Solid-Supported Synthesis of Bicyclic Peptides Containing Three Parallel Peptide Chains

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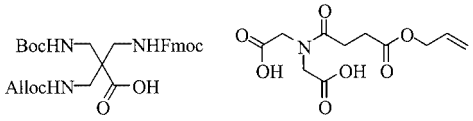
Four homodetic bicyclic peptides **9a**–**d**, containing three parallel peptide chains, were synthesized on a hydroxymethyl-functionalized polystyrene support. α,α-Bis(aminomethyl)-β-alanine, bearing orthogonal protections (Alloc, Boc, Fmoc) on the three amino groups (**1**), was attached to the support through an H_2N-Leu-Leu-Gly-OH spacer, and the peptide chains were assembled on the amino groups of **1** by either a stepwise coupling or coupling of a segment, keeping the amino protection within each chain unchanged. *N*-(4-Allyloxy-4-oxobutanoyl)iminodiacetic acid (**2**) was then coupled to the Fmoc-protected chain. The Boc protecting group was removed, and the exposed amino group was coupled with the remaining free carboxylic acid function of **2**. Finally, the Alloc and allyl ester protections were removed from the carboxylic and amino functions, and a second cyclization was performed. Release from the support gave **9a**–**d** as free carboxylic acids.

Introduction

Conformational rigidity may give biologically active homodetic cyclic peptides increased potency, receptor selectivity, metabolic stability and bioavailability.[1−4] Additional cyclization resulting in bicyclic homodetic peptides increases the rigidity further making such peptides interesting subjects of research.[5−9] In spite of this appeal, relatively few syntheses of homodetic bicyclic peptides either in solution[10−14] or on a solid support[15−17] have been described. Recently, we have widened their scope by reporting a synthesis of homodetic bicyclic spiropeptides on a solid support.[18]

To find out whether libraries of homodetic bicyclic peptides containing three parallel amino acid chains could be prepared conveniently on a solid support, we have synthesized four such peptides on hydroxymethyl-functionalized polystyrene. These peptides consist of two different branching units, viz. α,α-bis(aminomethyl)-β-alanine (cf. **1** in Figure 1) and *N*-succinyliminodiacetic acid (cf. **2**), used to cap the carboxylic and amino termini of the peptide chains, respectively. The synthesis is initiated by assembling the peptide chains one after another on the three orthogonally protected amino groups of solid-supported α,α-bis(aminomethyl)-β-alanine; *N*-succinyliminodiacetic acid is then coupled to one of the chains and cyclizations with the remaining two branches are carried out on the support. Release of the bicyclic peptides from the solid support as free carboxylic acids makes them suitable for possible further conjugation.



1 **2**

Figure 1. Branching units used in the synthesis of bicyclic peptides

Results and Discussion

Synthesis of Building Blocks

The preparation of the orthogonally protected α,α-bis(aminomethyl)-β-alanine branching unit **1**, used as a solid-supported scaffold for the peptide synthesis, has been described previously.[19] The second branching unit **2**, used to cap the amino termini of the peptide chains, was synthesized as depicted in Scheme 1. Succinic acid monoallyl ester[20] and the di-*tert*-butyl ester of iminodiacetic acid[21] were condensed by HOAt/DCC[22] activation. Subsequent removal of the *tert*-butyl protecting groups gave **2** in a moderate 40% overall yield. The amino-protected dipeptides were obtained by established methods based on HOSu/DCC chemistry.

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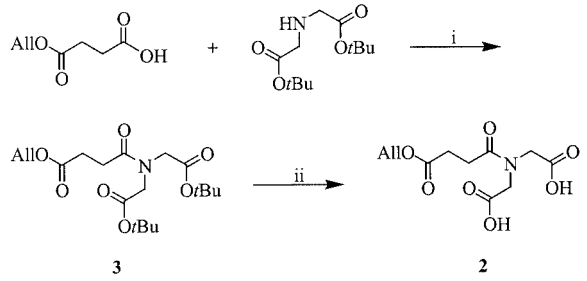


Scheme 1. Synthesis of the branching unit **2**: (i) HOAt, DCC, Py, DMF; (ii) TFA, CH_2Cl_2

