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Synthesis of a Hybrid Peptide with Both α- and β-Amino Acid Residues: Toward a New β-Sheet Nucleator

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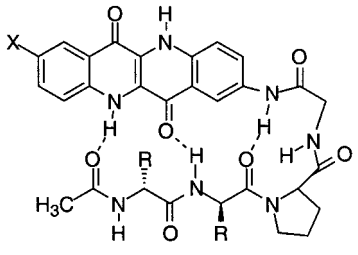
Abstract—A hybrid peptide was synthesized from a pentameric β-peptide (**5**) with two strands of a dimeric natural peptide (L-Val-L-Leu) attached to β-amino acid residues 1 and 4. The trifluoroacetate (**7**) of the hybrid peptide **6** was readily soluble in a variety of organic solvents indicating an ordered structure. All nine amide NH protons are separated between 7.4 and 8.8 ppm in $CDCl_3$ containing 5% DMSO($-d_6$). The downfield shift of the amide NH protons is an indication that hydrogen bonds are present. The CD spectrum of **7** in methanol is consistent with a β-sheet structure.

Introduction

Recent studies have shown that β-sheets play functionally important roles in many proteins. For examples, β-sheets provide the key element in protein-DNA,[1] protein-RNA,[2] and protein–protein recognition.[3] Similarly, aggregated protein fibrils exhibiting predominantly β-sheet structure have been implicated in amyloid diseases (such as Alzheimer's disease).[4,5]

Studies involving β-sheets are still an underexplored area. Unlike α-helices, β-sheets are known to aggregate easily and the aggregation makes the study of their properties difficult. Compounds that are designed to overcome some of these difficulties have been reported.[6] One of the early studies was done by Kemp and coworkers,[7] who used a 2,8-diaminoepindolidione to nucleate β-sheet formation. This planar aromatic compound serves as a template to induce an adjacent strand to form a β-sheet-like conformation. They were able to study the propensities for β-sheet formation of different amino acids by determining the stability of these induced β-sheet-like conformations.



Smith, Hirshmann, and coworkers studied 3,5-linked pyroline-4-ones as β-strand mimics.[8] This group of peptidomimetic compounds has a rigid conformation and duplicates the main chain conformation and sidechain placement of β-sheets. A study of their activities as HIV protease inhibitors was also carried out.[8] Several designs of β-hairpin structure have also been reported. Kelly and coworkers used 4-(2-aminoethyl)-6-dibenzofuranpropionic acid as a nucleator.[9] β-Sheet hydrogen bonding pattern was observed in the attached amino acid strands. The dibenzofuran moiety not only restricts the movement of the amino acid strands but also creates a hydrophobic cluster, which led to the formation of β-hairpin. More recently, a number of studies of β-hairpin structures have suggested the following conditions for their stability in solution: (1) inter-strand hydrogen bonding,[10] (2) hydrophobic interactions,[11,12] and (3) torsional and steric effects related to turn sequence.[13,14] Several short peptides have now been characterized to adopt β-hairpin conformations in solution.[10–20] However, these studies are limited to β-hairpins. In order to study parallel β-sheets or β-sheets of intermediate size, a proper molecular scaffold must be used.

Nowick and coworkers have reported the use of oligourea in combination with *m*-hydrazinobenzoic acid to serve as molecular scaffolds for the study of parallel β-sheets stability.[21] Their studies have established that this combination can duplicate the hydrogen-bonding functionality of one edge of a peptide strand in a β-strand conformation and can template β-sheet formation in an attached peptide strand. In addition, the β-sheet mimic is soluble in chloroform, which makes the identification of specific hydrogen bonds possible.

Background

We chose to study the influence of inter-strand hydrogen

Keywords: hybrid peptide; β-amino acid; β-sheets.

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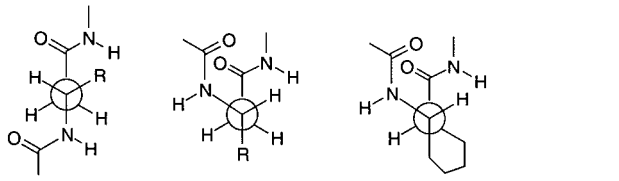
bonding on β-sheet stability by a new model system based on the recently discovered 3_{14} helical β-peptides (peptides made from β-amino acids).[22–24] This new model system is expected to (1) allow the control of solubility in either organic solvents or water, (2) encourage β-sheet formation, and (3) allow the deletion of inter-strand hydrogen bonds to study their influence on β-sheet stability.

