trt)-OH) [28c]).

3. Preparation of a β-Homoselenocysteine-Containing β-Peptide. – Boc-β³hSec(PMB)-OH was used for the preparation of the β-peptide **8**, which, in turn, was used for a (seleno)ligation to the undecapeptide **11** (*cf. Scheme 3*, below).



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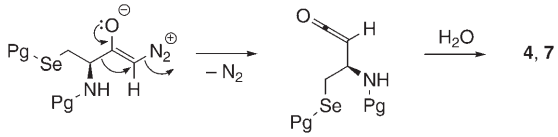
As mentioned in the *Introduction*, in α-peptide synthesis, incorporation of Sec is usually performed with the corresponding pentafluorophenyl esters and HOBt

[6]) After all, selenoethers, more so than thioethers, are expected to react with a carbene to form selenoylides [31]: *cf.* the catalytic enantioselective oxirane formation by *in-situ* trapping of a carbene (generated from a diazo compound) with a thioether and subsequent reaction with an aldehyde [32].

[7]) This may suggest that no free carbene is involved in the *Wolff* rearrangement of **3** and **6**, compatible with the view that migration and loss of nitrogen are concerted [33][34]:

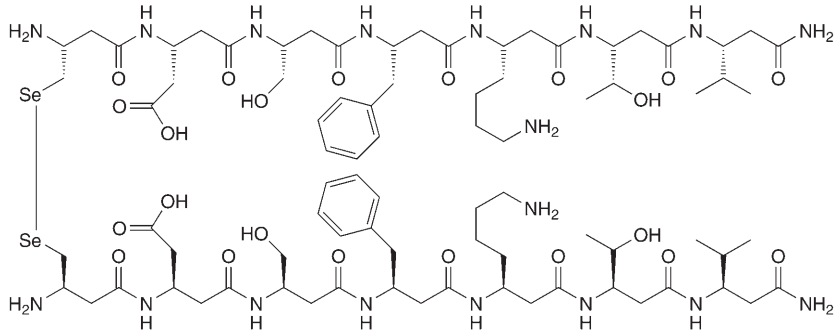


catalysis [16] to avoid racemization and elimination [17]. Neither of these processes is expected to occur with β-homoselenocysteine, since the side chain is in the β- and not in the α-position of the carbonyl group. For the synthesis of the β-heptapeptide **8** with an N-terminal β^3hSec residue, we used conventional solid-phase coupling on a *Rink-Amide AM* resin. The couplings were achieved with HATU and *Hünig*'s base in DMF. Prior to the incorporation of the β-homoselenocysteine moiety, the resin-bound β-peptide was divided into two equal portions.

The Boc (PMB-protected) β-homoselenocysteine was coupled to the first half of the resin-bound peptide by standard methods (HATU and DIPEA as tertiary base). The use of *N*-Boc-protected β-selenocysteine obviates the need for a final, N-terminal Fmoc deprotection. After this last coupling step, the peptide was removed from the resin under conditions that did not set the selenol groups free. After purification by preparative HPLC, the heptapeptide **8** was isolated as its TFA salt in a disappointingly low 21% yield.

Alternatively, β-homoselenocysteine was attached to the other half of the resin-bound peptide by activation as its pentafluorophenyl (PFP) ester[8]) in the presence of HOBt. After cleavage from the resin and purification by preparative HPLC, the heptapeptide **8** was isolated as its TFA salt in a similarly low 19% yield.

When samples of the heptapeptide **8** were treated with a mixture of *m*-cresol, thioanisol, TFA, and TMSBr (50:120:750:132) for 1 h at 0°, followed by preparative HPLC purification, the diselenide **9** was isolated as its TFA salt in 59% yield[9]).



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Thus, both methods of attaching the β^3hSec to the resin-bound β-hexapeptide afforded the β-heptapeptide **8** (in comparable overall yield). As expected, base treatment does not seem to have an adverse effect on the results in the case of β^3hSec derivatives.

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[8]) Boc-β^3hSec(PMB)-OPFP was prepared according to [35].

[9]) Additionally, 18% starting material **8** was recovered.

4. Selenoligation. – Coupling of the β-heptapeptide fragment derivative **9** with the β-tetrapeptide thiol ester[10]) **10** was achieved by Sec-mediated chemical ligation (selenoligation [11c][20][21]) as depicted in *Scheme 3*.

The TFA salts of the lyophilized peptides were transferred to a *Schlenk* flask, and degassed phosphate buffer (100 mM, pH 8.5) containing 6M GdmCl was added to dissolve them. After adjusting the pH of the solution to 7.5, the reaction was initiated with 5% thiophenol (*v*/*v*) under N_2. The course of the reaction was monitored by HPLC and LC/MS analyses. The conversion was judged to be complete after 8 h[11]). The disappearance of the starting materials was accompanied by formation of two new compounds (*Fig. 1*): the faster moving species corresponded to the diselenide **11c** of peptide **11a** ($[M+H]^+$, $C_{136}H_{205}N_{26}O_{32}Se_2^+$; found: 2874.0; calc. 2874.3 Da)[12]), whereas the slower moving species had a mass which is compatible with the selenosulfide **11b** formed from peptide **11a** and thiophenol ($[M+H]^+$, $C_{74}H_{108}N_{13}O_{16}SSe^+$; found: 1546.8; calc. 1546.7 Da).

After completion of the reaction, the ligation mixture was treated with dithiothreitol (DTT; 4 mg/ml reaction) for 30 min under N_2 in order to generate a homogenous solution that was subsequently washed twice with Et_2O. The resulting aqueous solution was diluted with 3 ml of MeCN/H_2O 1:1 containing 0.1% of TFA, and then directly injected into the preparative HPLC column. The diselenide product **11c** was isolated as the TFA salt in 50% yield and characterized by analytical HPLC and high-resolution MALDI-MS (*Fig. 2*).

The normalized CD spectra in MeOH of the β-peptidic diselenides **9** and **11c**, and of the mixed selenosulfide **11b**, are shown in *Fig. 3*. The starting diselenide **9** and the selenosulfide product **11b** exhibit the characteristic *Cotton* effect of a 3_{14}-helix: a minimum around 214 nm and a maximum near 200 nm. The diselenide **11c** of the ligation product shows, instead, a minimum near 208 nm but no positive *Cotton* effect at shorter wavelength.