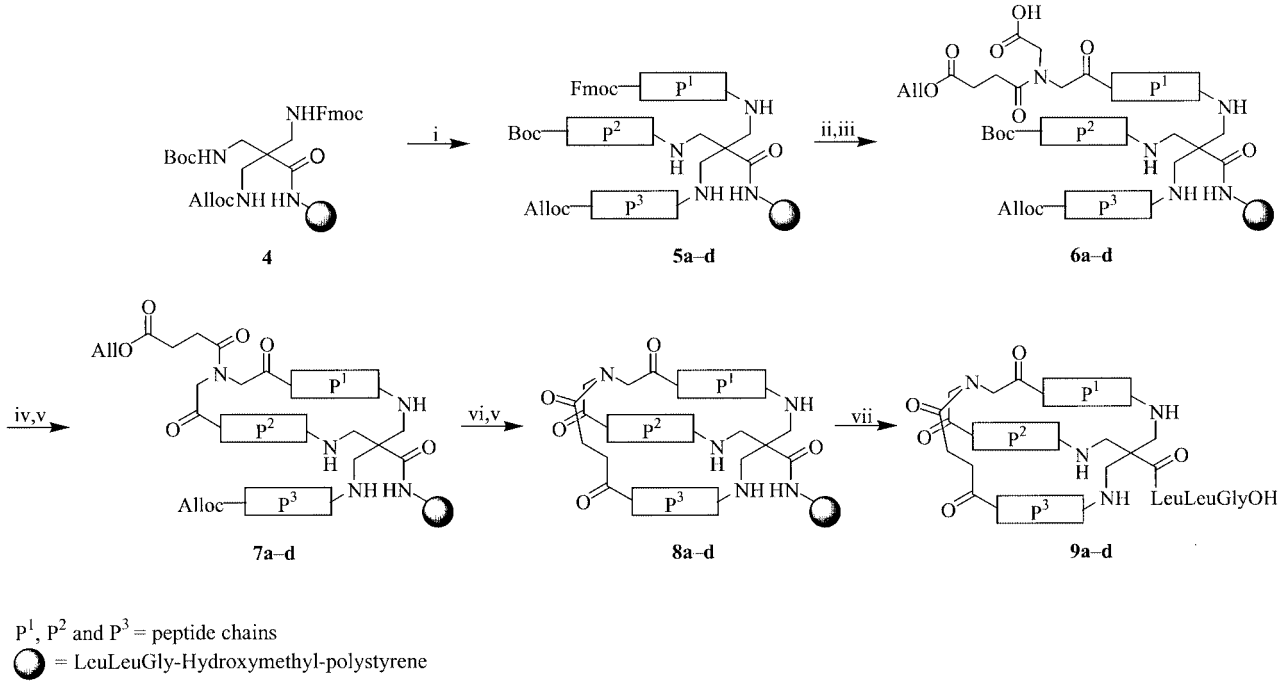
Table 1. Dipeptide branches used for the synthesized peptides (**5**–**9**, cf. Scheme 2)

Entry	P^1	P^2	P^3
5–**9a**	β-Ala-Gly	Gly-β-Ala	Phe-β-Ala
5–**9b**	Ser-β-Ala	Gly-β-Ala	Phe-β-Ala
5–**9c**	Ala-Gly	Asn-Gly	Phe-β-Ala
5–**9d**	Gly-Ala-Gly	Gly-Asn-Gly	Phe-β-Ala

Solid-Phase Synthesis of Bicyclic Peptides

The hydroxymethyl-functionalized polystyrene was first derivatized with an H_2N-Leu-Leu-Gly-OH spacer.[23] Accordingly, glycine was attached to the resin by the symmetrical anhydride method, and the chain was elongated by two leucine residues using Fmoc chemistry with HATU/DIEA[24,25] activation. The branching unit **1** was then coupled similarly to obtain tetrapeptide **4** (Scheme 2) bearing three orthogonally protected amino groups. Two approaches were used for the construction of the parallel peptide chains on these amino functions, which can be deprotected one after another. With peptides **5a** and **5b** (Table 1), the chains were introduced as dipeptide segments. With peptides **5c** and **5d**, the chains on the Fmoc- and Boc-protected amino groups were assembled by stepwise coupling using Fmoc- and Boc-protected amino acids, respectively. The Alloc-protected branch P^3 was introduced as a segment. Both approaches gave satisfactory coupling yields, but segment coupling was considered safer and more reproducible for manual SPPS since the number of steps on the solid support was smaller.

