Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Constrained amino acid β-Acc confers potency and selectivity to integrin ligands

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Abbreviations:

Acc – β-aminocyclopropanecarboxylic acid monomethyl ester, Boc – *tert*-butoxycarbonyl, DBU – 1,8-diazabicyclo[5.4.0]undec-7-ene, DEPT – distorsionless enhancement by polarization transfer, DIPEA – diisopropylethylamin, DIPSI – decoupling in the presence of scalar interactions, DMF – N,N-dimethylformamide, DMSO – dimethyl sulfoxide, EDC – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, Fmoc – fluorenylmethoxycarbonyl, HATU – *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt – 1-hydroxy-1*H*-benzotriazol, MD – molecular dynamics, MALDI-ToF MS – matrix-assisted laser desorption/ionization time of flight mass spectrometry, MEM – minimal essential medium Eagle, NMR – nuclear magnetic resonance, NOE – nuclear Overhauser effect, Pbf – 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, RP-HPLC – reversed phase HPLC, TBTU – 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, TFA – trifluoroacetic acid, TIS – triisopropylsilane. If the one-letter code is used for amino acid abbreviation, D-amino acids are symbolized by a non-capital letter.

Experimental Section

Materials and Methods

All chemicals were used as supplied without further purification. All organic solvents were distilled before use. Fmoc amino acids were purchased from ORPEGEN Pharma (Heidelberg, Germany). *o*-Chlorotritylchloride resin, TBTU and HATU were purchased from IRIS Biotech

(Marktredwitz, Germany). A dual-channel syringe pump (KD scientific Model 200) was used for slow reagent addition. MALDI-ToF MS analyses were performed on a Voyager-DE (Perseptive Biosystems) using 2,5-dihydroxybenzoic acid as the matrix. Preparative RP-HPLC was performed on a Thermo Separation Products apparatus equipped with a Vydac 218 TP 1022 efficiency column (22 x 250 µm, 10 µm) with water/acetonitrile gradients as the eluent and UV detection at 220 nm. Analytical RP-HPLC was performed on a Thermo Separation Products apparatus equipped with a Phenomenex Jupiter 4 µ Proteo 90 Å column (250 x 4.6 mm, 4 µm) with water/acetonitrile gradients as the eluent and UV detection at 220 nm.

Synthesis of Fmoc-Asp(O*t*Bu)-(+)-β-Acc-OBn

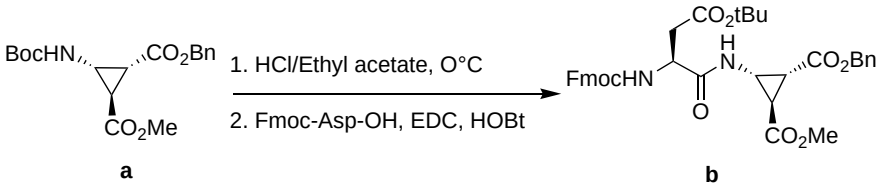

Figure 1: Synthesis of Fmoc-Asp(O*t*Bu)-(+)-β-Acc-OBn.

A solution of **a** (320 mg, 0.86 mM) in HCl 3 M in ethyl acetate (33 mL) was stirred at 0 °C for 3 h. The solvent was evaporated the solid was resospended in CH_2Cl_2 (30 mL) and a solution of Fmoc-Asp(O*t*Bu)-OH (708 mg, 1.72 mM, 2 eq.), EDC (330 mg, 1.72 mM, 2 eq) and HOBt (209 mg, 1.55 mM, 1.8 eq.) in CH_2Cl_2 (40 mL) (previously stirred 1 h at 0 °C) was added. Pyridine (123 µL, 1.55 mM, 1.8 eq.) was then added dropwise. The mixture was stirred overnight at room temperature. The solution was washed with saturated $NaHCO_3$ (50 mL), 1 M $KHSO_4$ (50 mL) and saturated $NaHCO_3$ (50 mL). The organic phase was dried over Na_2SO_4 and concentrated. The product was purified by chromatography (PE/EA 3:1 Rf = 0.1). 497 mg, 89%.