5. Discussion and Conclusions. – The *Arndt–Eistert*-homologation sequence of steps is readily applicable to selenocysteine (H-Sec-OH) for the preparation of Boc or Fmoc protected β^3hSec derivatives in good yields (57 and 40%, resp., for five steps from selenocystine). Furthermore Fmoc-β^3hSec(PMB)OH was successfully employed for selenoligation of a β-hepta- and a β-tetrapeptide to give derivatives **11b** and **11c** of the β-undecapeptide **11a**.

As noted in the *Introduction*, Sec residues are convenient precursors of the dehydroalanine moiety **A**, the C=C bond of which is captodatively [37] substituted. Analogous elimination with the β^3hSec residue would lead to a dehydro-β^3hAla unit **B** in which the C=C bond is substituted by an acylamido and a CH_2CO group. The C=C bond in **B** is expected to be a better donor for electrophiles than that in **A**. The product of electrophilic attack on **B**, an acyliminium ion **C**, is likely to lose a proton from the α-

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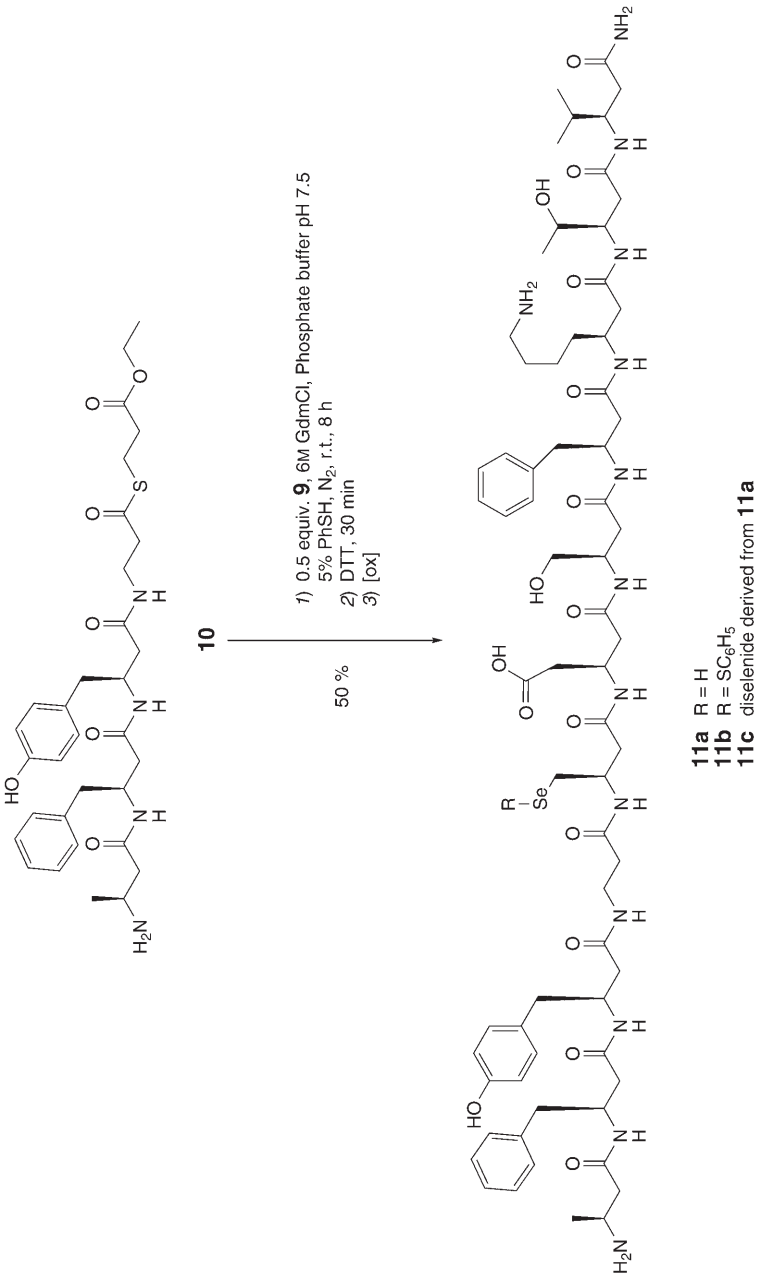
[10]) The preparation of the thioester **10** is described in [36]. We thank Dr. *Thierry Kimmerlin* for the preparation of the sample we used (*cf.* ETH Dissertation No. 15800, p. 83, Zürich, 2004).

[11]) For a comparison HPLC chromatograms after 0, 5 and 18 h see *Fig. 1*.

[12]) The masses reported in the discussion were obtained from LC/ESI-MS. The exact masses are reported in the *Exper. Part.*

Scheme 3. *Selenoligation between Diselenide* **9** *and Thioester* **10**. GdmCl: guanidinium chloride; DTT: dithiothreitol.



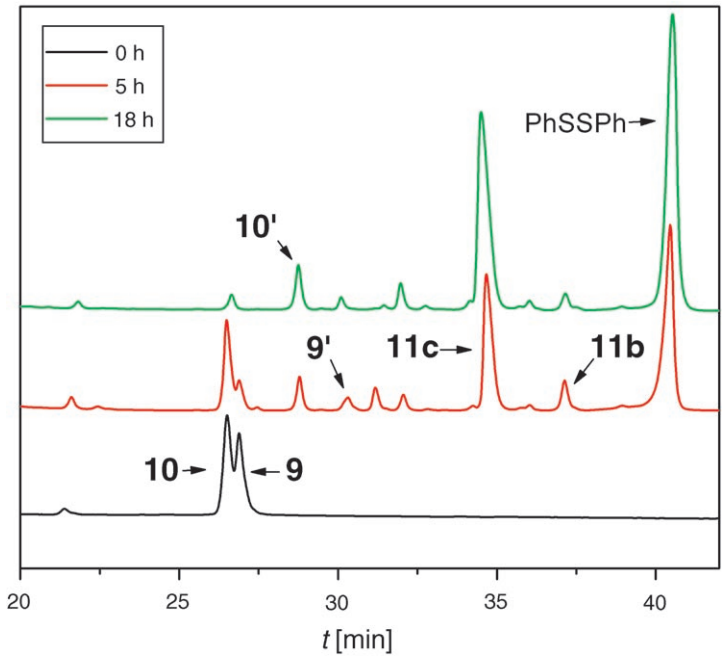
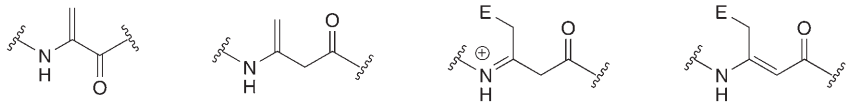