After completing the chain assembly, the Fmoc protection of chain P^1 was removed. The second branching unit **2** was converted into a cyclic anhydride with EDAC activation, and allowed to react with the exposed amino group. In this manner, protection of the carboxymethyl groups could be avoided. Cleavage of the Boc protecting group from chain P^2 then enabled the first cyclization reaction, which was performed by HATU/DIEA activation. After reaction at room temperature for 5 h, no starting material could be detected. Because **2** is not chiral, there is no risk for epimerization even though the cyclization is slow.[26] While the cyclization of **6a** and **6b**, which contain β-alanine residues in both of the peptide chains participating in the reaction, proceeded well, the corresponding reaction with **6c** and **6d**, having a glycine residue in place of β-alanine, yielded a more complex product mixture. The fact that 1,1,3,3-tetramethylguanidino derivatives[27] could be detected among the side products led us to use a phosphonium coupling reagent for the second cyclization with



Scheme 2. Synthesis of bis(aminomethyl)-β-alanine conjugates: (i) SPPS; (ii) piperidine, DMF; (iii) *N*-(4-allyloxy-4-oxobutanoyl)iminodiacetic anhydride, DMF; (iv) TFA, CH_2Cl_2; (v) HATU, DIEA, DMF; (vi) $Pd(OAc)_2$, PPh_3, Bu_3SnH, AcOH, CH_2Cl_2; (vii) 30% HBr/AcOH, thioanisole, pentamethylbenzene, TFA

these peptides. The Alloc and allyl ester protecting groups were removed simultaneously from the monocyclic peptides using palladium/tributyltin hydride chemistry.[28] The second cyclization to **8a** and **8b** was performed over 16 h using similar HATU/DIEA chemistry as before. To obtain **8c** and **8d**, HATU was replaced with PyBOP[20] and the solvent was changed from DMF to a 1:4 mixture of DMSO and NMP. Furthermore, the reaction time was extended to 22 h. The cyclization reaction was repeated with **7d** since not all the starting material had disappeared during the reaction. In spite of these changes, the second cyclization reaction of **7c** and **7d** led to a complex reaction mixture and markedly decreased yield, probably because of polymerization on the solid support. Acid-catalysed cleavage from the support gave **9a**−**d** as free carboxylic acids. Accordingly, the bicyclic peptides bear a functionalized side arm that may be exploited for further conjugation in solution.

All four peptides were obtained in pure states by HPLC, but in low yields. Only peptide **9a**, which had the simplest amino acid composition, was obtained as a reasonably pure crude product. Owing to the chiral centre of branching unit **1**, the compound appeared as a pair of diastereoisomers that could be separated by HPLC (Figure 2). In addition to these four bicyclic peptides, two attempts were made, unsuccessfully, to synthesize a bicyclic peptide having a smaller ring size and no β-alanine residues. Accordingly, the methodology described seems to allow preparation of homodetic bicyclic peptides on a solid support, but its applicability to library synthesis is severely limited. Only in favourable cases is the crude product reasonably pure, and usually HPLC purification is needed.

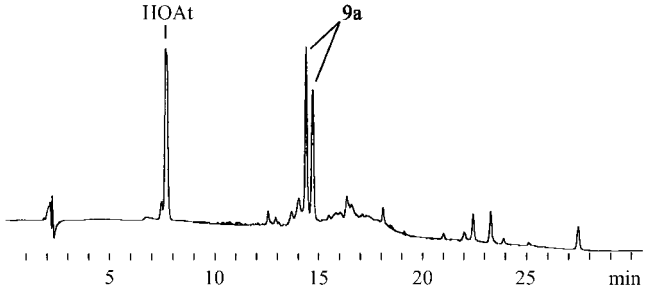


Figure 2. Chromatogram of the crude diastereoisomeric pair of **9a** recorded by using a Hypersil HyPurity Elite C18 column (150 × 4.6 mm, 5 μm), applying a linear gradient from 0.1% aq. TFA to acetonitrile over 30 min at a flow rate of 1.0 mL/min; the detection wavelength was 215 nm

