Synthesis of β^3-Peptides and Mixed α/β^3-Peptides by Thioligation

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Dedicated to Prof. *Waldemar Adam* on the occasion of his 65th birthday

Five β-peptide thioesters (**1**–**5**, containing 3, 4, 10 residues) were prepared by manual solid-phase synthesis and purified by reverse-phase preparative HPLC. A β-undecapeptide (**6**) and an α-undecapeptide (**7**) with *N*-terminal β^3-HCys and Cys residues were prepared by manual and machine synthesis, respectively. Coupling of the thioesters with the cysteine derivatives in the presence of PhSH (*Scheme* and *Fig. 1*) in aqueous solution occurred smoothly and quantitatively. Pentadeca- and heneicosapeptides (**8**–**10**) were isolated, after preparative RP-HPLC purification, in yields of up to 60%. Thus, the so-called native chemical ligation works well with β-peptides, producing larger β^3- and α/β^3-mixed peptides. Compounds **1**–**10** were characterized by high-resolution mass spectrometry (HR-MS) and by CD spectroscopy, including temperature and concentration dependence. β-Peptide **9** with 21 residues shows an intense negative *Cotton* effect near 210 nm but no zero-crossing above 190 nm, (*Figs. 2–4*), which is characteristic of β-peptidic 3_{14}-helical structures. Comparison of the CD spectra of the mixed α/β-pentadecapeptide (**10**) and a helical α-peptide (*Fig. 5*) indicate the presence of an α-peptidic 3.6_{13} helix.

1. Introduction. – The nonenzymatic coupling of unprotected peptides in aqueous solution is one of the most important recent developments in protein synthesis[3]). There are various types of reactions that can be used for this purpose[4]); these are referred as *chemical ligation*, *orthogonal ligation*, *native ligation*, Kent *ligation*, *etc*. The most common method is the reaction between a *C*-terminal thioester of one peptide (protein) and an *N*-terminal cysteine of another. The first observation of this process, occurring through an acyl shift from S to N, was reported by *Wieland et al*. in 1953 [3]; *Kemp* invented the use of 1-hydroxy-8-sulfenyldibenzofuran as a thiol-capture molecule to combine an intermolecular ligation with a subsequent intramolecular acyl transfer in 1981 [4]; and it was *Kent* and co-workers who popularized the method in a seminal article entitled '*Synthesis of Proteins by Native Chemical Ligation*' in 1994 [5]. An outline of the thioligation is shown in the *Scheme*.

In our work on β-peptides built of homologated proteinogenic amino acids [6], we have reached a stage where the synthesis of longer and longer peptide chains [7] would facilitate the search for tertiary structures and for enzyme mimics. Thus, we have

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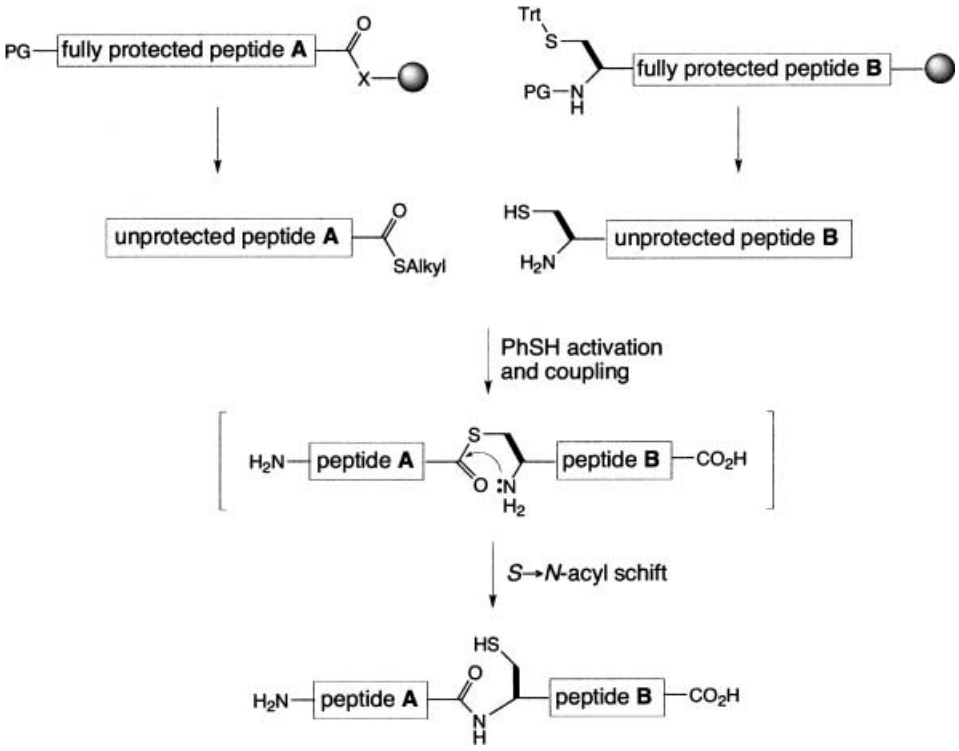
[1]) Part of the projected Ph.D. thesis of *T.K.*, ETH Zürich

[2]) Preliminary experiments for the coupling of β-peptides by thioligation have been carried out by *A. Sewing* (ETH-Dissertation No. 14326, 2001)

[3]) For recent review articles, see [1].

[4]) Besides the thioester/cysteine method discussed here, there is, *e.g.*, a perthioester/cysteine, a perthioester/histidine, a thioester/glycine, and a thioester/azidopeptide coupling [2].

Scheme. *Synthesis of Peptides by Chemical Ligation of Unprotected Peptide Segments in Aqueous Solution at Neutral pH*. The coupling process starts with a chemoselective *trans*-thioesterification reaction between the *C*-terminal thioester of peptide **A** and the sulfhydryl group of the *N*-terminal Cys of fragment **B**. The thioester-linked intermediate undergoes spontaneous and rapid intramolecular S → N acyl shift, forming the amide bond at the ligation site.



applied solid-phase [7][8] and machine [9] synthesis adapted to the particular structures and reactivities of the β-amino acid building blocks and peptide target sequences[5]). To efficiently construct larger β-peptides, we have now also tested the possibility of applying thioligation to these unnatural derivatives, and also to the coupling of α- with β-peptidic fragments.

2. Solid-Phase Synthesis of β-Peptide Thioesters and Peptides with *N*-Terminal β-HCys. – Two methods were applied to the assembly of β-peptides with *C*-terminal thioester groups: the (manual) solid-phase coupling of Fmoc-protected β-amino acids *a*) on an HMBA[6]) resin, with Me_2AlCl/EtSH cleavage from the solid support and

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[5]) *DeGrado*, *Gellman*, and *Gung*, and their respective co-workers, have recently also started to synthesize and investigate β-peptides consisting exclusively of β^3-amino acids with proteinogenic side chains [10–12]. Parallel to our studies, *Gellman* and co-workers have also investigated β-peptides consisting of or containing cyclic β-amino acids, such as 2-aminocyclopentane and -cyclohexane carboxylic acid [13]. For two recent review articles containing sections on β-peptides, see [14].

[6]) See *Exper. Part.*

CF_3CO_2H deprotection of the side-chain functional groups[7]), and *b*) on a sulfonamide resin, with Me_3SiCHN_2/$HS(CH_2)_2CO_2Et$ cleavage from the solid support and the same conditions for deprotection[8]). In this way, we have obtained the ethyl thioesters **1**, **2**, and **4**, and the ethyl (acylthio)propanoates **3** and **5**. They were purified by preparative reverse-phase HPLC (RP-HPCL) and identified by high-resolution mass spectrometry (MALDI or ESI). As a β-peptide with an *N*-terminal β-HCys residue, we chose the β-undecapeptide **6** (consisting of eleven different β-amino acid residues), which was prepared on a *Wang* resin, purified and characterized in the usual way (like **4** and **5**)[9]). The yields of the purified β-tri- and β-tetrapeptide derivatives **1**–**3** are in the range of 70%, those of the β-deca- and β-undecapeptide derivatives **4**–**6** are 20–30% (see *Exper. Part*). The α-undecapeptide **7** was prepared by standard Fmoc solid-phase synthesis.