Both Seebach and Gellman have shown that β-peptides have a strong tendency to form helical conformations.[22,23] We also demonstrated that such a tendency depended in part in the conformational preference of each substituted β-amino acid residue.[25,26]



A single β-amino acid residue peptide is depicted above in Newman projections along the α–β sp^3 carbons between the nitrogen atom and the carbonyl carbon. In general, hydrocarbon chains with sp^3 carbons prefer an *anti* rather than a *gauche* conformation. However, for chiral β-amino acids carrying a sterically-demanding side chain (i.e. R≠H), the *gauche* conformation with the R group *anti* to the main chain becomes favorable. As shown in the Newman projections, if the R group is more sterically demanding than that of the main chain amide function, it should drive the dihedral angle $NC(\beta)^3_{sp}–C(\alpha)^3_{sp}C(=O)$ to a *gauche* conformation, which is conducive for the formation of the 3_{14} helical conformation. The use of *trans*-2-aminocyclohexane carboxylic acid (*trans*-2-ACHC) by Gellman as β-peptide monomers provides a fixed *gauche* conformation for each residue, which leads to a robust 3_{14} helical conformation for the oligimers.[23]

The 3_{14} helical β-peptides have three residues per turn and have a pitch of 5 Å.[22] The inter-strand distance in β-sheets is about 4.8 Å. Therefore, inter-strand amide–amide hydrogen bonds are favorably aligned if two strands of natural peptides are attached to the side chains of residue *i* and residue *i*+3 of a helical β-peptide (see illustration below). Those hydrogen bonds would be characteristic of the H-bonds in β-sheets.

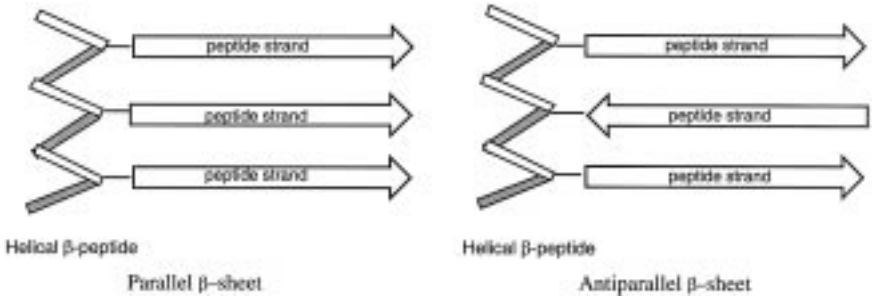
Given a strong tendency of forming the 3_{14} helical conformation, a β-peptide with appropriately attached natural peptides should allow a study of β-sheet properties. To study the tendency of intramolecular hydrogen bonding by the side chains of the *i*th and *i*+3 residues, the first step in our plan is to investigate a simple structural representative of a parallel β-sheet.

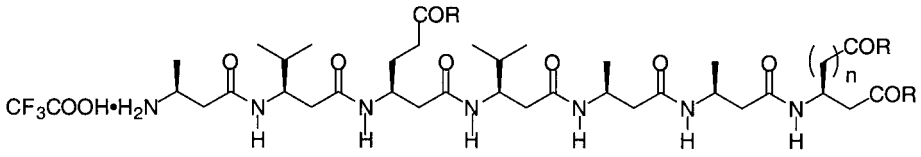
Various β-peptides have recently been prepared in our laboratories using modified procedures of previous reports.[24] The conformations of these β-peptides were studied by NMR and CD spectroscopy. The β-peptides with ester sidechains (**Ia** and **IIa**) are soluble in organic solvents and form helical conformations in organic solvents (chloroform and methanol).

The β-peptides containing carboxylic acid groups (**Ib** and **IIb**) are water-soluble. β-Peptide **IIb** containing one β-HGlu residue and one D-Asp residue (at the C-terminus) has an especially high water-solubility.[24] The results are consistent with our expectation that by varying the side chains of individual residues one can control the solubility of the peptides. The next step of our plan involves the examination of using β-peptides as β-sheet nucleators. This report describes our first effort in this area.

Results

After our initial successful experience in the identification of the conformations of β-peptides by multi-dimensional NMR spectroscopy, we set out to synthesize the β-sheet nucleator. Solution phase chemistry was employed in our





R = OMe: **Ia** (n = 2), **IIa** (n = 0); R = OH: **Ib** (n = 2); **IIb** (n = 0)

preparation of the model β-sheet. The synthesis is depicted in Scheme 1.