Experimental Section

General Remarks: The NMR spectra were recorded with a JEOL JNM-GX 400 or JNM-A 500 spectrometer. The chemical shifts are given in ppm downfield from that of internal $(CH_3)_4Si$. The mass spectra of low-molecular-weight compounds were recorded with a VG 7070E mass spectrometer. Finnigan Mat Lasermat was used for mass analyses of reactions on solid support. For high-resolution mass spectrometry of bicyclic peptides, an Applied Biosystems Mariner 5272 mass spectrometer was used. RP HPLC analyses and separations were performed with a Hypersil HyPurity Elite C18 column (150 × 4.6 mm, 5 μm), applying a linear gradient from 0.1% aq. TFA to acetonitrile over 30 min at a flow rate of 1.0 mL/min. The detection wavelength was 215 nm. For abbreviations used see ref.[22]

Di-*tert*-butyl *N*-(4-Allyloxy-4-oxobutanoyl)iminodiacetate (3): Monoallyl succinate (2.9 g, 18 mmol), HOAt (2.6 g, 19 mmol), DCC (3.9 g, 19 mmol), di-*tert*-butyl iminodiacetate (4.2 g, 17 mmol) and pyridine (3.1 mL, 38 mmol) were stirred in DMF (50 mL) overnight. The reaction mixture was filtered and concentrated by rotary evaporation. The residue was dissolved in CH_2Cl_2 and washed with 1 M aq. AcOH and saturated aq. $NaHCO_3$. The organic phase was dried with Na_2SO_4, and the solvent was evaporated. Silica gel chromatography (EtOAc/petroleum ether, 3:7, v/v) yielded **3** (3.4 g, 48%) as a clear oil. R_f = 0.49 (EtOAc/petroleum ether, 3:7). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.45 and 1.49 [2 s, 2 × 9 H, C(CH_3)$_3$], 2.67 (m, 4 H, CH_2CH_2CO$_2$), 4.01 and 4.05 (2 s, 2 × 2 H, CH_2CO$_2$*t*Bu), 4.59 (td, *J* = 1.4, 5.7 Hz, 2 H, 4 OCH_2CH=CH$_2$), 5.22 (tdd, *J* = 1.4, 2.7, 10.4 Hz, 1 H, CH=C*H*H), 5.31 (tdd, *J* = 1.5, 3.1, 17.2 Hz, 1 H, CH=CH*H*), 5.91 (tdd, *J* = 5.7, 10.5, 17.2 Hz, 1 H, C*H*=CH$_2$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 27.6 (CH$_2$*C*H$_2$CON), 27.9 (C*C*H$_3$), 29.1 (CH$_2$*C*H$_2$CO$_2$), 48.8 and 50.7 (N*C*H$_2$CO$_2$), 65.1 (CH$_2$=CH*C*H$_2$O), 81.7 and 82.6 (*C*CH$_3$), 118.0 (CH=*C*H$_2$), 132.1 (*C*H=CH$_2$), 167.8 and 168.3 (*C*O$_2$*t*Bu), 171.7 (*C*O$_2$All), 172.3 (*C*ON) ppm. MS (EI$^+$): *m*/*z* = 385 [M$^+$] (0.1), 329 (6), 328 (5), 312 (12), 273 (46), 256 (22), 228 (47), 141 (100), 57 (72). HRMS (EI$^+$): $C_{19}H_{31}NO_7$ requires 385.2101; found 385.2101 [M$^+$].