Fig. 1. *Analytical HPLC traces of the ligation reaction between diselenide* **9** *and thioester* **10**. At time zero (without thiophenol), the two starting materials are visible. Five hours after PhSH addition, the coupling products **11b** and **11c** accumulated. After 18 h, the starting diselenide **9** was almost completely consumed, and the thiophenol ester **10′** (SPh instead of $S(CH_2)_2CO_2Et$ in **10**) as well as the 'mixed' selenosulfide **9′** (PhSSe derivative formed from **9**) were identified by LC/ESI-MS. Chromatographic conditions: C_8 column (*Macherey-Nagel* 250 mm × 4.6 mm × 300 Å, 5 μ; 5–95% *B* in 60 min: 5 min 5% *B*, 50 min 60% *B*, 55 min 95% *B*).

carbonyl position to give an α,β-unsaturated β-acylamino-carbonyl system **D** ('vinylogous amide'). With E = H in **C** and **D**, this process would be a simple double-bond shift, and, with other electrophiles E, there would be a possibility of elaborating the structure further.



The CD spectrum of the diselenide **11c** (green curve in *Fig. 3*), as compared to those of the β-peptides **9** and **11b**, provides yet another example of the structural changes that are seen when long-chain β-peptides are investigated. Based on an analogy to many structurally characterized β-peptides that exhibit a negative *Cotton* effect near 215 nm and a positive *Cotton* effect near 200 nm, the CD spectra shown in *Fig. 3* suggest that β-peptides **9** and **11b**, which contain 14 and 11 β-amino-acid residues,

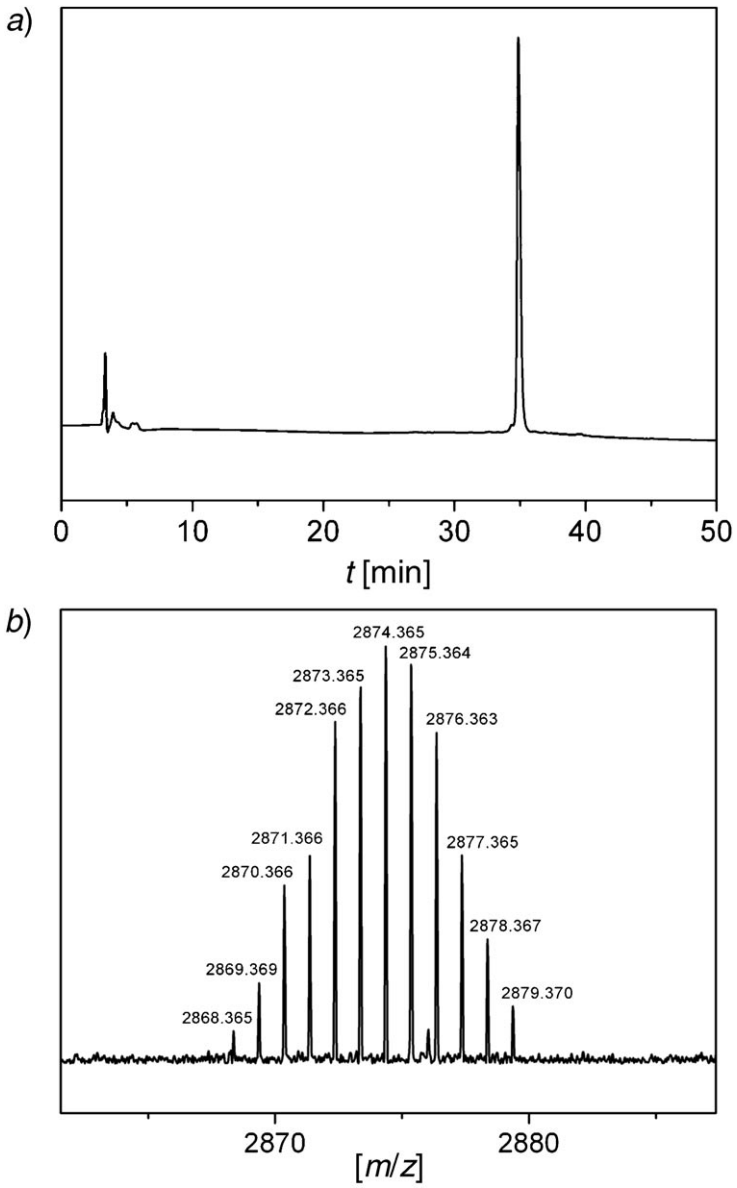


Fig. 2. *HPLC Trace and high-resolution MALDI mass spectrum of peptide diselenide* **11c**. *a*) Analytical HPLC chromatogram of purified **11c**; conditions: C_8 column (*Macherey-Nagel* 250 mm × 4.6 mm × 300 Å, 5 μ; 5–95% *B* in 60 min: 5 min 5% *B*, 50 min 60% *B*, 55 min 95% *B*). b) High resolution MALDI of **11c**. Shown is the isotopic pattern of $[M+H]^+$ between the sixth and the seventeenth isotopic peak. A value of 2874.365 was calculated for the twelfth isotopic peak based on a full width at half maximum resolution of 30,000 using the Exact Mass Calculator program from the *IonSpec Corporation*. The mass found for the corresponding peak was 2874.359.