The multiplicity of the carbon atoms was determined by ^{13}C-DEPT-NMR (+/−).

m.p. 44-46 °C. $[\alpha]_D^{21} = -15.0$ (c = 0.5 in methanol); ^{1}H NMR ($CDCl_3$, 300 MHz); $\delta = 1.46$ (s, 9H, O*t*Bu), 2.32 (dd, $J = 4.9$, 4.9 Hz, 1H, cyclopropyl-CH), 2.55 (dd, $J = 8.2$, 5.2 Hz, 1H, cyclopropyl-CH), 2.53-2,60 (m, 1H, Asp-CH_2), 2.89 (dd, $J = 16.9$, 4.5 Hz, 1H, Asp-CH_2), 3.70 (s, 3H, CH_3O), 4.02 (ddd, $J = 7.9$. 7.9, 4.7 Hz, 1H, cyclopropyl-CHN), 4.22-4.27 (m, 1H, Fmoc-CH), 4.31-4.37 (m, 1H, Fmoc-CH_2), 4.45-4.51 (m, 2H, Fmoc-CH_2, Asp-CH), 5.03 (d, $J = 12.3$ Hz, 1H, CH_2O), 5.10 (d, $J = 12.3$ Hz, 1H, CH_2O), 5.94 (d, $J = 8.5$ Hz, 1H, Asp-NH), 7.28-7.43 (m, 9H, Fmoc-Ar-CH, Bn-Ar-CH), 7.54-7.66 (m, 3H, Fmoc-Ar-CH, Acc-NH), 7.76 (pseudo d, $J = 7.4$ Hz, 2H, Fmoc-Ar-CH); ^{13}C NMR ($CDCl_3$, 75.5 MHz): $\delta = 26.2$ (+,

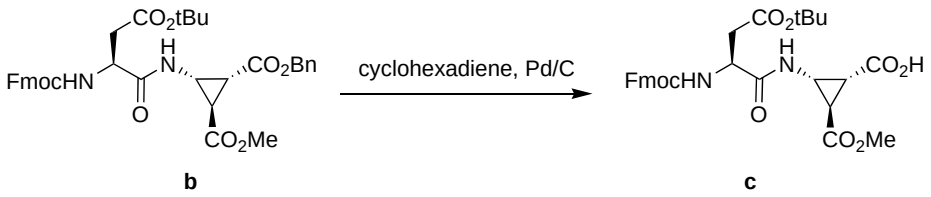

Figure 2: Synthesis of Fmoc-Asp(O*t*Bu)-(+)-β-Acc-OH.

cyclopropyl-CH), 28.1 (+, $(CH_3)_3C$), 28.7 (+, cyclopropyl-CH), 36.0 (+, cyclopropyl-CHN), 37.1 (−, Asp-CH2), 47.1 (+, Fmoc-CH), 51.2 (+, Asp-CHN), 52.5 (+, CH3O), 67.4 (−, Fmoc-CH_2, 1C), 68.8 (−, Bn-CH_2, 1C), 82.0 (Cquat, $(CH_3)C$), 120.0 (+, Ar-CH), 120.2 (+, Fmoc-Ar-CH), 124.4 (+, Ar-CH), 125.1 (+, Ar-CH), 125.2 (+, Ar-CH), 127.1 (+, Ar-CH), 127.8 (+, Ar-CH), 127.9 (+, Ar-CH),128.4 (+, Ar-CH), 128.5 (+, Ar-CH), 128.6 (+, Ar-CH, 2C), 129.4 (+, Ar-CH), 135.0 (Cquat, Ph-Ar-C), 139.7 (Cquat, Fmoc-Ar-C), 141.3 (Cquat, Fmoc-Ar-C), 143.6 (Cquat, Fmoc-Ar-C), 143.9 (Cquat, Fmoc-Ar-C), 156.0 (Cquat, N(CO)O), 169.7 (Cquat, C=O), 169.9 (Cquat, C=O), 171.1 (Cquat, C=O), 171.2 (Cquat, C=O); MS FAB (MeOH/Glycerine) 643 (MH^+, 86), 587 (100); IR: $\tilde{\nu} = 3329$, 2978, 1718, 1522, 1308, 1217, 1169, 1051, 741 cm^{-1}; HR MS calcd for $C_{36}H_{38}N_2O_9$ + H 643.2656, found 643.2650.