***N*-(4-Allyloxy-4-oxobutanoyl)iminodiacetic Acid (2):** Compound **3** (3.4 g, 8.8 mmol) was stirred in a mixture of TFA and CH_2Cl_2 (2:1, 21 mL) for 30 min. The reaction mixture was concentrated by rotary evaporation. The crude product was purified by silica gel chromatography (MeOH/CH_2Cl_2, 1:9) to give **2** (1.9 g, 79%) as a clear viscous oil. R_f = 0.28 (MeOH/CH_2Cl_2, 1:9). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.52 (m, 4 H, CH_2CH_2CO$_2$), 3.96 and 4.18 (2 s, 2 × 2 H, NCH_2CO$_2$), 4.51 (td, *J* = 1.5, 5.3 Hz, 2 H, OCH_2CH=CH$_2$), 5.18 (tdd, *J* = 1.4, 2.8, 10.5 Hz, 1 H, CH=C*H*H), 5.29 (tdd, *J* = 1.7, 3.4, 17.2 Hz, 1 H, CH=CH*H*), 5.88 (tdd, *J* = 5.3, 10.5, 17.3 Hz, 1 H, C*H*=CH$_2$), 12.70 (br. s, 1 H, CO$_2$*H*) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 27.1 (CH$_2$*C*H$_2$CON), 28.7 (CH$_2$*C*H$_2$CO$_2$), 48.2 and 49.7 (N*C*H$_2$CO$_2$), 64.3 (CH$_2$=CH*C*H$_2$O), 117.5 (CH=*C*H$_2$), 132.7 (*C*H=CH$_2$), 170.6 and 170.8 (*C*O$_2$H), 171.7 (*C*O$_2$All), 172.3 (*C*ON) ppm. MS (EI$^+$): *m*/*z* = 273 [M$^+$] (11), 255 (69), 229 (56), 216 (12), 141 (34), 101 (7), 41 (100). HRMS (EI$^+$): $C_{11}H_{15}NO_7$ requires 273.0849; found 273.0846 [M$^+$].

***N*-[*N*-(Allyloxycarbonyl)phenylalanyl]-β-alanine (10):** *N*-Fmoc-phenylalanine (1.1 g, 2.8 mmol), DCC (0.68 g, 3.3 mmol) and HOSu (0.38 g, 3.3 mmol) were dissolved in cooled dioxane and stirred for 2 h and the filtered. β-Alanine *tert*-butyl ester hydrochloride (0.50 g, 2.8 mmol) and K_2CO_3 (0.38 g, 2.8 mmol) were dissolved in aq. dioxane, and then added to the filtered solution. After stirring overnight, the solvent was evaporated. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with half-saturated brine. The organic layer was dried with Na_2SO_4 and the solvent was evaporated. The crude *N*-(*N*-Fmoc-phenylalanyl)-β-alanine *tert*-butyl ester was purified by silica gel chromatography (EtOAc/petroleum ether, 6:4). The Fmoc protecting group was removed by stirring for 30 min in piperidine/CH_2Cl_2 (1:4). The volatile components were removed by rotary evaporation. The product was purified by silica gel chromatography (EtOAc/petroleum ether, 3:7, then MeOH/CH_2Cl_2, 1:19), which gave *N*-phenylalanyl-β-alanine *tert*-butyl ester in an overall yield of 84% (0.69 g). The latter compound (0.68 g, 2.3 mmol) was dissolved in dioxane and NaOH (2.8 mmol) in H_2O (10 mL) was added. Allyl chloroformate (0.42 g, 2.8 mmol), diluted with diox-