3. Thioligations. – Coupling of the peptide thioesters with the peptides bearing an *N*-terminal Cys or HCys residue was carried out under modified standard conditions [17]: aqueous solution, pH 7.5 phosphate buffer, 4% (*v*/*v*) PhSH, with or without tris(2-carboxyethyl)phosphine as a reducing reagent. The products of ligation **8** (**3**+**6**), **9** (**5**+**6**), and **10** (**3**+**7**) were isolated and purified by preparative RP-HPLC. The reactions were monitored by HPLC and led to complete disappearance of the starting materials (*Fig. 1*). After purification, the coupling products were isolated in yields ranging from 40 to 60%; they gave correct high-resolution mass spectra. Thus, the thioligation is applicable to β-peptides[10]), under essentially the same conditions used for α-peptides and proteins. 'Mixed' α/β-peptides such as **10** are also available by this strategy.

4. Characterization of the β-Peptides. – The β-heneicosapeptide **9** contains 13 different proteinogenic side chains, with the segment 11 to 21 bearing all different groups (*cf.* **6**). The HPLC trace of a purified sample, and the corresponding CD spectra in MeOH and in H_2O[11]) are shown in *Fig. 2*.

The normalized CD spectra of the β-tetrapeptide thioester **3**, of the β-decapeptide thioester **5**, of the unprotected β-undecapeptide **6** with *N*-terminal β-HCys, and of the two thioligation products **8** and **9** in MeOH and in H_2O are compared in *Fig. 3*. Clearly, all but the tetrapeptide derivative exhibit a negative *Cotton* effect between 210 and 220 nm in MeOH, while only the β-heneicosapeptide **9** shows this effect in aqueous solution. As the chain length increases, the intensity of the negative *Cotton* effect (per amino acid) also increases, while the positive *Cotton* effect near 200 nm decreases in the same order. So far, we have correlated a trough near 215 and a peak near 200 with the β-peptidic 3_{14} helix [6][18] (*cf.* the CD spectrum of the β-decapeptide **5** in *Fig. 3*,*b*, for a typical example).

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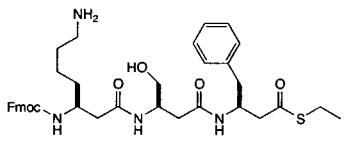
7) A method developed by one of our groups [15].
8) The method of *Ellman* and co-workers [16].
9) *Cf.* the direct preparation of longer β-peptides without thioligation [7].
10) So far, we have tested this ligation method only with β^3-peptides.
11) An NMR analysis of **9** is underway, and the results will be published.



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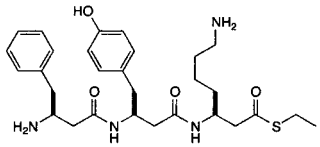

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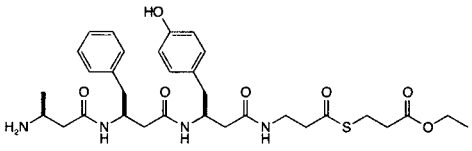

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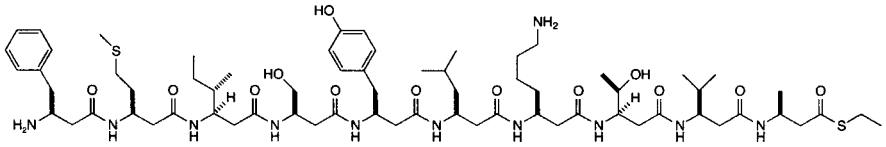

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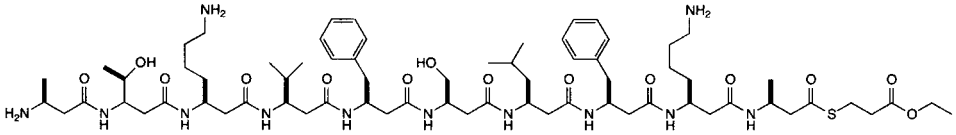

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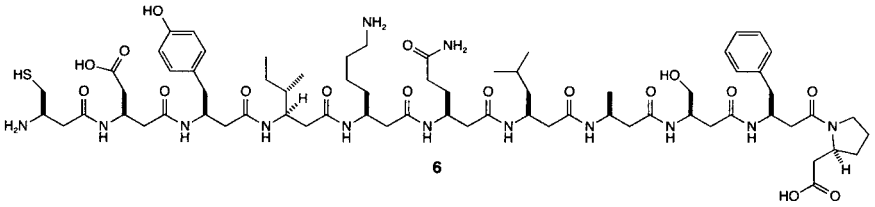

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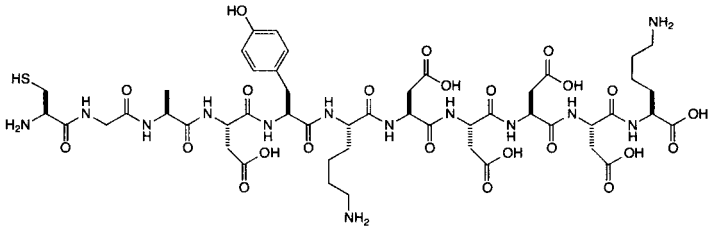

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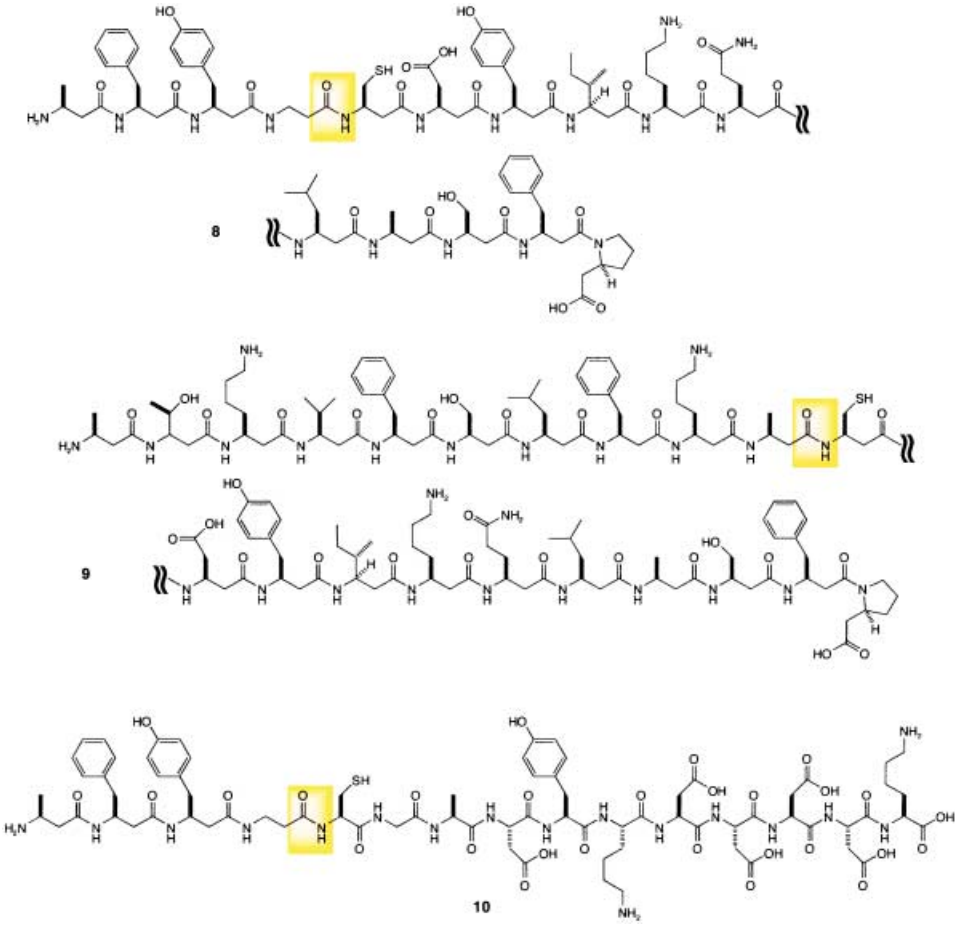

However, it is worth noting that the peak at shorter wavelength disappears with increasing chain length in the series of compounds shown here[12]). This result adds to the 'mysterious of CD spectra' of β-peptides [7][18], because a β-peptide of size and amphiphilic character similar to that of **9** had given rise to entirely different CD spectra [7]. If β-peptide **9** were to form a 3_{14} helix, the side chains would be arranged as shown in *Fig. 2*,*c*, with interactions between side chains as indicated. To see whether intermolecular interactions are involved in creating the *Cotton* effect, we have measured the spectrum of the β-peptide **9** at different concentrations (from 0.2 to 0.02 mM, *Fig. 4*,*a*) and at various temperatures (210 nm, 15 – 90°, up and down, *Fig. 4,b*) in aqueous solution.