Synthesis of Fmoc-Asp(O*t*Bu)-(+)-β-Acc-OH

Fmoc-Asp(O*t*Bu)-(+)-β-Acc-OBn (497 mg, 0.77 mM) was dissolved under nitrogen atmosphere in MeOH (20 mL), then 1,4-cyclohexadiene (0.49 mL, 4 M in pentane, 5 eq) and Pd/C 5 % (100 mg) were added. The reaction mixture was stirred under inert atmosphere overnight, then it was filtrated on a 2 cm celite pad and concentrated to afford the product as a white solid (353 mg, 83 %).

m.p. 88-90 °C. $[\alpha]_D^{21} = -21.7$ (c = 0.5 in methanol); ^{1}H NMR (CD_3OD, 300 MHz): δ =1.42 (s, 9H, O*t*Bu), 2.37 (m, 2H, cyclopropyl-CH), 2.53 (dd, $J = 16.2$, 9.2 Hz, 1H, Asp-CH_2), 2.75 (dd, $J = 16.2$, 4.7 Hz, 1H, Asp-CH_2), 3.65-3.70 (m, 1H, cyclopropyl-CHN) 3.71 (s, 3H, CH_3O), 4.20-4.24 (m, 2H, Fmoc-CH_2), 4.33-4.35 (d, $J = 6.6$ Hz, 1H, Fmoc-CH), 4.47-4.51 (m, 1H, Asp-CH), 7.29-7.40 (m, 4H, Fmoc-Ar-CH), 7.63-7.67 (m, 2H, Fmoc-Ar-CH), 7.77-7.79 (m, 2H, Fmoc-Ar-CH); ^{13}C NMR (CD_3OD, 75.5 MHz): δ = 28.5 (+, cyclopropyl-CH), 28.7 (+, cyclopropyl-CH), 36.7 (+, cyclopropyl-CHN), 38.6 (−, Asp-CH_2), 48.4 (+, Fmoc-CH), 53.0 (+, CH_3O), 53.1 (+, Asp-CHN), 68.3 (−, Fmoc-CH_2), 82.5 (Cquat, $(CH_3)C$), 121.0 (+, Fmoc-Ar-CH, 2C), 126.3 (+, Fmoc-Ar-CH, 2C), 128.2 (+, Fmoc-Ar-CH, 2C), 128.8 (+, Fmoc-Ar-CH, 2C), 142.6 (Cquat, Fmoc-Ar-C, 2C), 145.2 (Cquat, Fmoc-Ar-C), 145.4 (Cquat, Fmoc-Ar-C), 158.4 (Cquat, N(CO)O), 170.7 (Cquat, C=O), 171.3 (Cquat, C=O),

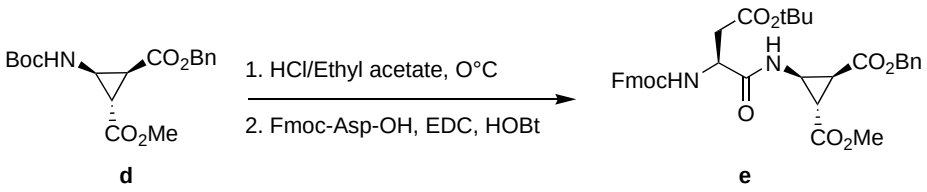

Figure 3: Synthesis of Fmoc-Asp(O*t*Bu)-(−)-β-Acc-OBn.

175.0 (Cquat, C=O), 172.1 (Cquat, C=O), 174.5 (Cquat, C=O); MS ESI (DCM/MeOH + 10 mM L^{-1} NH_4Ac) m/z (%) 570.3 (MNH_4^+, 100), 553.3 (MH^+, 78), 497.1 (MH^+-C_4H_8, 30); IR: $\tilde{\nu}$ = 3329, 2980, 1717, 1522, 1450, 1302, 1217, 1157, 756 cm^{-1}; HR MS calcd for $C_{29}H_{32}N_2O_9$+H 553.21861, found 553.21846.