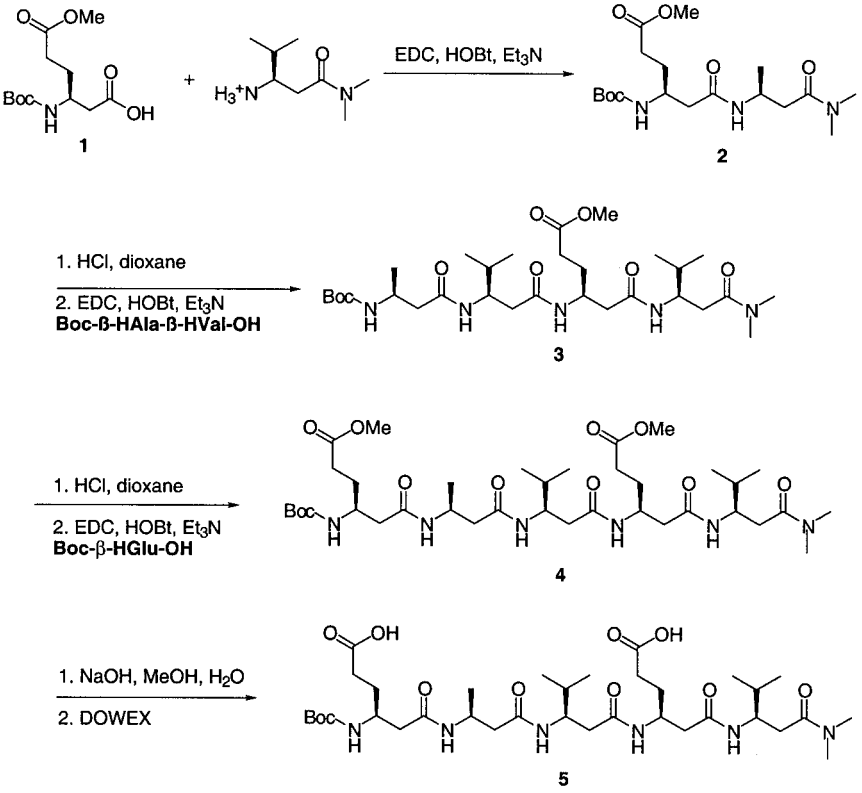
The β-sheet nucleator (**5**) was built from five β-amino acid residues. Two β-amino acid residues derived from glutamic acid (β-HGlu) are incorporated in the first and the fourth position. Their side chains serve as linkers for the attachment of two strands of natural peptides. Three hydrophobic β-amino acid residues, one derived from L-alanine and two from L-valine, were chosen to improve the solubility of the peptide in organic solvents. The C-terminus is capped with a *N*,*N*-dimethyl amide instead of a methyl ester so that the two methyl ester groups on the side chains of the two β-HGlu residues can be selectively hydrolyzed.

β-Peptide **4** was synthesized from C-terminus toward N-terminus. The C-terminus residue Boc-β-HVal-NMe_2 was prepared by using Arndt–Eistert method[27] starting with a *t*-Boc protected L-alanine using dimethyl amine as the nucleophile. After removing the Boc protecting group, the resulting H_3N^+-β-HVal-NMe_2 residue was coupled to a Boc protected glutamic acid residue, Boc-β-HGlu(OMe)-OH (**1**), using a combination of reagents including EDC and HOBt.[22] The resulting β-dipeptide (**2**) was obtained as a white solid, which was coupled to a pre-assembled β-dipeptide Boc-β-HAla-β-HVal-OH.[27] The resulting β-tetrapeptide (**3**) was soluble in $CHCl_3$ and its NMR spectrum was taken in $CDCl_3$. This was the first β-tetrapeptide in our hands to be soluble in $CHCl_3$. Most other β-terapeptides are soluble only in DMSO or DMF. After the *t*-Boc protecting group of **3** was removed by treatment with HCl in dioxane, the N-terminal glutamic acid residue, Boc-β-HGlu(OMe)-OH (**1**), was coupled to **3** to give the β-pentapeptide (**4**). The β-pentapeptide **4** was not soluble in $CHCl_3$ and precipitated at the end of the coupling reaction. After the *t*-Boc group of **4** was removed by treatment with CF_3COOH, the resulting trifluroacetate was soluble in $CHCl_3$. This is consistent with our previous observation for other oligomers of β-amino acids.[24] The structure of the trifluoroacetate of **4** was identified by NMR.

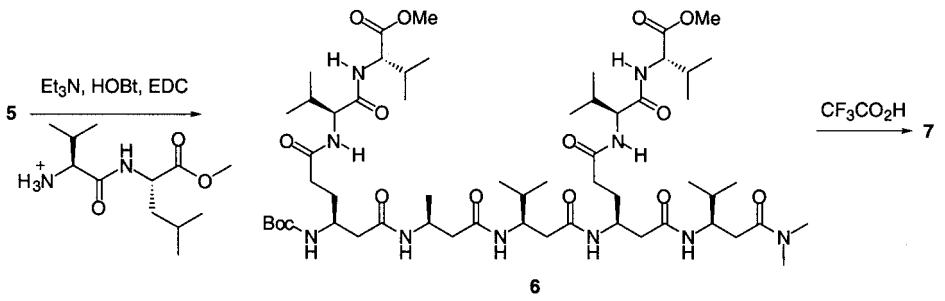
A solution of NaOH in methanol and water was used to hydrolyze the methyl ester groups on residues 1 and 4 of the β-pentapeptide **4**. After hydrolysis, the resulting diacid **5** was obtained as a white powder and was soluble in DMSO. With the desired β-peptide **5** in hand, the synthesis of the β-sheet model was successfully completed by attaching two strands of a dipeptide made of valine and leucine (NH_3^+-L-Val-L-Leu-OMe), Scheme 2.