Upon dilution from 0.2 to 0.1 mM the negative *Cotton* effect undergoes a blue shift of *ca*. 5 nm and remains at constant wavelength, with decreasing intensity, upon further

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12) *Gellman*'s β-hexapeptide consisting of *trans*-2-aminocyclohexanecarboxylic acid residues also does not show a short-wavelength *Cotton* effect (*cf.* Fig. 5 in [19]).

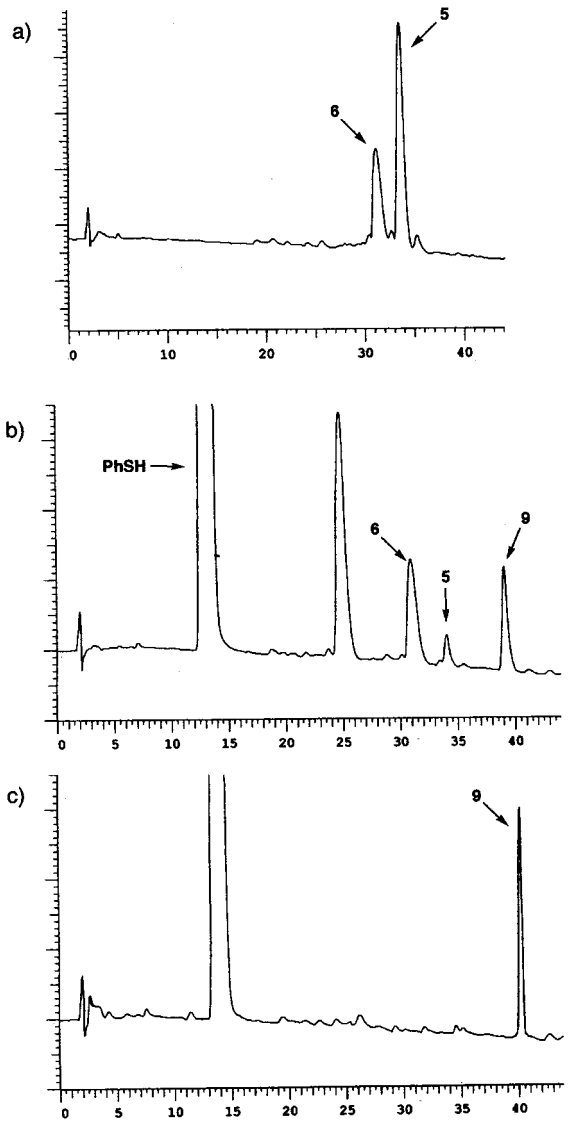

Fig. 1. *Analytical-HPLC traces of the ligation reaction between β-peptide* **6** *with an* N*-terminal* β^3*-HCys and* β^3*-peptide* **5** *with a* C*-terminal thioester group:* a) *time zero without thiophenol*, b) *reaction mixture after 3 h*, c) *after 11 h*. The peak at 40 min corresponds to the expected ligation product **9**, as judged by ESI-MS after purification by preparative RP-HPLC. The peak at 25 min is probably from the intermediate phenylthioester (*cf.* the *Scheme*). Chromatographic conditions: column *Macherey-Nagel* C_8 (250 × 4 mn), eluant *A:* H_2O (0.1% TFA), eluant *B:* MeCN, linear gradient 25–50% *B* over 50 min.

dilution. As the temperature is raised to 90°, the intensity of this *Cotton* effect decreases by only 25%, with no sign of 'melting' [19], an effect that is fully reversible. The increase in signal intensity upon concentration may be ascribed to aggregation, and the

red shifts that occur at the highest concentrations and in the absence of buffer suggest an alteration of the secondary structure in an aggregate. The normalized CD spectra of the mixed α/β-peptide **10** in MeOH and in H_2O are shown in *Fig. 5*,*a*. In MeOH, compound **10** exhibits a weak negative *Cotton* effect at 222 nm, a zero-crossing at 209, and a positive peak near 203 nm. The negative *Cotton* effect at 222 nm is characteristic

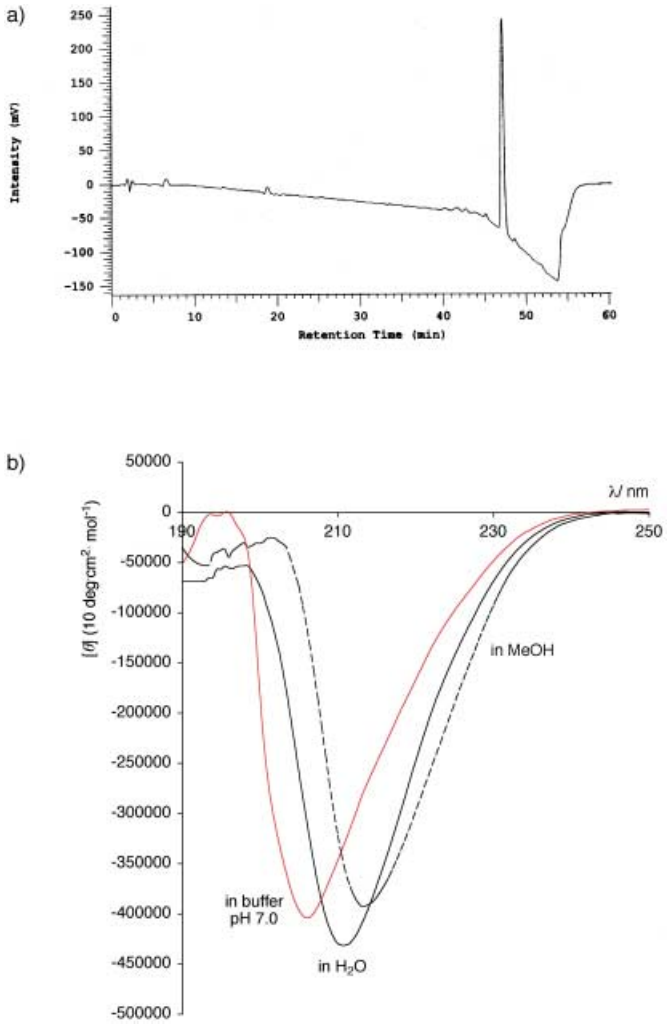

Fig. 2. *HPLC Trace, CD spectra, and schematic view of a hypothetical* 3_{14} *helical conformation of* β^3*-peptide* **9**. *a*) Analytical-HPLC profile of purified **9** (*RP-*C_8 column, linear gradient of 0.1% TFA in H_2O and MeCN, see *Exper. Part*). *b*) CD Spectra in MeOH, in H_2O, and in buffered aqueous solution (pH 7.0) of the β-peptide **9**. The spectra were recorded at 20° at a concentration of 0.2 mM. The peptide was measured as the TFA salt as obtained after lyophilization. The spectrum of **9** in MeOH and in aqueous solution is characteristic of the β-peptidic 3_{14} helix. *c*) Schematic presentation along the axis of an idealized 3_{14} helical secondary structure [7] of β-peptide **9**.