Synthesis of Fmoc-Asp(O*t*Bu)-(−)-β-Acc-OBn

A solution of **d** (300 g, 0.86 mM) in HCl 3 M in ethyl acetate (35 mL) was stirred at 0 °C for 3 h. The solvent was evaporated the solid was resuspended in CH_2Cl_2 (30 mL) and a solution of Fmoc-Asp(O*t*Bu)-OH (708 mg, 1.72 mM, 2 eq.), EDC (330 mg, 1.72 mM, 2 eq) and HOBt (209 mg, 1.55 mM, 1.8 eq.) in CH_2Cl_2 (40 mL) (previously stirred 1 h at 0 °C) was added. Pyridine (123 µL, 1.55 mM, 1.8 eq.) was then added dropwise. The mixture was stirred overnight at room temperature. The solution was washed with saturated $NaHCO_3$ (50 mL), 1 M $KHSO_4$ (50 mL) and saturated $NaHCO_3$ (50 mL). The organic phase was dried over Na_2SO_4 and concentrated. The product was purified by chromatography (PE/EA 3:1; Rf = 0.1). 408 mg, 74 %.

m.p. 75-77 °C. $[\alpha]_D^{21}$ = +5.0 (c = 0.5 in methanol); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.46 (s, 9H, O*t*Bu), 2.32 (dd, J = 5.1, 4.9 Hz, 1H, cyclopropyl-CH), 2.56 (dd, J = 8.2, 5.1 Hz, 1H, cyclopropyl-CH), 2.62 (dd, J = 16.9, 5.0 Hz, 1H, Asp-CH_2), 2.93 (dd, J = 17.0, 4.7 Hz, 1H, Asp-CH_2), 3.70 (s, 3H, CH_3O), 4.06-4.13 (m, 1H, cyclopropyl-CHN), 4.25-4.30 (m, 1H, Fmoc-CH), 4.33-4.39 (m, 1H, Fmoc-CH_2), 4.47-4.55 (m, 2H, Fmoc-CH_2, Asp-CH), 5.03 (d, J = 12.2 Hz, 1H, CH_2O), 5.12 (d, J = 12.2 Hz, 1H, CH_2O), 5.86 (d, J = 8.8 Hz, 1H, Asp-NH), 7.29-7.43 (m, 4H, Fmoc-Ar-CH), 7.52 (d, J = 8.0 Hz, 1H, Acc-NH), 7.61-7.64 (m, 2H, Fmoc-Ar-CH), 7.77 (pseudo d, J = 7.7 Hz, 2H, Fmoc-Ar-CH); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 26.1 (+, cyclopropyl-CH), 28.1 (+, $(CH_3)_3C$), 28.6 (+, cyclopropyl-CH), 36.1 (+, cyclopropyl-CHN), 36.8 (−, Asp-CH_2), 47.1 (+, Fmoc-CH), 51.3 (+, Asp-CHN), 52.5 (+, CH_3O), 67.4 (−, Fmoc-CH_2, 1C), 67.5 (−, Bn-CH_2, 1C), 81.9 (Cquat, (CH_3C), 120.0 (+, Fmoc-Ar-CH, 2C), 125.2 (+, Fmoc-Ar-CH, 2C), 127.1 (+, Fmoc-Ar-CH), 127.2 (+, Fmoc-Ar-CH), 127.8 (+, Ar-CH, 2C), 128.4 (+, Ar-CH, 2C), 128.5 (+, Ar-CH, 1C), 128.7 (+, Ar-CH, 2C), 135.0 (Cquat, Ph-Ar-C), 141.3 (Cquat, Fmoc-Ar-C, 2C), 143.7 (Cquat, Fmoc-Ar-C),

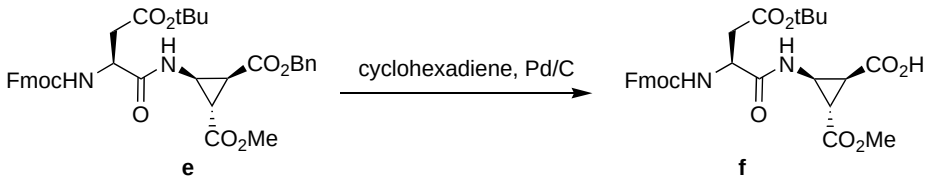

Figure 4: Synthesis of Fmoc-Asp(O*t*Bu)-(−)-β-Acc-OH.