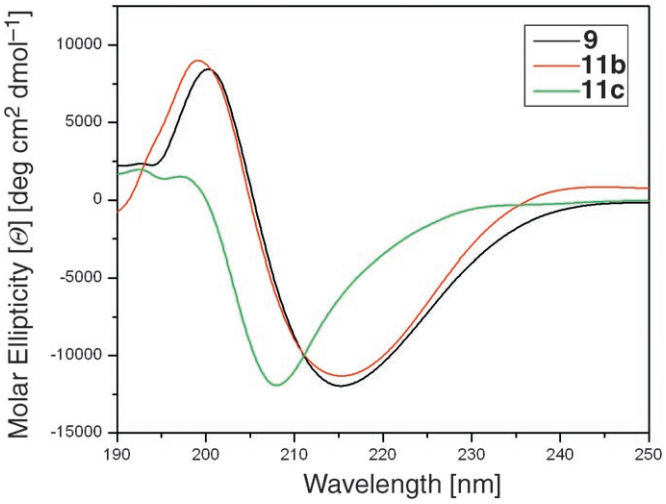


Fig. 3. *Normalized CD Spectra of* **9** *and* **11**. The spectra were recorded in MeOH, at 20°, with 0.25 mM **9**, 0.19 mM **11c**, and 0.18 mM **11b**. Solutions were prepared by weighing the peptide on a μg-precision balance and dissolving it in a volumetric flask.

respectively, adopt 3_{14}-helical structures [25]. Hypsochromic shifts of the negative and the 'loss' of the positive *Cotton* effect, as seen in the CD spectrum of the diselenide **11c**, have been observed previously with larger β-peptides [25a,b,e][36][38] but never interpreted. As we and others have noted [25e][38], assignment of β-peptidic secondary structure based on CD data alone must be done with considerable caution[13]). In the present case, the spectral changes observed upon dimerization of the β-peptide might be due to aggregation, but in the absence of detailed NMR studies other explanations cannot be ruled out.[14]) Aggregated peptides often show CD spectra, that are different from those of the corresponding monomers. A good example is the equilibrium between non-helical monomers that form ordered four-helix bundles [39].

Experimental Part

1. *General. Abbreviations:* Bn: benzyl, Boc: (*tert*-butoxy)carbonyl, DIPEA: diisopropylethylamine (EtN(i-Pr)$_2$), DMAP: 4-(dimethylamino)pyridine, DMF: dimethylformamide, DTT: dithiothreitol, ESI: electron spray ionisation, FC: flash chromatography, Fmoc: [(9 H-fluoren-9-ylmethoxy]carbonyl, Fmoc-OSu: *N*-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}oxy)succinimide, GdmCl: guanidinium hydrochloride, HATU: *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt: 1-hydroxy-1*H*-benzotriazole, HPLC: high-performance liquid chromatography, HR: high resolution, h.v.: high vacuum (0.01 – 0.1 Torr), MALDI :matrix-assisted laser desorption ionization, MeIm: 1-methyl-1*H*-imidazole, MSTN: 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole, NMM: *N*-methylmorpholine,

[13]) See Sect. 7.6 in the review article [25a,b]

[14]) The diselenide **11c** is rather poorly soluble in CD_3OH, but the NMR spectrum in this solvent shows sharp signals and well separated N*H* chemical shifts. This would indicate that the β-peptide **11c** is not aggregated. Aggregation normally gives rise to broadened NMR peaks (*cf.* [36])

PMBCl: *para*-methoxybenzyl chloride, SPS: solid-phase synthesis, TFA: trifluoroacetic acid, (CF_3COOH), TIS: triisopropylsilane ($(i\text{-}Pr)_3SiH$), TNBS: 2,4,6-trinitrobenzene sulfonic acid. Solvents for chromatography and workup procedures were distilled from *Sikkon* (anh. $CaSO_4$; *Fluka*) and from KOH (Et_2O). Amino acids were purchased from *Fluka* or *Senn*. CH_2N_2 was prepared according to [40]. All other reagents were used as received from *Fluka* or *Aldrich*. FC: *Fluka* silica gel 60 (40–63 μm), TLC: *Merck* silica gel *60 F_{254}* plates; detection with UV, anisaldehyde soln. (9.2 ml anisaldehyde, 12.5 ml conc. H_2SO_4, 3.75 ml AcOH, 340 ml EtOH), or 'Mo-stain' soln. (25 g phosphormolybdic acid, 10 g $Ce(SO_4)_2 \cdot H_2O$, 60 ml conc. H_2SO_4, 940 ml H_2O). Circular Dichroism (CD) Spectra: *Jasco J-710* spectropolarimeter from 190–250 nm at 20° in 1-mm rectangular cells, all spectra were corrected for the corresponding solvent spectrum, the molar ellipticity $[\theta]$ in $deg \cdot cm^2 \cdot dmol^{-1}$ calculated for the corresponding peptide. IR Spectra: *Perkin-Elmer 1600 FT-IR* spectrophotometer. NMR Spectra: *Bruker AMX-300* (^1H: 300 MHz, ^{13}C: 75 MHz), *Varian Mercury XL 300* (^1H: 300 MHz, ^{13}C: 75 MHz); chemical shifts δ in ppm downfield from internal Me_4Si (= 0 ppm); *J* values in Hz. MS: *IonSpec Ultima 4.7 T FT* Ion Cyclotron Resonance (ICR; HR-MALDI, in 2,5-dihydroxybenzoic acid matrix) spectrometer; in *m*/*z* (% of basis peak). Anal. and prep. RP-HPLC: *Merck* HPLC system (*LaChrom*, pump type *L-7150*, UV detector *L-7400*, interface *D7000*, HPLC manager *D7000*) or *Waters* HPLC system (pump type *515*, PDA-UV detector *996* or dual wavelength detector *2487*, HPLC manager *Empower Pro*). LC/MS was performed on a *Thermo Separation Products* (*TSP*) HPLC system connected to a PDA detector (*UV6000LB*, *TSP)* and to an ion-trap mass-spectrometry detector (*LCQdeca*, *Finnigan*). Linear gradients of 5–60% MeCN in H_2O/0.1% formic acid (0.2 ml/min) on a *Waters Atlantis dC18-3* 3 × 100 mm column were used. Elemental analyses were performed by the Microanalytical Laboratory of the Laboratorium für Organische Chemie, ETH-Zürich.