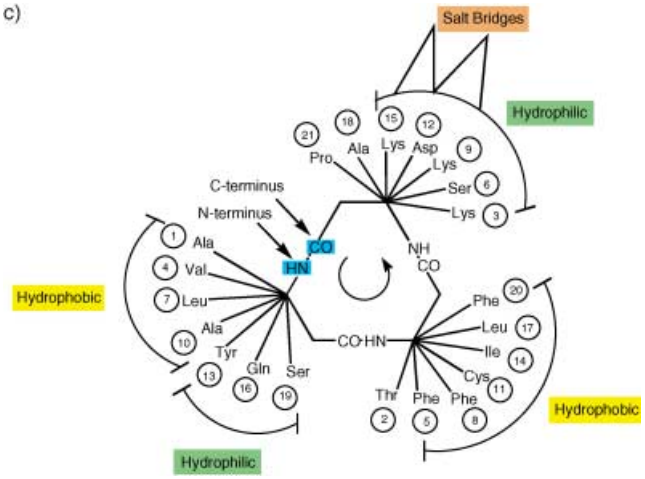

Fig. 2 (cont.)

of an α-peptidic 3.6_{13} helix, (*cf. Fig. 5*,*b* for a typical example), while it appears at shorter wavelength for a 3_{14} helix of β-peptides. Nevertheless, the spectrum is clearly different from that of an α-helix and likely reflects a more complex structure[13]).

5. Conclusions. – The successful thioligation of β-peptidic thioesters with α- or β-peptides carrying an *N*-terminal cysteine residue opens new possibilities *i*) for the construction of larger peptides containing β-amino acids, and *ii*) for the coupling of α- with β-peptides ('chimeric' structures). This will allow us (and others) to design more complex arrays of β-peptidic secondary structures and assist the search for β-peptides with novel tertiary structures and functions; it will also supply numerous compounds for CD-spectroscopic investigations, which will, hopefully, generate more reliable correlations between CD patterns [20] (*cf.* α-peptides and proteins [21]). Furthermore, the coupling of α- with β-peptides will enable us to study the influence of β-peptidic secondary structures on the folding properties, enzymatic activity, and proteolytic stability of α-peptides and proteins.

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Experimental Part

1. *General*. Abbreviations: DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene, DMPA: 4-(dimethylamino)pyridine, HBTU: 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt: *N*-hydroxybenzotriazole HMBA: 4-(hydroxymethyl)benzoic acid, h.v.: high vacuum, Melm: 1-methyl-imidazole, MSNT: 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole, PyBop: (benzotriazol-1-yl)oxytris(pyrrolidinophosphonium hexafluorophosphate), TFA: trifluoroacetic acid, TIS: triisopropylsilane, TNBS: 2,4,6-trinitrobenzenesulfonic acid. The β-amino acids were purchased from *Fluka* or prepared according to literature procedures [8]. Anal. HPLC: *Merck* HPLC system (*LaChrom*, pump type *L-7150*, UV detector *L-7400*, interface *D-7000*, HPLC manager *D-7000*). *Macherey-Nagel* C_8 column (*Nucleosil 100-5* C_8 250×4 mm)). Prep. HPLC: *Merck* HPLC

[13]) This speculative conclusion will have to be substantiated by an NMR investigation, which is underway.

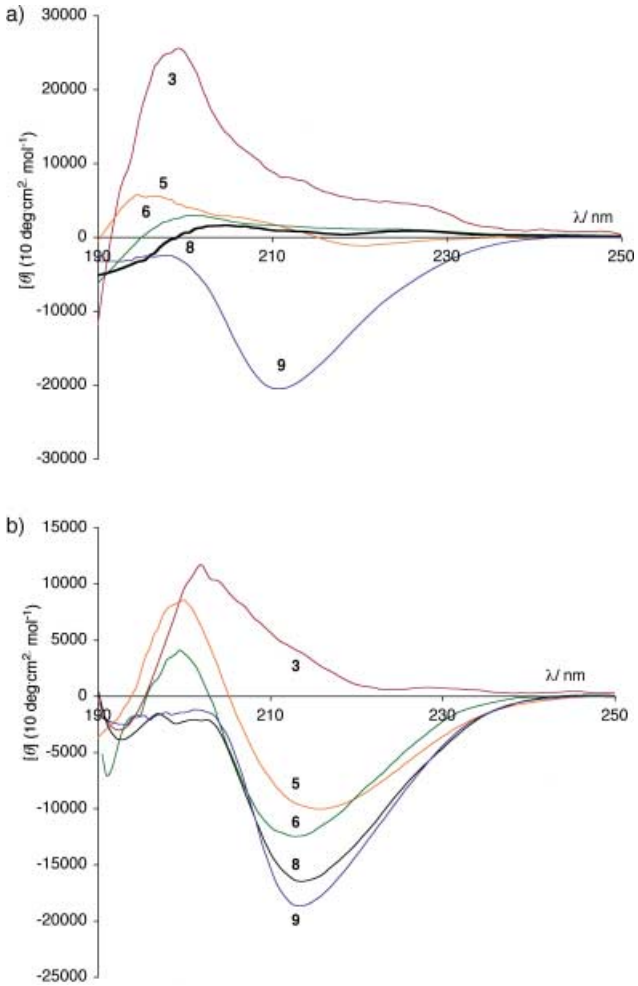

Fig. 3. *Normalized CD spectra* a) *in H_2O and* b) *in MeOH of the β^3-peptides* **3**, **5**, **6**, **8**, *and* **9**. The spectra were recorded at room temperature. The concentration was 0.2 mM. The peptides were submitted to measurements as their TFA salts as obtained after lyophilization. Except for **3**, all these β-peptides exhibit the pattern that we assign to a 3_{14} helix in MeOH. Only β-peptide **9** shows this pattern also in H_2O.

system (*LaChrom*, pump type *L-7150*, UV detector *L-7400*, interface *D-7000*, HPLC manager *D-7000*). *Macherey-Nagel C_8* column (*Nucleosil 100-7 C_8* (250 × 21 mm)). Circular dichroism (CD) spectra: CD spectra were recorded on an *Aviv Circular Dichroism* spectropolarimeter from 190 to 300 nm at 25° in 1-mm rectangular cells. All spectra were corrected for the corresponding solvent spectrum. Peptide concentrations were typically 0.2 mM. The molar ellipticity $[\theta]$ in 10 deg · cm^2 · mol^{-1} (λ in nm) was calculated for the corresponding peptide or normalized. Smoothing was done by *CDS V2.69* software. Solvents: MeOH (HPLC grade), aq. buffer pH 7.0: 0.1M KH_2PO_4/0.1M NaOH. NMR Spectra: *Bruker AMX-500* (^{1}H: 500 MHz, ^{13}C: 125 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 a 2.5-dihydroxybenzoic acid matrix), or *Finnigan MAT TSQ-700* (ESI) mass spectrometer; in m/z (% of basis peak).