Discussion

The neutral hybrid peptide **6** was not soluble in many solvents. However, the corresponding trifluoroacetate (**7**) obtained from a treatment of **6** with CF_3CO_2H was readily soluble in a variety of organic solvents, including chloroform and methylene chloride. A ^1H NMR spectrum was taken on a 500 MHz Bruker instrument using a solution of **7** in $CDCl_3$ (~0.5 mM) containing 5% DMSO($-d_6$). All nine amide NH protons are separated between 7.4 and 8.8 ppm (Fig. 1). This ^1H NMR spectrum with distinct NH proton peaks indicates that aggregation was minimal. The downfield shift of the amide NH protons is an indication that hydrogen bonds are present. Because the sample appears to be free of aggregation, the hydrogen bonds present should



Scheme 1.



Scheme 2.

be intramolecular rather than intermolecular. Two-dimensional NMR methods including COSY, TOCSY, and NOESY (see supporting materials) on a 500 MHz machine allowed us to assign all signals in the ^{1}H NMR spectra to specific residues (Table 1).

The large coupling constants observed for the NH protons of residues β-HAla[2], β-HVal[3], and β-HGlu[4], are consistent with a stable 3_{14} helical conformation in the main chain of the peptide. The α-CH proton is *anti* to the NH proton in 3_{14} helices of β-peptides and hence the large coupling

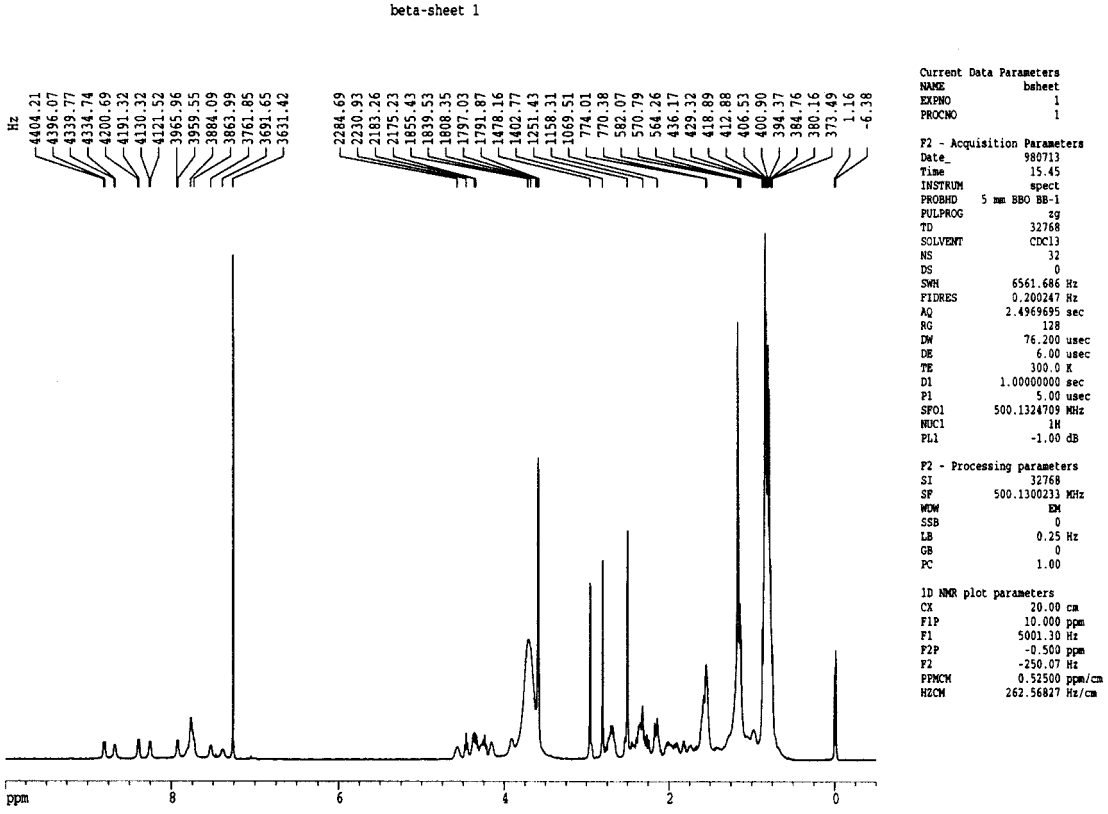


Figure 1. 500 MHz ^{1}H NMR spectrum for peptide **7** in $CDCl_3$.