143.9 (Cquat, Fmoc-Ar-C), 156.2 (Cquat, N(CO)O), 169.8 (Cquat, C=O), 169.9 (Cquat, C=O), 171.0 (Cquat, C=O), 172.2 (Cquat, C=O); MS FAB (MeOH/Glycerine) 643 (MH^+, 86), 587 (100); IR: $\tilde{\nu} = 3329$, 2978, 1718, 1522, 1308, 1217, 1169, 1051, 741 cm^{-1}; HR MS calcd for $C_{36}H_{38}N_2O_9$ + H 643.2656, found 643.2650.

Synthesis of Fmoc-Asp(O*t*Bu)-(−)-β-Acc-OH

Fmoc-Asp(O*t*Bu)-(−)-βAcc-OBn (300 mg, 0.47 mM) was dissolved under nitrogen atmosphere in MeOH (30 mL), then 1,4-cyclohexadiene 4 M in pentane (0.60 mL, 5 eq.) and Pd/C 5% (120 mg) were added. The reaction mixture was stirred under inert atmosphere overnight, then it was filtrated on a 2 cm celite pad and concentrated to afford the product as a white solid (227 mg, 88 %).

m.p. 78-80 °C. $[\alpha]_D^{21} = -10.4$ (c = 0.5 in methanol); ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 1.42$ (s, 9H, O*t*Bu), 2.32-2.39 (m, 2H, cyclopropyl-CH), 2.54 (dd, $J = 16.2$, 8.8 Hz, 1H, , Asp-CH_2), 2.78 (dd, $J = 16.2$, 5.2 Hz, 1H, Asp-CH_2), 3.65 (dd, $J = 7.9$, 4.9 Hz, 1H, cyclopropyl-CHN) 3.71 (s, 3H, CH_3O), 4.20-4.25 (m, 1H, Fmoc-CH), 4.33-4.38 (m, 2H, Fmoc-CH_2), 4.49 (dd, $J = 8.7$, 5.2 Hz, 1H, Asp-CH), 7.27-7.40 (m, 4H, Fmoc-Ar-CH), 7.66 (pseudo t, $J = 6.4$ Hz, 2H, Fmoc-Ar-CH), 7.66 (pseudo d, $J = 7.8$ Hz, 2H, Fmoc-Ar-CH); ^{13}C NMR: (CD_3OD, 75.5 MHz): $\delta = 28.4$ (+, cyclopropyl-CH), 28.8 (+, cyclopropyl-CH), 36.7 (+, cyclopropyl-CHN), 38.6 (−, Asp-CH_2), 48.4 (+, Fmoc-CH), 53.0 (+, CH_3O), 53.4 (+, Asp-CHN), 68.4 (−, Fmoc-CH_2), 82.5 (Cquat, $(CH_3)C$), 121.0 (+, Fmoc-Ar-CH, 2C), 128.3 (+, Fmoc-Ar-CH, 2C), 128.9 (+, Fmoc-Ar-CH, 2C), 129.9 (+, Fmoc-Ar-CH, 2C), 142.6 (Cquat, Fmoc-Ar-C, 2C), 145.2 (Cquat, Fmoc-Ar-C), 145.4 (Cquat, Fmoc-Ar-C), 158.4 (Cquat, N(CO)O), 171.3 (Cquat, C=O), 172.3 (Cquat, C=O), 172.8 (Cquat, C=O), 174.4 (Cquat, C=O), 174.5 (Cquat, C=O); MS FAB (MeOH/Glycerine): m/z (%) 553 (MH^+, 12), 497 (MH^+-*t*Bu, 62), 275 (100); IR: $\tilde{\nu} = 3337$, 3066, 2979, 1724, 1530, 1449, 1370, 1302 cm^{-1}; HR MS calcd for $C_{29}H_{32}N_2O_9$ + H 553.21861, found 553.21846.

Synthesis of c-(-Arg-Gly-Asp-(+)-β-Acc-Val-)

For loading of the o-chlorotritylchloride resin a 0.7 g (1.45 mM/g resin) portion of o-chlorotritylchloride resin, suspended in 10 mL of dry DCM was treated with 0.89 g of Fmoc-Gly-OH (3.0 mM) and 171 µL of DIPEA (1.0 mM) for 10 min and further with 256 µl of DIPEA (1.5 mM) for 1 hour. After adding 1 mL methanol the mixture was shaken for 15 min. The solvent was removed and the resin was washed several times with DCM (3x), DMF (3x), DCM (3x), methanol (1x).