2. *Synthesis of the β-Amino-Acids* **4** *and* **7**. *Fmoc-Sec(PMB)-OH* (**2**) [11b,e]. $NaBH_4$ (400 mg, 15 mmol) was added in portions to an ice-cooled soln. of *selenocystine* (**1**) (500 mg, 1.50 mmol) in 0.5N NaOH soln. (8 ml) and EtOH (2 ml), and the mixture was stirred at r.t. until the yellow color disappeared. After cooling again to 0°, degassed 2N NaOH soln. (4 ml) and PMBCl (0.82 ml, 6.00 mmol) were added. The mixture was stirred 30 min at 0° and 3 h at r.t. Then, the pH was adjusted to 1–2 with 6N HCl, and the mixture was kept at 0° overnight. The product was collected by filtration, washed with cold H_2O, and dried under h.v. overnight. Then, a soln. of Fmoc-OSu (972 mg, 2.88 mmol) in MeCN (4 ml) was added to an ice-cooled suspension of the crude product in a mixture of H_2O (7 ml) and Et_3N (0.42 ml, 2.99 mmol). After the addition of Et_3N (0.42 ml, 2.99 mmol), the mixture was stirred at r.t. for 1 h. Then, the pH was adjusted to 1–2 with 1N HCl, and the aq. phase was extracted with AcOEt (3 × 30 ml). The combined org. layers were washed with 1N HCl (20 ml) and brine (20 ml), and dried (Na_2SO_4) and concentrated under reduced pressure. FC (SiO_2; hexane/AcOEt/AcOH 25:25:1) yielded **2** (1.173 g, 77%). Anal. data are in accordance with those in [11b].

Boc-Sec(PMB)-OH (**5**) [11b,e]. $NaBH_4$ (400 mg, 15 mmol) was added in portions to an ice cooled soln. of **1** (500 mg, 1.50 mmol) in 0.5N NaOH soln. (8 ml) and EtOH (2 ml), and the mixture was stirred at r.t. until the yellow color disappeared. After cooling again to 0°, degassed 2N NaOH soln. (4 ml) and PMBCl (0.82 ml, 6.00 mmol) were added. The mixture was stirred 30 min at 0° and 4 h at r.t. Then, the pH was adjusted to 1–2 and the mixture was kept at 0° overnight. The product was collected by filtration and washed with cold H_2O. After drying under h.v. overnight, the crude product was suspended in MeCN/sat. aq. $NaHCO_3$ soln. (20 ml/20 ml), and Boc_2O (753 mg, 3.45 mmol) was added. After stirring overnight citric acid soln. (10% in H_2O, 60 ml) was added, and the aq. phase was extracted with AcOEt (3 × 50 ml). The combined org. layers were washed with brine (2 × 50 ml), dried ($MgSO_4$), and concentrated under reduced pressure. FC (SiO_2; hexane/AcOEt/AcOH 66:33:2) provided **5** (925 mg, 79%). Colorless oil. R_f (hexane/AcOEt/AcOH 25:25:1) 0.48. $[\alpha]_D^{rt} = -23.7$ ($c = 0.5$, MeOH). IR ($CHCl_3$): 2980*m*, 1710*s*, 1610*m*, 1510*s*, 1370*m*, 1035*m*. ^1H-NMR (300 MHz, CD_3OD): 1.44 (*s*, *t*-Bu); 2.80–2.91 (*m*, CH_2); 3.73 (*s*, Me); 3.78 (*s*, CH_2); 4.19 (*t*, $J = 6.3$, CH); 6.81 (*d*, $J = 8.7$, 2 arom. H); 7.20 (*d*, $J = 8.7$, 2 arom. H). ^{13}C-NMR (75 MHz, CD_3OD): 26.1, 28.0 (CH_2); 29.0 (Me_3C); 55.3 (CH); 55.9 (CH_2); 81.0 (C); 115.1, 131.4 (CH); 132.3, 157.6, 159.9, 174.5 (C). ESI-MS: 412.2 (100, $[M + D]^+$), 529.9 (23), 304.2 (38). MALDI-HR-MS: 558.0894 ($[M + Na]^+$, $C_{27}H_{25}N_3O_4SeNa^+$; calc. 558.0903).

*1-Diazo-3-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-4-[(4-methoxybenzyl)selanyl]butan-2-one* (*Fmoc-Sec(PMB)-CHN$_2$*; **3**). Compound **2** (510 mg, 1.00 mmol) was dissolved in THF (5 ml) under

Ar, and the mixture was cooled to $-25°$. After addition of Et_3N (0.15 ml, 1.07 mmol) and $ClCO_2{}^iBu$ (0.14 ml, 1.07 mmol), the mixture was stirred at $-25°$ for 30 min. The resulting white suspension was allowed to warm to 0°, and a soln. of CH_2N_2 in Et_2O was added until the yellow color persisted (*ca.* 3.00 mmol). Stirring was continued for 4 h as the mixture was allowed to warm to r.t. Excess CH_2N_2 was destroyed by the addition of a few drops of AcOH and vigorous stirring for 5 min. The mixture was diluted with Et_2O (50 ml), and washed with sat. aq. $NaHCO_3$ soln. (20 ml), sat. aq. NH_4Cl (20 ml) and brine (20 ml). The org. layer was dried (Na_2SO_4) and concentrated under reduced pressure. FC (SiO_2; hexane/AcOEt 2:1 to 1:1) gave **3** (392 mg, 73%). Yellow solid. M.p. 90–92°. R_f (hexane/AcOEt 2:1) 0.20. $[\alpha]_D^{r.t.} = -36.1$ ($c = 0.8$, $CHCl_3$). IR ($CHCl_3$): 3005*w*, 2110*s*. 1720*s*, 1640*m*, 1610*m*, 1510*s*, 1450*m*, 1365*s*, 1035*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 2.70–2.86 (*m*, CH_2); 3.75 (br. *s*, CH_2, Me); 4.17 (*t*, $J = 6.3$, CH); 4.32–4.56 (*m*, CH, CH_2); 5.31 (*s*, CH); 5.72 (br. *d*, $J = 7.4$, NH); 6.82 (*d*, $J = 8.5$, 2 arom. H); 7.21 (*d*, $J = 8.5$, 2 arom. H); 7.30–7.43 (*m*, 4 arom. H); 7.61 (br. *s*, 2 arom. H); 7.77 (*d*, $J = 7.4$, 2 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): 24.8, 27.4 (CH_2); 47.0, 54.3 (CH); 55.0 (Me); 57.1 (CH); 66.6 (CH_2); 113.8, 119.8, 124.9, 126.9, 127.5, 129.8 (CH); 130.3, 141.1, 143.4, 155.5, 158.3, 193.1 (C). ESI-MS: 557.8 (100, $[M + Na]^+$), 529.9 (23), 304.2 (38). MALDI-HR-MS: 558.0894 ($[M + Na]^+$, $C_{27}H_{25}N_3NaO_4Se^+$; calc. 558.0903).