2. *Reversed-Phase (RP) HPLC Analysis and Purification.* RP-HPLC Analysis was perfomed on a *Macherey-Nagel C_8* column (*Nucleosil 100-5 C_8* (250 × 4 mm)) with a linear gradient of *A* (0.1% TFA in H_2O)

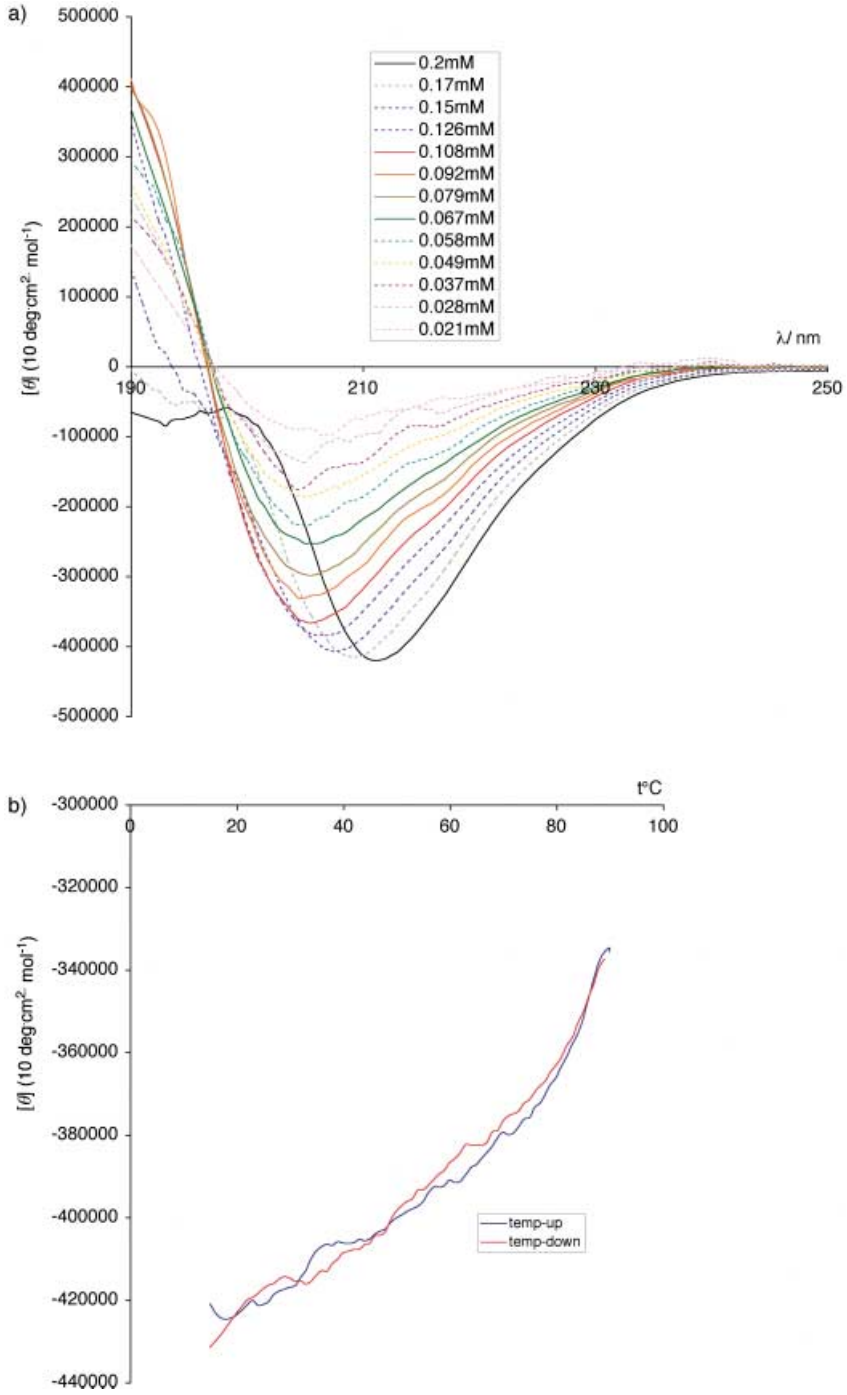

Fig. 4. *Concentration and temperature dependence of the CD spectrum of β-heneicosapeptide* **9**). *a*) CD Spectra of **9** in H_2O, recorded at room temperature and different concentrations. The peptide was submitted to measurement as the TFA salt as obtained after lyophilization. *b*) Molar ellipticity at 210 nm of *β*-peptide **9** as a function of temperature; blue curve: increasing, red curve: decreasing temperature.

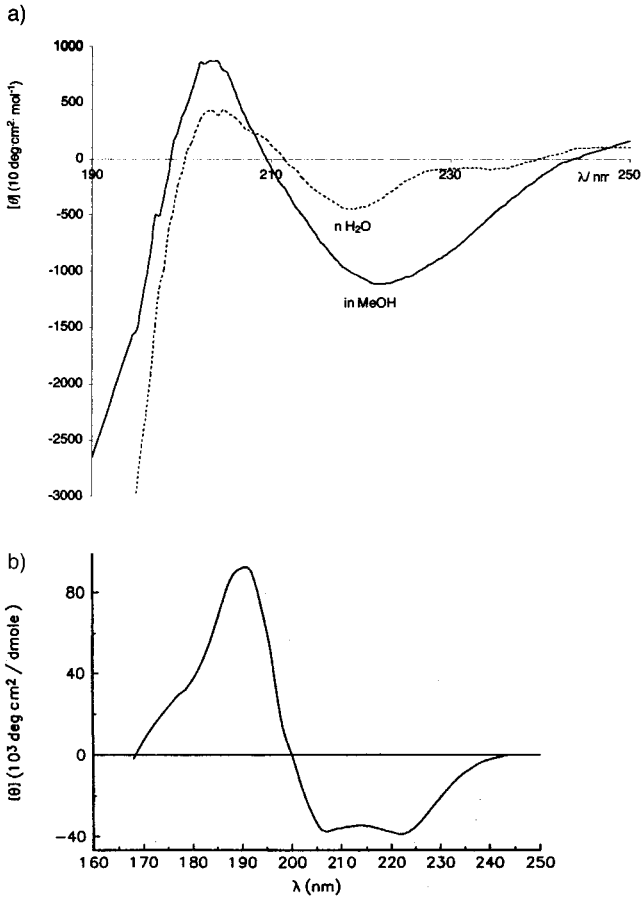

Fig. 5. a) *Normalized CD spectra of mixed α/β-peptide* **10** *in MeOH and H_2O, and comparison with a typical CD spectrum of an α-peptidic helix*. The spectra were recorded at room temperature. The concentration was 0.2 mM. The peptide was submitted to measurement as the TFA salt as obtained after lyophilization. b) *Typical CD* spectra of an α-helical peptide. Reproduced from [21].

and *B* (MeCN) at a flow rate of 1.2 ml/min with UV detection at 220 nm; t_R in min. RP-HPLC Purification was performed on a *Macherey-Nagel C_8* column (*Nucleosil 100-5 C_8* (250 × 21 mm)) with a linear gradient of *A* and *B* at a flow rate of 18 ml/min (*Merck* HPLC system).

3. *Anchoring of* N-*Fmoc-Protected β-Amino Acid on the 4-Sulfamylbutanoyl* AM *Resin: General Procedure 1* (*GP 1*). The loading of the sulfamoylbutanoyl resin was performed according to [16]. A soln. of the Fmoc-β^3-amino acid (6 equiv.) and DIPEA (10 equiv.) in CH_2Cl_2 was added to the resin that had been preswelled in CH_2Cl_2 for 30 min. The suspension was mixed by Ar bubbling for 10 min at r.t. before addition of PyBop (6 equiv.) as a solid, and the mixture was stirred for 10 h. Subsequently, the resin was filtered off, washed with DMF (4 ml, 4 × 1 min), CH_2Cl_2 (4 ml, 4 × 1 min), and dried under h.v. overnight. The loading was determined by measuring the absorbance of the benzofulvene-piperidine adduct according to [8].

4. *Anchoring of* N-*Fmoc-Protected β-Amino Acid on the* Wang *Resin or the* HMBA *Resin: General Procedure 2* (*GP 2*). Esterification of the Fmoc-β^3-amino acid with the *Wang* resin or the HMBA resin was performed according to [22]. To a soln. of the Fmoc-β^3-amino acid (5 equiv.) in dry CH_2Cl_2 (3 ml/mmol) was added Melm (3.75 equiv.) followed by MSNT (5 equiv.). The mixture was stirred until the MSNT dissolved. The soln. was then transferred to a vessel containing the preswelled resin, and mixed by Ar bubbling for 1 h at r.t.

The resin was then filtered off, washed with DMF (4 ml, 4 × 1 min), CH_2Cl_2 (4 ml, 4 × 1 min), and dried under h.v. overnight. The loading was determined by measuring the absorbance of the benzofulvene piperidine adduct according to [8].