Linear Precursor Peptide Synthesis

The synthesis was carried out according to standard Fmoc coupling protocols, starting with 0.33 g of Fmoc-Gly-2-Cl-Trt resin (substitution 1.2 mM amino acid/g resin). Deprotection of the N-terminal Fmoc group was achieved by using a solution of 2% piperidine and 2% DBU in DMF. Coupling of the amino acid was carried out using 1.5 eq. Fmoc-amino acid (0.3 M in DMF), 1.5 eq. TBTU (0.3 M in DMF) and 3.0 eq. DIPEA (0.6 M in DMF). Each coupling step was performed in duplicate. The Acc derivatives were incorporated by fragment condensation employing Fmoc-Asp(O*t*Bu)-β-Acc-OH dipeptides. For the coupling of dipeptide Fmoc-Asp(O*t*Bu)-β-Acc-OH (100 mg,0.18 mM) to H-Val-Arg(Pbf)-Gly-2-Cl-Trityl resin, HATU was applied as the coupling reagent and DIPEA as the base. Coupling reactions were monitored by MALDI-ToF mass spectrometry.

Peptide Cleavage

The resin bound peptide was treated 10 times with 5 mL 1% trifluoroacetic acid (TFA) in dichloromethane for 5 min each time. The combined solution was evaporated in vacuo.

Cyclization

100 µmol of linear precursor were dissolved in 20 mL DMF and transferred into a syringe. 114 µL HATU (300 µmol) was dissolved in the same volume of DMF and transferred into a second syringe. These two solutions were added slowly using a dual channel syringe pump to a stirred solution of 103 µL DIPEA (600 µmol) and 3.8 µg HATU (10 µmol) in 10 mL DMF at a rate of 1.00 mL h^{-1}. Once the addition was complete, the mixture was stirred for another 15 minutes. The solvent was evaporated in vacuo at a temperature lower than 30 °C and the peptide was purified using preparative RP-HPLC. [1]

Side Chain Deprotection

The cyclic peptide was treated with 3 mL of a solution of trifluoroacetic acid (95%), triisopropylsilane (2.5%) and water (2.5%). The solution was shaken at room temperature for 8 h, the solvent was evaporated and cold diethyl ether 30 mL was added to the residue. The system was centrifuged for 1 h at 0 °C with 3220 × g, and diethyl ether was decanted. The residue was dissolved in water and purified by preparative RP-HPLC.

Synthesis of c-(-Arg-Gly-Asp-(−)-β-Acc-Val-)

For loading of the o-chlorotritylchloride resin, a 0.7 g (1.45 mol/g resin) portion of o-chlorotritylchloride resin suspended in 10 mL of dry DCM was treated with 0.89 g of Fmoc-Gly-OH (3.0 mM) and 171 µL of DIPEA (1 mM) for 10 min and further 256 µL of DIPEA (1.5 mM) for 1 hour. After adding 1 mL methanol the mixture was shaken for 15 min. The solution was removed and the resin was washed several times with DCM (3x), DMF (3x), DCM (3x), methanol (1x).

Peptide Synthesis

Starting with 0.85 g of Fmoc-Gly-2-Cl-Trt resin (substitution 1.2 mM amino acid/g resin), the synthesis was carried out according to standard Fmoc coupling protocols. Deprotection of the N-terminal Fmoc group was achieved by using a solution of 20% piperidine in DMF. Coupling of the amino acid was carried out using 3.0 eq. Fmoc–amino acid (1 M in DMF), 3.0 eq. TBTU (1 M in DMF) and 6.0 eq. DIPEA. Fmoc–amino acid was preactivated by TBTU in the presence of DIPEA for 5-10 min. Each coupling step was performed during 45 min. After the sequence Fmoc-Val-Arg(Pbf)-Gly was assembled on the resin, peptide quantification was carried out by cleaving the Fmoc group and detecting the formed piperidine-dibenzofulvene adduct at 290 nm. Dipeptide Fmoc-Asp(O*t*Bu)-(−)β-Acc-OH(154 mg/0.28 mM) was preactivated by 3.0 eq. HATU in the presence of 6.0 eq. DIPEA for 10 min before adding to 1.1 eq. H-Val-Arg(Pbf)-Gly-2-Cl-Trityl resin (1.19 g, 0.31 mM). Coupling reactions were monitored by MALDI-ToF mass spectrometry.