*3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-4-[(4-methoxybenzyl)selanyl]butanoic Acid* (Fmoc-β^3hSec(PMB)-OH; **4**). Compound **3** (392 mg, 0.73 mmol) was dissolved in THF/H_2O (8 ml/2 ml), and $PhCO_2Ag$ (34 mg, 0.15 mmol) was added with exclusion of light. The mixture was ultrasonicated at r.t. After 4 h, H_2O (20 ml) was added, and the pH was adjusted to 1–2 with 1N HCl. The aq. phase was extracted with AcOEt (3 × 20 ml), and the combined org. layers were dried (Na_2SO_4) and concentrated under reduced pressure. FC (SiO_2; hexane/AcOEt/AcOH 66:33:2) yielded **4** (273 mg, 71%). White solid. M.p. 120–122°. R_f (hexane/AcOEt/AcOH 66:33:2) 0.42. $[\alpha]_D^{r.t.} = -11.0$ ($c = 0.5$, $CHCl_3$). IR ($CHCl_3$): 3010*w*, 1720*s*, 1610*w*, 1510*s*, 1450*w*, 1040*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 2.40–2.83 (*m*, 2 CH_2); 3.75 (br. *s*, CH_2, Me); 4.17 (br. *s*, CH); 4.22 (*t*, $J = 6.5$, CH); 4.40 (br. *s*, CH_2); 5.37 (br. *d*, $J = 9.1$, NH); 6.80 (*d*, $J = 7.9$, 2 arom. H); 7.20 (*d*, $J = 7.9$, 2 arom. H); 7.26–7.48 (*m*, 4 arom. H); 7.59 (*d*, $J = 7.3$, 2 arom. H); 7.75 (*d*, $J = 7.3$, 2 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): 27.2, 27.7, 37.9 (CH_2); 47.1, 47.8 (CH); 55.2 (Me); 66.8 (CH_2); 113.9, 119.9, 125.0, 127.0, 127.7, 129.9 (CH); 130.6, 141.2, 143.7, 155.7, 158.4, 176.4 (C). MALDI-MS: 548 (100, $[M + Na]^+$), 526 (16, $[M + H]^+$), 346 (48). MALDI-HR-MS: 548.0938 ($[M + Na]^+$, $C_{27}H_{27}NNaO_5Se^+$; calc. 548.0947). Anal. calc. for $C_{27}H_{27}NO_5Se$ (524.47): C 61.38, H 5.19, N 2.67; found: C 61.58, H 5.21, N 2.71.

*1-Diazo-3-{[(*tert*-Butoxy)carbonyl]amino}-4-[(4-methoxybenzyl)selanyl]butan-2-one* (*Boc-Sec(PMB)-CHN$_2$*, **6**). Compound **5** (1.55 g, 4.00 mmol) was dissolved in THF (20 ml) under Ar and cooled to $-25°$. After addition of Et_3N (0.60 ml, 432 mg, 4.27 mmol) and $ClCO_2{}^iBu$ (0.56 ml, 585 mg, 4.28 mmol), the mixture was stirred at $-25°$ for 30 min. The resulting white suspension was allowed to warm to 0°, and a soln. of CH_2N_2 in Et_2O was added until the yellow color persisted (*ca.* 12.0 mmol). Stirring was continued for 4 h as the mixture was allowed to warm to r.t. Excess CH_2N_2 was destroyed by the addition of a few drops of AcOH and vigorous stirring for 5 min. The mixture was diluted with Et_2O (100 ml), and washed with sat. aq. $NaHCO_3$ soln. (20 ml), sat. aq. NH_4Cl (20 ml), and brine (20 ml). The org. layer was dried (Na_2SO_4) and concentrated under reduced pressure. FC (SiO_2; hexane/AcOEt 2:1) provided **6** (1.36 g, 82%). Yellow oil. R_f (hexane/AcOEt 2:1) 0.44. $[\alpha]_D^{r.t.} = -30.4$ ($c = 0.9$, $CHCl_3$) IR ($CHCl_3$): 3010*w*, 2110*s*. 1710*s*, 1640*m*, 1510*s*, 1490*s*, 1370*s*, 1035*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.43 (*s*, *t*-Bu); 2.76 (*d*, $J = 6.0$, CH_2); 3.76 (*m*, CH_2, Me); 4.34 (br. *s*, CH); 5.33, 5.48 (2*s*, CH, NH); 6.81 (*d*, $J = 8.4$, 2 arom. H); 7.20 (*d*, $J = 8.4$, 2 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): 25.1, 27.4 (CH_2); 28.2 (*Me*$_3$C); 54.2 (CH); 55.1 (Me); 56.8 (CH); 80.1 (C); 113.9, 129.9 (CH); 130.5, 155.0, 158.4, 192.7 (C). ESI-MS: 436.1 (100, $[M + Na]^+$), 414.2 (6). MALDI-HR-MS: 436.0742 ($[M + Na]^+$, $C_{17}H_{23}N_3NaO_4Se^+$; calc. 436.0746).