ane (1.0 mL) was slowly added to the stirred reaction mixture. After the reaction had reached completion, the mixture was concentrated. The residue was dissolved in CH_2Cl_2 and washed with water. The organic layer was then dried with Na_2SO_4, the solvent was evaporated, and the residue was purified by silica gel chromatography (EtOAc/petroleum ether, 3:7), yielding *N*-(*N*-alloc-phenylalanyl)-β-alanine *tert*-butyl ester (0.78 g, 90%). The product was stirred for 45 min in TFA/CH_2Cl_2 (1:1; 8 mL), after which time the CH_2Cl_2 and some TFA was evaporated, and some diethyl ether was added. The product was left to crystallize in a refrigerator overnight. The crystals that formed were suction-filtered and washed with cold diethyl ether. Silica gel chromatography (MeOH/CH_2Cl_2, 1:9) gave *N*-[*N*-(allyloxycarbonyl)phenylalanyl]-β-alanine (0.45 g, 68%). R_f = 0.47 (MeOH/CH_2Cl_2, 1:9). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.33 (t, *J* = 6.8 Hz, 2 H, C*H*$_2$CO$_2$), 2.71 (dd, *J* = 10.3, 13.7 Hz, 1 H, C*H*HPh), 2.91 (dd, *J* = 4.5, 13.7 Hz, 1 H, CH*H*Ph), 3.24 (m, 2 H, C*H*$_2$CON), 4.14 (m, 1 H, NC*H*CO$_2$), 4.36 (d, *J* = 5.1 Hz, 2 H, OC*H*$_2$CH=CH$_2$), 5.11 (dd, *J* = 1.3, 10.5 Hz, 1 H, CH=C*H*H), 5.18 (dd, *J* = 1.7, 17.3 Hz, 1 H, CH=CH*H*), 5.81 (tdd, *J* = 5.1, 10.5, 17.3 Hz, 1 H, C*H*=CH$_2$), 7.15−7.25 (m, 5 H, Ar), 7.39 (d, *J* = 8.5 Hz, 1 H, Phe NH), 8.04 (t, *J* = 5.4 Hz, 1 H, β-Ala NH), 12.17 (br. s, CO$_2$*H*) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 33.8 (CH$_2$*C*H$_2$N), 34.8 (CH$_2$*C*H$_2$CO$_2$), 37.6 (*C*H$_2$Ph), 56.1 (N*C*HCO), 64.3 (CH$_2$=CH*C*H$_2$O), 116.8 (CH=*C*H$_2$), 126.2 (*C*4 Ph), 128.0 (*C*2,6 Ph), 129.1 (*C*3,5 Ph), 133.5 (*C*H=CH$_2$), 138.1 (*C*1 Ph), 155.7 (N*C*O$_2$CH$_2$), 171.4 (*C*ON), 172.9 (*C*O$_2$H) ppm. MS (EI$^+$): *m*/*z* = 320 [M$^+$] (1), 219 (8), 204 (9), 160 (9), 91 (65), 41 (100). HRMS (EI$^+$): $C_{16}H_{20}N_2O_5$ requires 320.1372; found 320.1368 [M$^+$].

***N*-(*N*-Fmoc-serinyl)-β-alanine (11):** *N*-Fmoc-*O*-*t*Bu-serine (0.84 g, 2.2 mmol), DCC (0.54 g, 2.6 mmol) and HOSu (0.30 g, 2.6 mmol) were dissolved in cooled dioxane (25 mL) and stirred for 2 h and then filtered. β-Alanine *tert*-butyl ester hydrochloride (0.40 g, 2.2 mmol) and K_2CO_3 (0.30 g, 2.2 mmol) were dissolved in aq. dioxane (4 mL), and then added to the filtered solution. After stirring overnight, the solvent was evaporated. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with half-saturated brine. The organic layer was dried with Na_2SO_4 and the solvent was evaporated. Crude *N*-(*N*-Fmoc-*O*-*tert*-butylserinyl)-β-alanine *tert*-butyl ester was purified by silica gel chromatography (EtOAc/petroleum ether, 3:7). The *tert*-butyl protecting groups were removed by stirring for 2 h in TFA, after which time the TFA was evaporated. Silica gel chromatography (MeOH/CH_2Cl_2, 1:9) gave the title compound (0.72 g, 82%). R_f = 0.28 (MeOH/CH_2Cl_2, 1:9). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.36 (m, 2 H, C*H*$_2$CO$_2$), 3.25 (m, 2 H, C*H*$_2$CON), 3.54 (m, 2 H, C*H*$_2$OH), 3.99 (m, 1 H, NC*H*CO$_2$), 4.21 (m, 1 H, Fmoc CH), 4.25 (m, 2 H, Fmoc CH$_2$), 7.32 (m, 3 H, Fmoc Ar, Ser NH), 7.41 (m, 2 H, Fmoc Ar), 7.73 (m, 2 H, Fmoc Ar), 7.88 (m, 2 H, Fmoc Ar), 7.94 (m, 1 H, β-Ala NH) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 33.9 (CH$_2$*C*H$_2$N), 34.9 (CH$_2$*C*H$_2$CO$_2$), 46.6 (*C*H Fmoc), 57.2 (N*C*HCO), 61.8 (*C*H$_2$OH), 65.8 (*C*H$_2$ Fmoc), 120.1, 125.4, 127.1, 127.7, 140.7, 143.8, and 143.9 (Ar Fmoc), 155.9 (N*C*O$_2$CH$_2$), 170.1 (*C*ON), 173.0 (*C*O$_2$H) ppm. MS (EI$^+$): *m*/*z* = 398 [M$^+$] (1), 354 (1), 178 (100), 165 (25). HRMS (EI$^+$): $C_{21}H_{22}N_2O_6$ requires 398.1478; found 398.1480 [M$^+$].