Table 1. ^{1}H NMR chemical shifts (ppm, 5% DMSO in $CDCl_3$) for the β-sheet model **7**

Residue	β-HGlu[1]	β-HAla[2]	β-HVal[3]	β-HGlu[4]	β-HVal[5]	α-Val[6]	α-Leu[7]	α-Val[8]	α-Leu[9]
NH, NH_3	7.77 Broad	8.4 *J*=9.3 Hz	8.26 *J*=8.89.3 Hz	8.81 *J*=8.19.3 Hz	8.68 *J*=5.09.3 Hz	7.73	7.52	7.38	7.93 *J*=6.49.3 Hz
H–C(NH)	3.71	4.57	3.9	4.25	4.19	4.46	4.37	4.26	4.35
CH_2(CO)	2.50 2.78	2.26 2.76	2.32 2.34	2.36 2.57	–	–	–	–	–
$CH(Me)_2$	–	–	1.54	–	1.85	1.95	–	2.05	–
CH_2	2.32	–	–	2.14	–	–	–	–	–
CH_3O	–	–	–	–	–	–	3.58	–	3.59
CH_3(CN)	–	1.16	–	–	–	–	–	–	–
$(CH_3)_2N$	–	–	–	–	2.8, 2.96	–	–	–	–

Table 2. NOE Observed in 5% DMSO in $CDCl_3$ for the β-sheet model

Residue *x*	Proton(s)	Residue *y*	Proton(s)	Cross-peak
2	NH	5	NH	m
2	CH_3	5	H–C(NH)	m
2	CH_3	5	H–CH(C═O)	m
2	CH_3	1	H–CH(C═O)	m

for the sidechain interactions was also complicated by the overlapping peaks of the isopropyl groups in the peptide strands.

The trifluoacetate of the hybrid peptide **7** was then studied by circular dichroism (CD) spectroscopy in methanol at 0.2 mM, Fig. 3. A negative ellipticity at 216 nm, a zero-crossing at 205 nm, and a positive ellipticity at 201 nm are observed. This characteristic of the CD absorption did not change when the concentration of the sample was varied from 0.1 to 1.0 mM. The CD spectrum of **7** in methanol is consistent with a β-sheet structure.[11] However, we are aware the fact that helical β-peptides also give similar CD spectra.[22] Therefore, although the CD spectrum does not provide evidence against a β-sheet structure, it cannot be used to confirm a β-sheet structure.

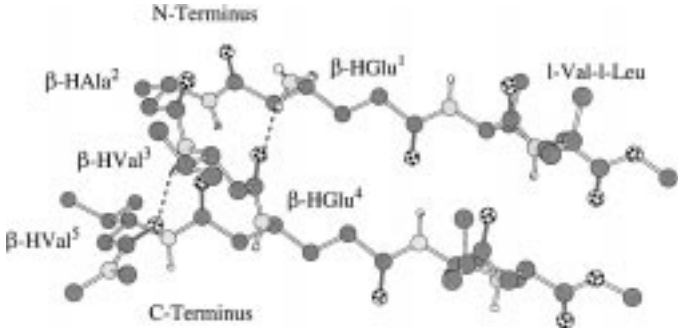


Figure 2. Model of hybrid peptide **7**. The graphic is generated by Chem3D Pro and the energy of the structure is minimized using MM2 force field. All C-bonded hydrogen atoms have been omitted for clarity.

constants. The smaller coupling of the C-terminal residue (β-$HVal^5$) suggests fraying of the helix at the C-terminus.

The 3_{14} helical conformation of the mainchain is also supported by the cross NOE peaks observed in the NOESY spectrum (Table 2). NOE cross peaks were observed between the NH proton of the main chain residue 2 (β-$HAla^2$) and residue 5 (β-$HVal^5$) and also between the methyl protons of residue 2 and the α- and β-CH protons of residue 5. As a visual aid, a 3-D model is shown in Fig. 2 for the suggested conformation of the hybrid peptide **7**. Given the relative small size of this hybrid peptide, the depicted conformation should be considered as one major contributor among many other similar conformations.

An accurate coupling constant reading could not be achieved for the NH protons of the dipeptide L-Val-L-Leu due to broadened peaks in the ^1H NMR spectrum. This broadening of the L-Val-L-Leu NH proton peaks suggests a dynamic equilibrium between H-bonded and free NH species in this region. Identification by NOE cross peaks

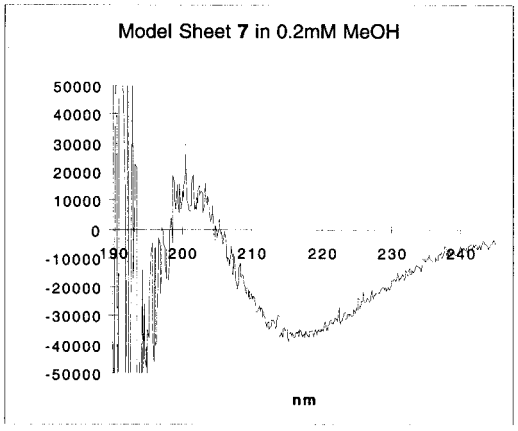


Figure 3. CD Spectrum of peptide **7** in MeOH. Molar ellipticity [Θ] in 10° cm^2 mol^{-1}.