*3-{[(*tert*-Butoxy)carbonyl]amino}-4-[(4-methoxybenzyl)selanyl]butanoic Acid* (Boc-β^3hSec(PMB)-OH, **7**). Compound **6** (1.24 g, 3.00 mmol) was dissolved in THF/H_2O (32 ml/8 ml), and $PhCO_2Ag$ (137 mg, 0.60 mmol) was added with exclusion of light. The mixture was ultrasonicated at r.t. After 4 h, H_2O (20 ml) and citric acid soln. (10% in H_2O, 30 ml) were added, and the aq. phase was extracted with AcOEt (1 × 100 ml, 2 × 50 ml). The combined org. layers were washed with brine (40 ml), dried (Na_2SO_4), and concentrated under reduced pressure. FC (SiO_2; hexane/AcOEt/AcOH 25:25:1) gave **7** (1.06 g, 88%). Yellow oil, which slowly crystallized to a pale yellow solid. M.p. 85–87°. R_f (hexane/

AcOEt/AcOH 25:25:1) 0.54. $[\alpha]_D^{r.t.} = +8.5$ ($c = 0.65$, $CHCl_3$). IR ($CHCl_3$): 3010*m*, 1710*s*, 1610*w*, 1510*s*, 1370*m*, 1035*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.43 (*s*, *t*-Bu); 2.50–2.79 (*m*, 2 CH_2); 3.75 (br. *s*, CH_2, Me); 4.07 (br. *s*, CH); 5.14 (br. *s*, NH); 6.81 (*d*, $J = 8.6$, 2 arom. H); 7.19 (*d*, $J = 8.6$, 2 arom. H). ^{13}C-NMR (75 MHz, CD_3OD): 21.8 (CH_2); 28.4 (Me_3C); 29.4, 40.1 (CH_2); 49.7 (CH); 55.9 (Me); 80.4 (Me_3C); 115.1, 131.4 (CH); 132.9, 157.8, 160.1, 175.0 (C). MALDI-MS: 426.1 (100, $[M + Na]^+$), 404.1 (16, $[M + H]^+$). MALDI-HR-MS: 426.0783 ($[M + Na]^+$, $C_{17}H_{25}NNaO_5Se^+$; calc. 426.0790). Anal. calc. for $C_{17}H_{25}NO_5Se$ (402.35): C 50.75, H 6.26, N 3.48; found: C 50.96, H 6.34, N 3.51.

*Pentafluorophenyl 3-{[(*tert-*Butoxy)carbonyl]amino}-4-[(4-methoxybenzyl)selanyl]butanoate* (*Boc-β³hSec(PMB)-OPFP*; **7a**) [35]. To a stirred ice-cooled soln. of **7** (402 mg, 1.00 mmol) and pentafluorophenol (202 mg, 1.10 mmol) in dry THF (10 ml) DCC (227 mg, 1.1 mmol) was added. Stirring was continued for 1 h at 0° and for 1 h at r.t. The suspension was cooled in an ice bath, filtered, and the filtrate was washed with cold THF. The solvent was evaporated. The crude product was treated with Et_2O (60 ml), filtered, and the filtrate was evaporated to dryness. Recrystallization of the crude product from hexane yielded **7a** (411 mg, 72%). Light yellow solid. M.p. 108°. R_f (hexane/AcOEt 4:1) 0.40. $[\alpha]_D^{r.t.} = 3.6$ ($c = 0.5$, $CHCl_3$). IR ($CHCl_3$): 2980*w*, 1785*m*, 1710*m*, 1610*w*, 1520*s*, 1515*m*, 1370*m*, 1100*m*, 1005*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.46 (*s*, *t*-Bu); 2.69–2.80 (*m*, CH_2); 2.95–3.06 (*m*, CH_2); 3.78 (*s*, Me); 3.81 (*s*, Me); 4.21 (br. *s*, CH); 4.90 (br. *s*, NH); 6.82 (*d*, $J = 8.7$, 2 arom. H); 7.23 (*d*, $J = 8.7$, 2 arom. H). ESI-MS: 591.9 (100, $[M + Na]^+$). Anal. calc. for $C_{23}H_{24}F_5NO_5Se$ (568.40): C 48.60, H 4.26, N 2.46; found: C 48.33, H 4.26, N 2.53.

3. *Peptide Synthesis.* 3.1. *Fmoc Deprotection. General Procedure 1* (*GP 1*). The Fmoc group was removed by treating the resin with 20% piperidine in DMF (4 ml, 3 × 10 min) under Ar bubbling. After filtration, the resin was washed with DMF (5 ml, 4 × 1 min).

3.2. *Coupling of the β-Amino Acids on* Rink-Amide AM *Resin. General Procedure 2* (*GP 2*). *GP 2a:* The Fmoc deprotection was carried out according to *GP 1*. Solid-phase synthesis was continued by sequential incorporation of Fmoc-protected amino acids. For each coupling step, the resin was treated with a soln. of the Fmoc-protected β^3-amino acid (3 equiv.), HATU (2.8 equiv.), and DIPEA (6 equiv.) dissolved in the minimal amount of DMF for 1 h. Monitoring of the coupling reaction was performed with the TNBS test [41]. In the case of a positive TNBS test (indicating incomplete coupling), the suspension was allowed to react further for 1 h, or, after filtration, the peptide-resin was treated again with the same Fmoc-protected amino acid (1–3 equiv.), and with the coupling reagents. The resin was then filtered and washed with DMF (20 ml/g resin, 5 × 1 min) prior to the following deprotection step.

GP 2b: As described in *GP 2a*, but without Fmoc deprotection (*GP 1*).

3.3. Rink-Amide AM *Resin Cleavage and Final Deprotection. General Procedure 3* (*GP 3*). The cleavage from the resin and the peptide deprotection were performed according to [42]. The dry peptide-resin was suspended in a soln. of TFA/H_2O/CH_2Cl_2/TIS 89:5:5:1 (10 ml) for 2 h. The resin was removed by filtration, washed with TFA (2×), and the org. phase was concentrated under reduced pressure. The resulting oily residue was treated with cold Et_2O, and the formed precipitate was separated. The isolated crude β-peptide was dried under h.v. and stored at −20° before purification.

3.4. *HPLC Analysis and Purification of β-Peptides. General Procedure 4* (*GP 4*). RP-HPLC Analysis was performed on a *Macherey-Nagel C_{18}* column (*Nucleosil 100-5 C_{18}* (250 × 4 mm)) by using a linear gradient of *A:* 0.1% TFA in H_2O and *B:* MeCN, at a flow rate of 1 ml/min with UV detection at 220 nm. t_R in min. Crude products were purified by prep. RP-HPLC on a *Macherey-Nagel C_{18}* column (*Nucleosil 100–7 C_{18}* (250 × 21 mm) using gradient of *A* and *B* at a flow rate of 17 ml/min with UV detection at 220 nm, and then lyophilized.