Solid-Phase Syntheses: Fmoc protecting groups were removed by treatment with piperidine/DMF (1:4) for 20 min. After deprotection, the solid support was washed with DMF, CH_2Cl_2 and MeOH. Boc protecting groups were removed by treatment with TFA/CH_2Cl_2 (1:3) for 1 h. The support was then neutralized with pyridine in CH_2Cl_2 and washed with CH_2Cl_2 and MeOH. Alloc and allyl ester deprotection was performed by palladium/tributyltin hydride chemistry.[28] Palladium(II) acetate, reduced with triphenylphosphane (6 equiv.), was used as the catalyst. HATU/DIEA activation[24,25] was utilized for coupling reactions using amino acid (2.5 equiv.), HATU (2.5 equiv.) and DIEA (5 equiv.). The resin beads were swelled with the reaction solvent before addition of the coupling reagents. The total volume of the solvent was 10 μL/mg of resin. Reaction time was 2 h. The solid support was then washed with DMF, CH_2Cl_2 and MeOH. The resin was always dried under reduced pressure after washes. Coupling reactions were monitored either by determining the Fmoc loading or by removing small resin aliquots that were cleaved and analysed by HPLC to evaluate the quality of the on-resin product. The theoretical loading was calculated by Equation (1), where *L* stands for the loading [mol g^{-1}] and Δ*M* for the change in the molecular mass of the product. The yield was then calculated by Equation (2).

$$L_{theoretical} = L_{initial}/(\Delta M \times L_{initial} + 1) \quad (1)$$

$$\text{Yield} = (L_{measured}/L_{theoretical}) \times 100\% \quad (2)$$

Synthesis of Bicyclic Peptide 9a: Hydroxymethyl-functionalized polystyrene resin was first loaded with Fmoc-glycine (loading 340 μmol g^{-1}) by the symmetrical anhydride method. The unchanged hydroxy groups were acetylated (Ac$_2$O/2,6-lutidine/1-methylimidazole/THF, 5:5:8:100; 30 min), and the support was washed with CH_2Cl_2 and MeOH. Two Fmoc-leucine units and branching unit **1** were subsequently attached in a stepwise manner to the glycine-loaded resin (120 mg, 42 μmol). Boc-Gly-β-AlaOH[30] and Fmoc-β-Ala-GlyOH[31] were coupled to the branching unit as dipeptide segments after removal of corresponding protecting groups. At this point, the Fmoc loading was 95% of the theoretical loading. The Alloc protecting group was then removed, and Alloc-Phe-β-AlaOH (**10**) was coupled. The Fmoc protecting group was removed from the branched peptide **5a**. Branching unit **2** (45 mg, 160 μmol) was dissolved in DMF and converted into its anhydride by treatment with EDAC·HCl (30 mg, 160 μmol) for 40 min. The solution was added to the peptide-derivatized solid support (130 mg, 32 μmol) that had been swollen in DMF. After 40 min of shaking, the resin was washed with DMF, CH_2Cl_2 and MeOH. This anhydride coupling was repeated. After removal of the last Boc protecting group, the first cyclization was performed by shaking the resin overnight with a mixture of HATU and DIEA (5 and 10 equiv., respectively) in DMF. Alloc and allyl ester protections were removed from the solid-supported peptide (**7a**; 110 mg, 29 μmol) and the second cyclization was carried out in a similar manner. The product was cleaved from the support with 0.1 M TBAF in THF containing 0.1 M water (6 h, 55 °C). After cleavage, the resin was rinsed with THF (5 mL), neutralized with TFA and the solvent was evaporated under reduced pressure. The cleavage reaction was repeated four times. HPLC purification yielded the two diastereoisomeric products (1.5 mg and 0.5 mg, combined yield of 4.7% calculated from the original Fmoc loading). **Diastereoisomer A:** ^{1}H NMR (500 MHz, [D$_6$]acetone/D$_2$O): δ = 0.86−0.94 (CH$_3$ Leu), 1.61−1.75 (CH$_2$, CH Leu), 2.30−2.90 (CH$_2$CO β-Ala, CH$_2$CO succinyl), 2.86 (C*H*H Phe), 3.20−4.10 (CH$_2$N β-Ala, Gly, branching unit), 3.25 (CH*H* Phe), 4.05−4.45 [CON(C*H*$_2$CO)$_2$], 4.36 (C*H*N Leu), 4.52 (C*H*N Leu), 4.55 (C*H*N Phe), 7.15−7.30 (H-Ar Phe), 7.30−7.37 (NH) and 7.80−8.15 (NH) ppm. **Diastereoisomer B:** ^{1}H NMR (500 MHz, [D$_6$]acetone/D$_2$O): δ = 0.88−0.95 (CH$_3$ Leu), 1.68−1.75 (CH$_2$, CH Leu), 2.18−2.80 (CH$_2$CO β-Ala, CH$_2$CO succinyl), 2.83 (C*H*H Phe), 3.20−4.25 (CH$_2$N β-Ala, Gly, branch), 3.37 (CH*H* Phe), 4.07−4.47 [CON(C*H*$_2$CO)$_2$], 4.29 (C*H*N Leu), 4.54 (C*H*N Leu), 4.57 (C*H*N Phe), 7.19−7.31 (H-Ar Phe),