5. *Solid-Phase Peptide Synthesis (SPPS) on the 4-Sulfamylbutanoyl* AM *Resin*, *the* Wang *Resin or the* HMBA *Resin: General Procedure 3* (*GP 3*). The Fmoc group of the first amino acid attached to the resin was removed with 20% piperidine in DMF (4 ml, 4 × 10 min) under Ar bubbling. After filtration, the resin was washed with DMF (4 ml, 4 × 1 min). Solid-phase synthesis was continued by sequential incorporation of *N*-Fmoc-protected β^3-amino acids. For each coupling step, the resin was treated with a soln. of Fmoc-β^3-amino acid (4 equiv.), HBTU (3.8 equiv.), and HOBt (4 equiv.) in DMF (3 ml). After mixing by Ar bubbling for 5 min, DIPEA (7.8 equiv.) in DMF (0.5 ml) was added, and the suspension was further mixed by Ar bubbling for 45–60 min. Monitoring of the coupling reaction was performed with TNBS [23]. In the case of a positive TNBS test (indicating incomplete coupling), the suspension was allowed to react further for 1–2 h or, after filtration, the peptide resin was treated again with the same Fmoc-β^3-amino acid (2 equiv.) and coupling reagents. The resin was then filtered off and washed with DMF (4 ml, 4 × 1 min) prior to the subsequent Fmoc deprotection step with 20% piperidine in DMF (4 ml, 4 × 10 min). The Fmoc group of the sixth amino acid was removed with 20% piperidine in DMF (4 ml, 2 × 10 min), DBU/piperidine/DMF (1:1:48, 4 ml, 2 × 10 min) under Ar bubbling. After filtration, the resin was washed with DMF (4 ml, 3 × 1 min), and solid-phase synthesis was continued by sequential incorporation of Fmoc-β^3-amino acids. For each coupling step, the resin was treated as described above. The resin was then filtered off, washed with DMF (4 ml, 4 × 1 min), CH_2Cl_2 (4 ml, 4 × 1 min), and MeOH (4 ml, 4 × 1 min), dried under h.v. for 24 h, and used for the cleavage step.

6. *Alkylation of the Peptide-Acylsulfonamide Resin: General Procedure 6* (*GP 6*). The *4-Sulfamylbutanoyl AM* resin was activated according to [16]. After swelling the resin in THF, 4 ml of a soln. of TMS-CHN_2 (2M in hexane) was added, and the suspension was mixed by Ar bubbling for 2 h. Subsequently, the resin was filtered off, washed with THF (4 ml, 4 × 1 min) and DMF (4 ml, 4 × 1 min), and used in the displacement reaction (see below) or further washed with CH_2Cl_2 (4 ml, 4 × 1 min) and dried under h.v. until further use.

7. *Thioesterification, Deprotection, and Purification of the Alkylated Peptide-Acylsulfonamide Resin: General Procedure 7* (*GP 7*). The activated *N*-acylsulfonamide resin was swollen in DMF and filtered off. A soln. of ethyl-3-sulfanylpropionate (50 equiv.) and sodium thiophenate (0.5 equiv.) in DMF (1M) was added. The suspension was mixed by Ar bubbling for 24 h, then the resin was filtered and washed with DMF (4 × 4 ml). The combined filtrate and washings were collected, evaporated, and dried under h.v. Removal of side-chain protecting groups was accomplished in soln. by treatment of the protected β-peptide thioester with a soln. of TFA/H_2O/TIS (95:2.5:2.5). Solvents were concentrated under reduced pressure. The precipitate, which formed upon addition of cold Et_2O to the oily residue, was collected by centrifugation. After drying, the crude thioester was purified by prep. RP-HPLC and lyophilized.

8. *Cleavage of the Peptide from the* HMBA *Resin to Give the Corresponding Thioester: General Procedure 8* (*GP 8*). According to [15], a soln. of Me_2AlCl (20 equiv.) in CH_2Cl_2 was cooled at 0° under Ar, then EtSH (60 equiv.) was added dropwise, and the resulting mixture was stirred for 15 min at 0°. This soln. was added to the preswelled resin and mixed under Ar bubbling for 5 h. The resin was then filtered off, and the filtrate was poured into 3 ml of a soln. of TFA/H_2O (97.5:2.5) and evaporated. Then, 3 ml of TFA/TIS/EtSH/H_2O (92.5:2.5:2.5:2.5) soln. was added to the oily residue and stirred 2.5 h at r.t. The soln. was then evaporated, and the crude peptide was precipitated by addition of cold Et_2O (0°), isolated by centrifugation, and purified by prep. RP-HPLC.

9. *Chemical Ligation Reaction of Unprotected β^3-Peptides: General Procedure 9* (*GP 9*). Typically, the *C*-terminal β^3-peptide thioester (1 equiv.) and the β^3-peptide containing an *N*-terminal Cys (1.1 equiv.) were dissolved in 100 mM sodium phosphate buffer (pH 7.5) to give a final concentration of 2 mM. PhSH (4% *v*/*v*) was added, and the mixture was stirred under Ar at r.t. The reaction was monitored by anal. C_8 RP-HPLC. Following the ligation, the product was purified by prep. C_8 RP-HPLC and lyophilized.

Fmoc-β^3-HLys-β^3-HSer-β^3-HPhe–SEt (**1**). The *HMBA* resin (300 mg, 0.346 mmol) was derivatized with Fmoc-β^3-HPhe–OH (695 mg, 1.73 mmol) according to *GP 2* to give a loading of 0.735 mmol/g (64%), corresponding to 0.220 mmol of anchored Fmoc-β^3-HPhe–OH. The unreacted OH groups were capped with Ac_2O (0.65 ml, 6.93 mmol) in DMF (3 ml) and DMAP (15 mg, 0.12 mmol, added in 0.5 ml of DMF) for 2 h. Solid-phase synthesis according to *GP 3*, cleavage according to *GP 8*, and purification by RP-HPLC (10–60% *B* in 40 min, C_8) yielded **1** (94 mg, 62%) as a colorless solid. Homogeneity > 95% (RP-HPLC). Anal. RP-HPLC (10–60% B in 40 min, C_8): t_R 25.14. ^{1}H-NMR (300 MHz, CD_3OD): 1.17 (*t*, *J* = 7.4, Me); 1.28–1.63 (*m*, 3 CH_2); 2.28–2.34 (*m*, 2 CH_2); 2.63–2.90 (*m*, 8 CH_2); 3.31–3.41 (*m*, CH_2); 3.91–4.13 (*m*, CH); 4.15–4.19 (*m*, 2 CH);

4.33 (*d*, *J* = 6.5, CH_2); 4.43–4.51 (*m*, CH); 7.15–7.40 (*m*, 9 arom. H); 7.60–7.62 (*m*, 2 arom. H); 7.78 (*d*, *J* = 7.4, 2 arom. H). ESI-MS (positive mode): 689.1 (100, $[M+H]^+$), 711.1 (40, $[M+Na]^+$).

H_2N-β³-HPhe-β³-HTyr-β³-HLys–SEt (**2**). The *HMBA* resin (300 mg, 0.348 mmol) was derivatized with Fmoc-β³-HLys(Boc)–OH (840 mg, 1.74 mmol) according to *GP 2* to give a loading of 0.715 mmol/g (62%), corresponding to 0.219 mmol of anchored Fmoc-β³-HLys(Boc)–OH. The unreacted OH groups were capped with Ac_2O (0.65 ml, 6.93 mmol) in DMF (3 ml) and DMAP (15 mg, 0.12 mmol, added in 0.5 ml of DMF) for 2 h. Solid-phase synthesis according to *GP 3*, cleavage according to *GP 8*, and purification by RP-HPLC (10–50% *B* in 35 min, C_8) yielded **2** (89 mg, 74%) as a colorless fluffy solid. Homogeneity >95% (RP-HPLC). Anal. RP-HPLC (10–50% *B* in 35 min, C_8): t_R 21.36. ^{1}H-NMR (300 MHz, CD_3OD): 1.19 (*t*, *J* = 7.4, Me); 1.32–1.70 (*m*, 3 CH_2); 2.27–2.40 (*m*, 3 CH_2CO); 2.53–2.92 (*m*, 2 PhCH_2, CH_2S, CH_2NH_2); 3.53–3.60 (*m*, CH); 4.23–4.37 (*m*, CH); 4.38–4.41 (*m*, CH); 6.65 (*d*, *J* = 6.5, 2 arom. H); 6.98 (*d*, *J* = 8.7, 2 arom. H); 7.20 (*d*, *J* = 6.8, 2 arom. H); 7.22–7.39 (*m*, 5 arom. H); 8.03 (br., NH). ESI-MS (positive mode): 565.1 (8, $[M+Na]^+$), 543.2 (20, $[M+H]^+$), 272.2 (100, $[M+2H]^{2+}$).