In summary, we have successfully synthesized a hybrid peptide by attaching two strands of a natural peptide to a five-residue β-peptide nucleator. We have shown that the five-residue β-peptide in the mainchain maintains a 3_{14} helical conformation. However, the sidechains of the attached natural peptides appear to be in a dynamic equilibrium. This result opens a new possibility for larger β-sheet to be synthesized and studied. In our future plan, the main chain β-peptides will have longer chain length to improve the stability of the 3_{14} helix. β-Amino acid residues with a covalent stabilizing linker, such as *trans*-2-ACHC,[23] may be incorporated into the main chain to enhance the stability of the 3_{14} helix. The attached natural peptide strands in our future plan should have different α-amino acid residues to facilitate the identification of the conformation by NMR spectroscopy.

Experimental

THF was freshly distilled from sodium and benzophenone under N_2. Hexanes were freshly distilled from calcium hydride under N_2. Routine ^1H NMR spectra were obtained on a Bruker Avance-200 MHz and 300 MHz spectrometers with XWIN-NMR and ICON-NMR for Iris version 2.0 software. Commercially obtained starting materials were used without further purification. Column chromatography was performed using 230–400 mesh silica gel from VWR Scientific as the stationary phase. Columns were eluted

with mixtures of EtOAc and hexanes with up to 5-psi air pressure. All glassware was dried in an oven at 120°C.

For the characterization of the hybrid peptide **7**, two-dimensional NMR experiments were performed using $CDCl_3$ as solvent at room temperature on a Bruker Avance-500 MHz spectrometer. COSY(500 MHz, 95% $CDCl_3$ and 5% DMSO-d_6) spectra were run with pulse program 'cosy90'; NOESY (500 MHz, $CDCl_3$ and 5% DMSO-d_6) spectra were run with pulse program 'noesytp' and a mixing time (D8) of 300 ms was used; TOCSY (500 MHz, $CDCl_3$ and 5% DMSO-d_6) spectra were run with pulse program 'mlevgpst19.cec'.

Circular dichroism (CD) spectra were acquired on a Jasco 715 CD spectrometer, using a 0.1-cm length cell. Stock peptide solutions were prepared by weighing sufficient peptide to give 1 mL of 1 mM solution. Samples were prepared by diluting the stock solution with methanol to a concentration of 0.2 mM. All experiments were run at room temperature. Typically 20 scans were acquired over the wavelength range of 190–250 nm with a resolution of 1 nm. Unless specifically noted otherwise a sensitivity of 50 mdeg and a response time of 64 ms were used. The speed of scanning was set to 500 nm/min.

t-Boc-β-HVal-NMe$_2$

The *t*-Boc-protected L-valine diazoketone[22] (2 g, 8.3 mmol) was dissolved in 10 mL of THF and put into a sealed tube. The sealed tube was cooled to −78°C by a dry-ice/acetone bath. Dimethylamine (5 mL) was allowed to condense into the tube. A solution of 0.28 g of AgOBz in 8 mL of Et_3N was added to the mixture. The sealed tube was covered with aluminum foil and stirred at room temperature overnight. The excess dimethylamine was evaporated by stirring at room temperature in a fume hood and the volatile solvent was removed under reduced pressure. *t*-Boc-β-HVal-NMe$_2$ was obtained as an oil (2.0 g, 7.75 mmol, 93%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.81–0.90 (m, 6H, *i*-Pr), 1.35 (s, 9H, *t*-Bu), 2.48 (m, 2H, CH_2), 2.86 (s, 3H, NCH_3), 2.96 (s, 3H, NCH_3), 3.52 (m, 1H, CH), 5.35 (d, 1H, J=10 Hz, NH).

t-Boc-β-HGlu(OMe)-OH (1). A mixture of AgOAc (0.097 g, 0.58 mmol) in 2.1 mL of Et_3N was added into a solution of the *t*-Boc protected L-glutamic diazoketone (1.5 g, 5.3 mmol) in 22 mL of THF and 2.2 mL of at −25°C in the dark. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solution was extracted by aq $NaHCO_3$ and the aq solution was adjusted to pH=2. After the solution was extracted by Et_2O and the solvent was evaporated, the *t*-Boc-β-HGlu(OMe)-OH was obtained as a white solid (0.94 g, 65%), mp 92°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.42 (s, 9H, *t*-Bu), 1.88 (m, 2H, CH_2), 2.39 (m, 2H, CH_2), 2.57 (m, 2H, CH_2), 3.66 (s, 3H, OMe), 3.92 (m, 1H, CH), 5.02 (d, 1H, J=10 Hz, NH). Anal. for $C_{12}H_{21}NO_6$: calcd C 52.35, H 7.69, N 5.09; found C 52.19, H 7.78, N 4.98.