7.34 (NH) and 7.95−8.35 (NH) ppm. HRMS (ESI$^+$): $[M + H]^+$ found 1084.5442; $C_{49}H_{73}N_{13}O_{15}$+H requires 1084.5422.

Synthesis of Bicyclic Peptide 9b: The synthesis was conducted as described for **9a**, with the exception that the initial loading was 210 μmol g^{-1}. Fmoc-Ser-β-AlaOH (**11**) was used instead of Fmoc-β-Ala-GlyOH, and the reaction time for the first cyclization was 5 h. The peptide was cleaved from the resin with acid (30% HBr in AcOH/pentamethylbenzene/thioanisole/TFA, 4:5:6:100; room temperature, 3 h). The resin was rinsed with CH_2Cl_2 and MeOH. The solvents were evaporated under reduced pressure, and the residue was extracted with EtOH/0.1% aq. TFA (1:4). The extract was washed with diethyl ether before HPLC purification. The diastereoisomers could not be separated by the HPLC system used. HRMS (ESI$^+$): $(C_{50}H_{75}N_{13}O_{16} + 2\ H)/2$ requires 557.7800; found 557.7813 $[M + 2\ H]^{2+}$.

Synthesis of Bicyclic Peptide 9c: The synthesis was conducted as described for **9a**, except that the initial loading was 440 μmol g^{-1}. Instead of segment coupling, the Fmoc- and Boc-protected branches were assembled by stepwise couplings. The first cyclization was performed by shaking the resin with a mixture of HATU and DIEA (5 and 10 equiv., respectively) in DMF for 5 h. The second cyclization was carried out by shaking the resin with PyBOP and DIEA (2 and 4 equiv., respectively) in DMSO/NMP (1:4) for 22 h. Acid was used to release the product as described for **9b**. The diastereoisomers could not be separated by the HPLC system. HRMS (ESI$^+$): $(C_{50}H_{74}N_{14}O_{16} + 2\ H)/2$ requires 564.2776; found 564.2764 $[M + 2\ H]^{2+}$.

Synthesis of Bicyclic Peptide 9d: The synthesis was conducted as described for **9c**, except that the second cyclization was repeated. The diastereoisomers could not be separated by the HPLC system. HRMS (ESI$^+$): $(C_{54}H_{80}N_{16}O_{18} + H)$ requires 1241.5909; found 1241.5861 $[M + H]^+$.

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[22] Abbreviations: Alloc, allyloxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDAC·HCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HATU, 1-[bis(dimethylamino)methylene]-1*H*-[1,2,3]triazolo[4,5-*b*]pyridinium hexafluorophosphate 3-oxide; HOAt, 1-hydroxy-7-azabenzotriazole; HOSu, *N*-hydroxysuccinimide; NMP, *N*-methylpyrrolidone; PyBOP, (1-benzotriazolyl)-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TBAF, tetrabutylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran.
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