H_2N-β³-HAla-β³-HPhe-β³-HTyr-HGly–S-*Ethylpropanoate* (**3**). The *4-Sulfamylbutyryl AM* resin (400 mg, 448 mmol) was derivatized with Fmoc–HGly–OH (837 mg, 2.69 mmol) according to *GP 1* to give a loading of 0.79 mmol/g (70%), corresponding to 0.316 mmol of anchored Fmoc–HGly–OH. The unreacted OH groups were capped with Ac_2O (0.42 ml, 4.48 mmol) in DMF (4 ml) and DMAP (15 mg, 0.12 mmol, added in 0.5 ml of DMF) for 2 h. Solid-phase synthesis was performed according to *GP 3*, followed by activation of the resin with 4 ml of a sol. of 2M TMS-CHN_2 in THF according to *GP 6*. The activated *N*-acylsulfonamide resin was then treated with a soln. of ethyl-3-sulfanylpropanoate (2.90 ml, 22.4 mol), and sodium thiophenate (30 mg, 224 mmol) in DMF (5 ml) for 24 h according to *GP 7*. Removal of side-chain protecting groups was accomplished in soln. by treatment of the protected β-peptide thioester with a soln. of TFA/H_2O/TIS (95 : 2.5 : 2.5) according to *GP 7*. Purification by RP-HPLC (10–50% *B* in 40 min, C_8) yielded **3** (150 mg, 75%) as a colorless fluffy solid. Anal. RP-HPLC (10–50% *B* in 40 min, C_8): t_R 22.09. CD (0.2 mM in MeOH): $+11.698 \cdot 10^3$ (202 nm). CD (0.2 mM in H_2O, pH 5.6): $+25.497 \cdot 10^3$ (199 nm). ^{1}H-NMR (300 MHz, CD_3OD): 1.17–1.25 (*m*, 2 Me); 2.28–2.39 (*m*, 2 CH_2); 2.58 (*t*, *J* = 6.9, CH_2); 2.61–2.78 (*m*, 3 CH_2); 3.09 (*t*, *J* = 6.9, CH_2); 3.41–3.53 (*m*, 2 CH_2, CH); 4.11 (*q*, *J* = 7.1, CH_2); 4.37–4.41 (*m*, 2 CH) 6.66 (*d*, *J* = 8.4, 2 arom. H); 7.01 (*d*, *J* = 8.4, 2 arom. H); 7.16–7.25 (*m*, 5 arom. H); 7.41–7.43 (br., NH); 7.82–7.84 (br., NH); 7.89–8.05 (br., NH). MALDI-MS: 651.3 (94, $[M+Na]^+$), 629.3 (100, $[M+H]^+$).

H_2N-β³-HPhe-β³-HMet-β³-HIle-β³-HSer-β³-HTyr-β³-HLeu-β³-HLys-β³-HThr-β³-HVal-β³-HAla–SEt (**4**). The *HMBA* resin (400 mg, 464 mmol) was derivatized with Fmoc-β³-HAla–OH (754.8 mg, 2.32 mmol) according to *GP 2* to give a loading of 0.863 mmol/g (75%), corresponding to 0.345 mmol of anchored Fmoc-β³-HAla–OH. The unreacted OH groups were capped with Ac_2O (0.43 ml, 4.64 mmol) in DMF (3 ml) and DMAP (10 mg, 0.08 mmol, added in 0.5 ml DMF) for 2 h. Solid-phase synthesis according to *GP 3*, cleavage according to *GP 8*, and purification by RP-HPLC (30–70% *B* in 60 min, C_8) yielded **4** (145 mg, 31%) as a colorless fluffy solid. Homogeneity >95% (RP-HPLC). Anal. RP-HPLC (30–70% *B* in 60 min, C_8): t_R 30.77. ^{1}H-NMR (300 MHz, CD_3OD): 0.87–0.96 (*m*, 5 Me); 1.15 (*d*, *J* = 6.5, Me); 1.18–1.20 (*m*, 2 CH_2); 1.22 (*d*, *J* = 6.9, Me); 1.24 (*m*, 3 CH); 1.26 (*t*, *J* = 7.4, Me)); 1.49–1.88 (*m*, 9 H); 2.11 (*s*, MeS); 2.53–3.3 (*m*, 2 PhCH_2, CH_2S, CH_2NH_2, 10 CH_2CO); 3.53–3.60 (*m*, CH_2O, CHO, 2 CH_2S); 4.23–4.37 (*m*, 6 CH); 4.38–4.41 (*m*, 4 CH); 6.67 (*d*, *J* = 8.4, 2 arom. H); 7.25 (*d*, *J* = 8.4, 2 arom. H); 7.27–7.36 (*m*, 5 arom. H); 7.65–7.68 (br., NH); 8.18–8.25 (br., NH); 8.30–8.33 (br., NH); 8.50–8.62 (br., NH). ESI-MS (positive mode): 1379.1 (27, $[M+Na]^+$), 1357 (100, $[M+H]^+$), 679.3 (100, $[M+2H]^{2+}$).

H_2N-β³-HAla-β³-HThr-β³-HLys-β³-HVal-β³-HPhe-β³-HSer-β³-HLeu-β³-HPhe-β³-HLys-β³-HAla–S-*Ethylpropanoate* (**5**). The *4-Sulfamoylbutyryl AM* resin (400 mg, 480 mmol) was treated with Fmoc-β³-HAla–OH (875 mg, 2.69 mmol) according to *GP 1* to give a loading of 0.91 mmol/g (75%), corresponding to 0.364 mmol of anchored Fmoc-β³-HAla–OH. The unreacted OH groups were capped with Ac_2O (0.45 ml, 4.8 mmol) in DMF (4 ml) and DMAP (15 mg, 0.12 mmol, added in 0.5 ml of DMF) for 2 h. Solid-phase synthesis was performed according to *GP 3*, followed by activation of the resin with 4 ml of a soln. of 2M TMS-CHN_2 in THF according to *GP 6*. The activated *N*-acylsulfonamide resin was then treated with a soln. of ethyl-3-sulfanylpropanoate (3.10 ml, 24 mol), and sodium thiophenate (31 mg, 240 mmol) in DMF (5 ml) for 24 h according to *GP 7*. Removal of side-chain protecting groups was accomplished in soln. by treatment of the protected β-peptide thioester with a soln. of TFA/H_2O/TIS (95 : 2.5 : 2.5) according to *GP 7*. Purification by RP-HPLC (10–50% *B* in 40 min, C_8) yielded **5** (72 mg, 19%) as a colorless fluffy solid. Anal. RP-HPLC (10–50% *B* in 40 min, C_8): t_R 38.67. CD (0.2 mM in MeOH): $-10.068 \cdot 10^3$ (215.5 nm); $+0.456 \cdot 10^3$ (205 nm); $+8.536.10^3$ (200 nm). CD (0.2 mM in H_2O): $+5.518 \cdot 10^3$ (197 nm). HR-MALDI-MS: 1390.8236 (34, $[M+Na]^+$, $C_{69}H_{115}O_{14}N_{12}NaS^+$; calc. 1390.8273), 1367.8360 (100, $[M+Na]^+$, $C_{69}H_{116}O_{14}N_{12}S^+$; calc. 1367.8371).