Peptide Cleavage

The resin bound peptide was treated 10 times with 5 mL 1% trifluoroacetic acid (TFA) in dichloromethane, 5 min each time. The combined solution was evaporated in vacuo and then purified by RP-HPLC.

Cylization

16.7 µg of linear precursor (18.7 µmol) was dissolved in 20 mL DMF and transferred into a syringe. 21.3 µg HATU (56.0 µmol) was dissolved in the same volume of DMF and transferred into a second syringe. These two solutions were slowly added with a dual channel syringe pump to a stirred solution of 12.0 µL DIPEA (112.0 µmol) in 20 mL DMF at a rate of 1.00 mL h^{-1}. Once the addition was complete, the mixture was stirred for further 15 minutes. The solvent was evaporated in vacuo at a temperature lower than 30 °C and the peptide was applied in the next step without purification.

Side Chain Deprotection

The cyclic peptide analogue was treated with 3 mL of a solution of trifluoroacetic acid (95%), triisopropylsilane (2.5%) and water (2.5%). The solution was shaken at RT for 8 h, the solvent was evaporated and cold diethyl ether (30 mL) was added to the residue. The system was centrifuged for 1 h at 0 °C with 3220 ×g, and diethyl ether was decanted. The residue was dissolved in water and purified by preparative RP-HPLC.

Biological tests

Cell Culture

The wild type of K562 cells was obtained from the Institut für Angewandte Zellkultur (I.A.Z., München, Germany). Monolayer cells WM115 were obtained from the American Type Culture Collection (ATCC, Manassas, USA). The K562 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 µg mL^{-1} normocin™. The WM115 cells were cultivated in MEM medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM natrium pyruvate and normocin™ 100 µg mL^{-1}. The cell lines were cultivated under 5.3% (*v*/*v*) atmosphere of CO_2 at 37 °C.

Assays with K562 Human Erythroleukemia Cancer Cells

The 96-wells microtiterplate with modified surface (maxisorp™, Nunc) was coated with 100 µL human vitronectin at a concentration of 1 µg mL^{-1} (Chemicon, England) and incubated for 18 h at 37 °C. Free binding sites on the plate were saturated with 1% bovine serum albumin in water for 1 h at 37 °C. The expression of the integrins in the K562 cells was activated 18 h before the experiment with 10 mM phorbol-12-myristat-13-acetate. On the day of the experiment the K562 cell suspension was centrifuged for 6 min at 150 ×g, washed with Hank's

Buffered Salt Solution (HBSS) and resuspended with Puck's Salt Solution containing divalent cations 2 mM Ca^{2+} and 2 mM Mg^{2+} to obtain a cell density of $1.1 \cdot 10^6$ cells mL^{-1}. Cells were incubated for 30 min on ice, mixed with peptide solutions and shaken 30 min at 37 °C. In the following step cells were dispensed on the plate ($1.0 \cdot 10^5$ cells $well^{-1}$) and incubated for 1 h at 37°C. After incubation the unbound cells were aspirated and the bound cells were washed three times with Puck's Salt Solution containing divalent cations. The cells were fixed with 100 µL of the 5% glutaraldehyde in water (*w/v*) for 30 min at RT and stained with 0.1% (*w/v*) crystal violet in 0.2 M MES for 1 h at RT. Subsequently, the staining solution was aspirated, the cells were washed twice with water (200 µL) and destained with 100 µL of 0.1 M citric acid in ethanol for 30 min at RT. Finally, the bound cells were quantified by measurement of the absorbance at 560 nm. Each probe was measured in triplicate and each test was repeated three times.

Assays with WM115 human epithelial cancer cells

The 96-wells microtiterplate with modified surface (maxisorp™, Nunc) was coated with 100 µL human fibronectin (20 µg mL^{-1}) (Gibco, France) and incubated for 18 h at 37 °C. WM115 cells were cultivated up to 70% confluence, detached with 2 mM EDTA in phosphate buffered solution (PBS), washed once with HBSS and resuspended with Puck's Salt Solution containing divalent cations to obtain a cell density of $1.1 \cdot 10^6$ cells mL^{-1}. The next steps of the test were carried out as described for the K562 cell line.