t-Boc-β-HGlu(OMe)-HVal-NMe$_2$ (2). In an oven-dried round bottom flask, Boc-β-HVal-NMe$_2$ (0.9 g, 3.5 mmol) was dissolved in 8 mL of HCl/dioxane (4 M) at 0°C. The mixture was allowed to warm to room temperature and stirred for 1.5 h. The solvent was evaporated under reduced pressure and a white solid was obtained. The residue was dried under high vacuum for 30 min. The HCl salts were used without further purification. In an oven-dried round bottom flask, the resulting HCl salt was dissolved in 8 mL of dry $CHCl_3$ and cooled to 0°C. After stirred for 5 min, 17.5 mmol of Et_3N was injected by syringe and the mixture was stirred for 10 min. To this solution, 4.2 mmol of HOBt, a solution of the Boc-β-HGlu(OMe)-OH (**1**) (0.9 g, 3.5 mmol) in 8 mL of dry $CHCl_3$ and 4.2 mmol of EDC were added successively. The mixture was allowed to warm to room temperature and stirred for 16 h. The mixture was diluted with 50 mL of $CHCl_3$ and washed with 100 mL of 1N HCl solution three times followed by 100 mL of saturated $NaHCO_3$ and 100 mL of saturated NaCl solution. The organic phase was dried with anhydrous $MgSO_4$ and the solvent was evaporated under reduced pressure. After purification by flash column chromatograph using 50% EtOAc in hexane, the corresponding dimer Boc-β-HGlu(OMe)-β-HVal-NMe$_2$ (**2**) was obtained as a solid (1.29 g, 3.11 mmol, 89%). Mp 133–136°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.82–0.89 (m, 6H, *i*-Pr), 1.61 (s, 9H, *t*-Bu), 1.83–1.91 (m, 1H, *i*-Pr), 1.91–2.00 (m, 2H, CH_2), 2.29–2.38 (m, 2H, CH_2), 2.41 (m, 2H, CH_2), 2.50 (m, 2H, CH_2), 2.86 (s, 3H, NCH_3), 2.96 (s, 3H, NCH_3), 3.60 (s, 3H, OCH_3), 3.81–3.88 (m, 4H, CH), 5.45 (d, 1H, J=7.6 Hz, NH), 6.77 (d, 1H, J=9.2 Hz, NH). Anal. for $C_{18}H_{33}N_3O_6$: calcd C 55.80, H 8.58, N 10.84; found C 55.76, H 8.69, N 10.76.

t-Boc-β-HAla-β-HVal-β-HGlu(OMe)-β-HVal-NMe$_2$ (3). The *t*-Boc protecting group of Boc-β-HGlu(OMe)-β-HVal-NMe$_2$ (**2**) (1.29 g, 3.11 mmol) was removed by treatment with HCl in dioxane (4 M) according to the procedure described above. The resulting compound was coupled to Boc-β-HAla-β-HVal-OH[27] (0.98 g, 3.11 mmol) according to the coupling procedure described above. The corresponding tetramer *t*-Boc-β-HAla-β-HVal-β-HGlu(OMe)-β-HVal-NMe$_2$ (**3**) was obtained as a solid (1.23 g, 2.01 mmol, 65%). Mp 193–196°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85–0.93 (m, 12H, *i*-Pr), 1.17–1.20 (m, 3H, CH_3), 1.39 (s, 9H, *t*-Bu), 1.75–2.00 (m, 4H, CH_2), 1.86 (m, 2H, *i*-Pr), 2.30–2.36 (m, 4H, CH_2), 2.53–2.55 (m, 2H, CH_2), 2.89 (s, 3H, NCH_3), 3.63 (s, 3H, OCH_3), 3.93–4.00 (m, 4H, CH), 5.4 (d, 1H, J=6 Hz, NH), 6.7 (d, 1H, J=15 Hz, NH), 7.14 (d, 1H, J=9 Hz, NH), 7.25 (d, 1H, J=9 Hz, NH). Anal. for $C_{30}H_{55}N_5O_8$: calcd C 58.70, H 9.03, N 11.41; found C 58.65, H 9.12, N 11.21.

t-Boc-β-HGlu(OMe)-β-HAla-β-HVal-β-HGlu(OMe)-β-HVal-NMe$_2$ (4). The *t*-Boc protecting group of *t*-Boc-β-HAla-β-HVal-β-HGlu-β-HVal-NMe$_2$ (**3**) (1.3 g, 2.01 mmol) was removed by treatment with HCl in dioxane according to the procedure described above. The resulting compound was coupled to Boc-β-HGlu(OMe)-OH (**1**) (0.61 g, 2.21 mmol) according to the coupling procedure described above. The corresponding pentamer *t*-Boc-β-HGlu(OMe)-β-HAla-β-HVal-β-HGlu(OMe)-β-HVal-NMe$_2$ (**4**) was obtained as a solid (800 mg, 1 mmol, 50%). Mp 232°C. Anal. for $C_{37}H_{66}N_6O_{11}$: calcd C 57.64, H 8.63, N 10.90; found C 57.56, H 8.72, N 10.86. Upon treatment with CF_3CO_2H, the corresponding trifluoroacetate became soluble in organic solvents. ^{1}H NMR (300 MHz, DMSO-*d*): δ 0.83–0.89 (m, 12H, *i*-Pr), 1.23–1.28 (m, 3H, CH_3),

2.33–2.67 (m, 10H, CH_2), 3.01 (s, 3H, NCH_3), 2.85 (s, 3H, NCH_3), 3.62 (s, 3H, OMe), 3.73 (s, 3H, OMe), 3.9–4.2 (m, 5H, CH), 7.6–7.7 (m, 3H, NH), 8.45 (d, J=9.9 Hz, 1H, NH), 8.7 (br, 1H, NH).