H-β³hSec(PMB)-β³hAsp-β³hSer-β³hPhe-β³hLys-β³hThr-β³hVal-NH_2 (**8**). *Rink-Amide AM* resin (282 mg, 0.2 mmol, loading 0.71 mmol/g) was preswelled in DMF (10 ml) for 1 h by N_2 bubbling. Subsequently the resin was filtered. SPS was performed according to *GP 2a* by sequential incorporation of Fmoc-β^3hVal-OH, Fmoc-β^3hThr(^{t}Bu)-OH, Fmoc-β^3hLys(Boc)-OH, Fmoc-β^3hPhe-OH, Fmoc-β^3hSer-OH, and Fmoc-β^3hAsp(^{t}Bu)-OH. Then, the resin was Fmoc-deprotected (*GP 1*), washed with CH_2Cl_2 (4 ml, 4 × 1 min), and dried under h.v. overnight. The dried resin was divided in two equal parts (2 × 221 mg, 2 × 0.10 mmol).

The first part (221 mg, 0.10 mmol) was preswelled in DMF (10 ml) for 1 h by N_2 bubbling. The resin was filtered and Fmoc-β^3hSec(PMB)-OH was coupled according to *GP 2b*. Then the resin was washed

with CH_2Cl_2 (4 ml, 4 × 1 min) and dried under h.v. overnight. Treatment of the peptide-resin according to *GP 3* afforded the crude peptide **8** (168 mg) as a TFA salt. Purification of this crude peptide by RP-HPLC (30–95% *B* in 30 min: 5 min 30% *B*; 25 min 40% *B*; 30 min 95% *B*; t_R 17.1 min) according to *GP 4* yielded the TFA salt of **8** (27.1 mg) in >98% purity.

The second part (221 mg, 0.10 mmol) was preswelled in DMF (10 ml) for 1 h by N_2 bubbling. After filtration, a mixture of Fmoc-β^3hSec(PMB)-OPFP (**7a**) (171 mg, 0.30 mmol) and HOBt (41 mg, 0.30 mmol) in DMF was added to the resin. The suspension was mixed by N_2 bubbling for 2 h. Subsequently, the resin was filtered, washed with DMF (4 ml, 4 × 1 min), with CH_2Cl_2 (4 ml, 4 × 1 min), and dried under h.v. overnight. Treatment of the peptide-resin according to *GP 3* afforded the crude peptide **8** (127 mg) as a TFA salt. Purification of this crude product by RP-HPLC (30–95% *B* in 30 min: 5 min 30% *B*; 25 min 40% *B*; 30 min 95% *B*; t_R 17.1 min) according to *GP 4* yielded the TFA salt of **8** (24.7 mg) in >98% purity.

RP-HPLC (5–99% *B* in 60 min: 45 min 50% *B*, 60 min 99% *B*): t_R 33.79, purity >98%. MALDI-HR-MS: 1064.5281 ($[M+H]^+$, $C_{49}H_{78}N_9O_{12}Se^+$; calc. 1064.5299).

[H-β³hSec-β³hAsp-β³hSer-β³hPhe-β³hLys-β³hThr-β³hVal-NH₂]₂ (**9**). Compound **8** (25.8 mg, 20 μmol) was dissolved in 1 ml of a mixture of *m*-cresol, thioanisol, TFA, and TMSBr 50:120:750:132 under Ar in an ice bath. After stirring for 1 h, cold Et_2O was added, and the precipitate formed was washed with cold Et_2O (3 ×). This crude product was dried under h.v. Purification of the crude peptide by RP-HPLC (20–95% B in 70 min: 5 min 20% *B*; 65 min 50% *B*; 70 min 95% *B*; 17 ml/min) yielded the TFA salt of **9** (13.9 mg, 59%; t_R 25.8 min) and the TFA salt of **8** (4.7 mg, 18%, t_R 37.9 min).

RP-HPLC (5–99% *B* in 60 min: 45 min 50% *B*, 60 min 99% *B*): t_R 28.11, purity >98%. MALDI-HR-MS: 1885.855 ($[M+H]^+$, $C_{82}H_{136}N_{18}O_{22}Se_2^+$; calc. 1885.8508).

3. *Selenoligation. [H-β³hAla-β³hPhe-β³hTyr-β³hGly-β³hSec-β³hAsp-β³hSer-β³hPhe-β³hLys-β³hThr-β³hVal-NH₂]₂* (**11c**) *and H-β³hAla-β³hPhe-β³hTyr-β³hGly-β³hSec(SPh)-β³hAsp-β³hSer-β³hPhe-β³hLys-β³hThr-β³hVal-NH₂* (**11b**). Degassed buffer (2.0 ml, 100 mM phosphate, 6M guanidinium HCl) was added to a mixture of the peptide **10** (3.0 mg, 4.06 μmol) and the diselenide of **9** (5 mg, 2.14 μmol) in a *Schlenk* flask under N_2. The reaction was initated by the addition of 5% thiophenol (100 μl). The heterogeneous mixture was vigorously stirred at r.t. Aliquots were withdrawn, diluted 10 times, and then analyzed by HPLC and LC/MS. After the reaction was judged to be over, the mixed selenosulfide was reduced with DTT (10 mg, 65 μmol) for 30 min, and then it was transferred to a 15-ml *Falcon* tube and extracted with Et_2O (2 × 10 ml). The resulting soln. was diluted with 3 ml of 50% MeCN in H_2O (0.1% TFA), and that was directly purified by prep. HPLC. The desired product was isolated as the diselenide of product **11c** in 50% yield (3.4 mg). RP-HPLC (5–95% *B* in 60 min: 5 min 5% *B*, 50 min 60% *B*, 55 min 95% *B*): t_R 35.94, purity >99%. MALDI-HR-MS for the $[A+12]$ peak: 2874.365 ($[M+H]^+$; calc. for $C_{136}H_{205}N_{26}O_{32}Se_2^+$: 2874.359). Following an alternative workup where Et_2O was omitted and the crude mixture was directly injected onto the RP-HPLC, the product peak contained a large amount of thiophenol in addition to **11b**. Most of the thiophenol contaminant could be removed by lyophilization, allowing isolation of **11b** in 66% yield (5 mg). RP-HPLC as for **11c**: t_R 36.74, purity 90%. LC/ESI-MS: 1546.8 ($[M+H]^+$, $C_{74}H_{108}N_{13}O_{16}SSe^+$; calc. 1546.7).

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