H_2N-β^3-HCys-β^3-HAsp-β^3-HTyr-β^3-HIle-β^3-HLys-β^3-HGln-β^3-HLeu-β^3-HAla-β^3-HSer-β^3-HPhe-β^3-HPro-OH (**6**). The *Wang* resin (400 mg, 452 mmol) was modified with Fmoc-β^3-HPro–OH (1.18 g, 3.61 mmol) according to *GP 2* at a loading of 0.915 mmol/g (81%), corresponding to 0.366 mmol of anchored Fmoc-β^3-HPro–OH. The unreacted OH groups were capped with Ac_2O (0.34 ml, 3.6 mmol) in DMF (4 ml) and DMAP (10 mg, 0.08 mmol, added in 0.5 of ml DMF) for 2 h. Solid-phase synthesis was performed according to *GP 3*. The dry peptide resin was treated for 2 h with 10 ml of a TFA/H_2O/EDT/TIS (94 : 2.5 : 2.5 : 1) soln. The resin was removed by filtration, washed with TFA, and the org. phase containing the peptide was concentrated under reduced pressure. The precipitate, which formed upon addition of cold Et_2O to the oily residue, was collected by centrifugation. The crude peptide was purified by RP-HPLC (15–10% *B* in 40 min, 50–99% *B* in 20 min, C_8) to yield the TFA salt of **6** (173 mg, 33%) as a colorless fluffy solid. Homogeneity >95% (RP-HPLC). Anal. RP-HPLC (15–50% *B* in 40 min, 50–99% *B* in 20 min, C_8): t_R 51.60. CD (0.2 mM in MeOH): $-12.498 \cdot 10^3$ (213 nm); $-0.075 \cdot 10^3$ (203 nm); $+4.074 \cdot 10^3$ (199.5 nm). CD (0.2 mM in H_2O, pH 4.0): $+2.9 \cdot 10^3$ (201 nm). ^{1}H-NMR (300 MHz, CD_3OD): 0.84–1.28 (*m*, 17 CH); 1.36–2.26 (*m*, 19 CH); 2.31–3.05 (*m*, 39 CH); 3.42–3.57 (*m*, 5 CH); 3.69–3.73 (*m*, CHN); 4.16–4.27 (*m*, CHN); 4.40–4.83 (*m*, 9 CHN); 6.69 (*d*, $J = 8.4$, 2 arom. H); 7.04 (*d*, $J = 8.4$, 2 arom. H); 7.20–7.31 (*m*, 5 arom. H); 7.53 (*d*, $J = 9.8$, NH);7.80 (*d*, $J = 8.1$, NH); 7.92 (*d*, $J = 8.1$, NH); 8.05 (*d*, $J = 9.6$, NH); 8.24–8.42 (*m*, 4 NH); 8.75 (*d*, $J = 9.03$, NH). ESI-MS (positive mode): 1438.8 (20, $[M+H]^+$), 730.7 (25, $[M+H+Na]^{2+}$), 719.7 (100, $[M+2H]^{2+}$).

H_2N-β^3-HAla-β^3-HPhe-β^3-HTyr-HGly-β^3-HCys-β^3-HAsp-β^3-HTyr-β^3-HIle-β^3-HLys-β^3-HGln-β^3-HLeu-β^3-HAla-β^3-HSer-β^3-HPhe-β^3-HPro-OH (**8**). β-^{3}-Peptide fragment **6** (9.27 mmol, 15.14 mg) and the *C*-terminal-thioester β^3-peptide **3** (7.73 mmol, 5.61 mg) were ligated in 3 ml of buffer (100 mM phosphate, pH 7.5) containing thiophenol (4% *v*/*v*) according to *GP 9*. The ligation reaction was performed at r.t. and monitored by anal. RP-HPLC. Following completion of the ligation, the mixture was diluted with H_2O (2 ml) containing 0.1% TFA and purified by prep. RP-HPLC (10–50% *B* in 40 min, 50–99% *B* in 10 min, C_8) to yield the TFA salt of **8** (9.2 mg, 61%) as a colorless fluffy solid. Homogeneity >95% (RP-HPLC). Anal. RP-HPLC (10–50% *B* in 40 min, 50–99% *B* in 10 min, C_8): t_R 44.23. CD (0.2 mM in MeOH): $-16.483 \cdot 10^3$ (214 nm). CD (0.2 mM in H_2O, pH 4.3): $+1.576 \cdot 10^3$ (205 nm). HR-MALDI-MS: 1956.0352 (13, $[M+Na]^+$, $C_{97}H_{145}N_{17}NaO_{22}S^+$; calc. 1956.0331), 1933.0533 (82, $[M+H]^+$, $C_{97}H_{146}N_{17}O_{22}S^+$; calc. 1933.0544).

H_2N-β^3-HAla-β^3-HThr-β^3-HLys-β^3-HVal-β^3-HPhe-β^3-HSer-β^3-HLeu-β^3-HPhe-β^3-HLys-β^3-HAla-β^3-HCys-β^3-HAsp-β^3-HTyr-β^3-HIle-β^3-HLys-β^3-HGln-β^3-HLeu-β^3-HAla-β^3-HSer-β^3-HPhe-β^3-HPro-OH (**9**). β^3-Peptide fragment **6** (3.33 mmol, 5.76 mg) and the *C*-terminal thioester β^3-peptide **5** (3.33 mmol, 5.53 mg) were ligated in 1 ml of buffer (100 mM phosphate, pH 7.5) containing PhSH (4% *v*/*v*) according to *GP 9*. The ligation reaction was performed at r.t. and monitored by anal. RP-HPLC. Following completion of the ligation, the reaction mixture was diluted with H_2O (2 ml) containing 0.1% TFA and purified by prep. RP-HPLC (25–50% *B* in 40 min, 50–99% *B* in 10 min, C_8) to yield the TFA salt of **9** (4.62 mg, 52%) as a colorless fluffy solid. Homogeneity >95% (RP-HPLC). Anal. RP-HPLC (25–50% *B* in 40 min, 50–99% *B* in 10 min, C_8): t_R 47.33. CD (0.2 mM in MeOH): $-18.703 \cdot 10^3$ (213 nm). CD (0.2 mM in H_2O, pH 3.9): $-20.558 \cdot 10^3$ (210.5 nm). ESI-MS (positive mode): 2672.0 (15, $[M+H]^+$), 1348.1 (20, $[M+H+Na]^{2+}$), 1337.2 (100, $[M+2H]^{2+}$), 891.5 (42, $[M+3H]^{3+}$).

H_2N-β^3-HAla-β^3-HPhe-β^3-HTyr-HGy-Cys-Gly-Ala-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-OH (**10**). Peptide fragment **7** (3.34 mmol, 5.13 mg) and the *C*-terminal thioester β^3-peptide **3** (3.39 mmol, 2.46 mg) were ligated in 1 ml of buffer (100 mM phosphate, pH 7.5) containing PhSH (4% *v*/*v*) according to *GP 9*. The ligation reaction was performed at r.t. and monitored by anal. RP-HPLC. Following completion of the ligation, the reaction mixture was diluted with H_2O (1 ml) containing 0.1% TFA and purified by prep. RP-HPLC (10–50% *B* in 40 min, 50–99% *B* in 10 min, C_8) to yield the TFA salt of **10** (3.25 mg, 56%) as a colorless fluffy solid. Anal. RP-HPLC (10–50% *B* in 40 min, 50–99% *B* in 10 min, C_8): t_R 18.83. CD (0.2 mM in MeOH): $-1.111 \cdot 10^3$ (222 nm); $+0.010 \cdot 10^3$ (209.5 nm); $+0.867 \cdot 10^3$ (203 nm). CD (0.2 mM in H_2O, pH 3.6): $-0.436 \cdot 10^3$ (218 nm); $+0.044 \cdot 10^3$ (211 nm); $+0.426 \cdot 10^3$ (203 nm). ESI-MS (positive mode): 1760.9 (5, $[M+Na]^+$); 1739.1 (10, $[M+H]^+$); 881.0 (20, $[M+H+Na]^{2+}$); 870.1 (100, $[M+2H]^{2+}$); 580.3 (36, $[M+3H]^{3+}$).

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