***t*-Boc-β-HGlu(OH)-β-HAla-β-HVal-β-HGlu(OH)-β-HVal-NMe_2 (5).** In a round bottom flask, the pentapeptide (100 mg, 0.13 mmol) (**4**) was dissolved in 9 mL of MeOH at room temperature. An aqueous solution of NaOH (4.5 mL, 5 M) was added. The reaction mixture was heated to 70°C with an oil bath and stirred for 20 h. The reaction flask was removed from the oil bath and DOWEX 50WX8-100 ion-exchange resin (washed with MeOH three times) was added until pH=7. The mixture was filtered and the filtrate was concentrated under reduced pressure to give a wet residue, which was dried by the addition and co-evaporation with MeOH until a white solid was obtained. The residue was put under high vacuum overnight and the corresponding diacid *t*-Boc-β-HGlu(OH)-β-HAla-β-HVal-β-HGlu(OH)-β-HVal-NMe_2 (**5**) was obtained as a solid (95 mg, 0.128 mmol, 98%). Mp 213°C. NMR (300 MHz, DMSO-*d*): δ 0.83 (m, 12H, *i*-Pr), 1.04 (d, J=6.5 Hz, 3H, CH_3), 1.42 (s, 9H, *t*-Bu), 1.5–1.6 (m, 1H, *i*-Pr), 1.65–1.9 (m, 4H, CH_2), 2.14–2.32 (m, 8H, CH_2), 2.42 (m, 2H, CH_2), 2.83 (s, 3H, NCH_3), 3.02 (s, 3H, NCH_3), 3.78–4.04 (m, 5H, CH), 6.73 (d, 1H, J=8.7 Hz, NH), 7.61–7.67 (br, 3H, NH), 7.81 (d, 1H, J=8.1 Hz, NH). Anal. for $C_{35}H_{62}N_6O_{11}$: calcd C 56.59, H 8.41, N 11.31; found C 56.41, H 8.53, N 11.12.

***t*-Boc-β-HGlu(l-Val-l-Leu-OMe)-β-HAla-β-HVal-β-HGlu(l-Val-l-Leu-OMe)-β-HVal-NMe_2 (6).** The pentapeptide (**5**) (100 mg, 0.13 mmol) was dissolved in DMF and a $CHCl_3$ solution of 2.5 equiv. of the dipeptide L-Val-L-Leu was added. Three equivalents of EDC and HOBt were added and the reaction mixture was stirred at room temperature for 16 h. The reaction mixture started as a clear solution and became a gel-like mixture after 16 h of stirring. The solvent was removed under reduced pressure and a solid was obtained, which was washed with water and MeOH successively. After MeOH was removed by filtration, a white solid (**6**) was collected on the filter and put under high vacuum for 24 h (85 mg, 0.73 mmol, 56%). Mp 273°C (decompose). Anal. for $C_{57}H_{102}N_{10}O_{15}$: calcd C 58.64, H 8.81, N 12.00; found C 58.53, H 8.90, N 11.88. The neutral hybrid peptide **6** was not soluble in many solvents. However, the corresponding trifluoroacetate (**7**) obtained from a treatment of 6 with CF_3CO_2H is readily soluble in a variety of organic solvents. A ^1H NMR spectrum was taken on a 500 MHz Bruker instrument using a solution of 7 in $CDCl_3$ containing 5% DMSO-d_6. ^1H NMR (500 MHz, $CDCl_3$): δ 0.76–0.85 (m, 12H, *i*-Pr), 1.19 (m, 3H, CH_3), 1.58 (m, 2H, CH), 1.7–2.53 (m, 18H, CH_2), 2.83 (s, 3H, NCH_3), 2.98 (s, 3H, NCH_3), 4.1–4.65 (m, 5H, CH), 7.4 (d, 1H, J=5 Hz, NH), 7.55 (d, 1H, J=3.2 Hz, NH), 7.8 (m, 2H, NH), 7.95 (d, 2H, J=6.4 Hz, NH), 8.25 (d, 1H, J=8.8 Hz, NH), 8.4 (d, 1H, J=9.4 Hz, NH), 8.7 (d, 1H, J=5 Hz, NH), 8.8 (d, 1H, J=8.1 Hz, NH).

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