Determination of IC_{50} Values

IC_{50} values (inhibition concentration of 50% cell adhesion) for the tested peptides were evaluated with GraphPad Prism Software 4.0.

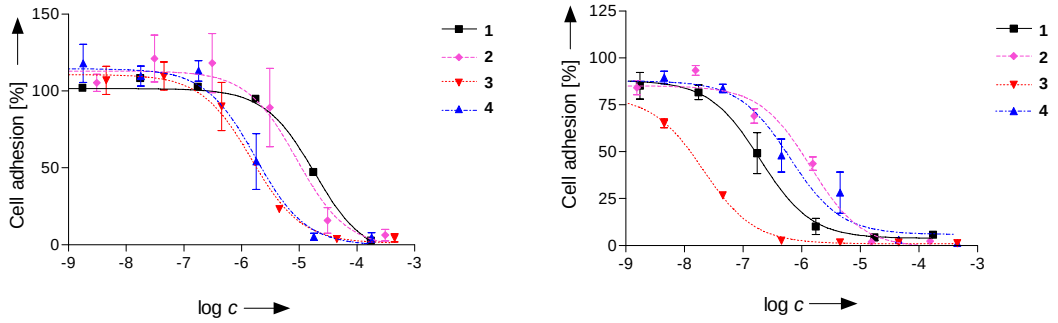

Figure 5: Influence of RGD peptides (**1-4**) on the adhesion of K562 (left) and WM115 (right) cells to fibronectin and vitronectin, respectively.

NMR Studies

NMR measurements of c-(-Arg-Gly-Asp-(+)-β-Acc-Val-) and c-(-Arg-Gly-Asp-(−)-β-Acc-Val-) were carried out at 300 K using 5-10 mM solutions in DMSO-D_6 on a Bruker Avance spectrometer at a ^{1}H resonance frequency of 600.13 MHz and a ^{13}C resonance frequency of 150.90 MHz. For the assignment of hydrogen atoms, a phase sensitive, double quantum filtered correlation spectrum COSY [2] and a total correlation spectrum TOCSY [3] with a mixing time of 60 ms using the DIPSI2 [4] pulse sequence for homonuclear Hartmann-Hahn transfer were recorded. The assignment of the carbon atoms was achieved using ^{1}H-^{13}C heteronuclear single-quantum correlation techniques HSQC as well as ^{1}H-^{13}C heteronuclear multiple-bond correlation spectroscopy HMBC. For the two-dimensional spectra, data matrices of usually 256×4096 points and 32-64 scans per increment were chosen. Zero-filling was performed in F1 to 1024×4096 points. Fourier transformation was generally followed by baseline correction in both dimensions. In order to obtain distance restraints for the molecular dynamics calculations, nuclear Overhauser enhancement spectroscopy [5] was performed. NOE spectra were recorded with mixing times between 320 and 440 ms in 40 ms steps for c-(-Arg-Gly-Asp-(+)-β-Acc-Val-) and with 300 to 450 ms in 50 ms for c-(-Arg-Gly-Asp-(−)-β-Acc-Val-). For each peptide the intensity of 4-5 NOE was plotted against the mixing time and in order to examine, whether and in which range the increase of the intensity of the NOEs was linear. For the structure determination of c-(-Arg-Gly-Asp-(+)-β-Acc-Val-), the NOE spectrum

Table 1: ^{1}H NMR shifts of c-(-Arg-Gly-Asp-(+)-β-Acc-Val-) in DMSO-D_6.

Residue	δ [ppm] H^N	δ [ppm] H^α	δ [ppm] H^β	δ [ppm] H^γ	δ [ppm] H^δ	δ [ppm] H^ϵ
Arg	8.13	3.95	$\beta^{2/3}$ 1.89/1.60	$\gamma^{2/3}$ 1.46/1.41	$\delta^{2/3}$ 3.09	7.48
Gly	8.05	$\alpha^{2/3}$ 3.93/3.35				
Asp	8.33	4.59	$\beta^{2/3}$ 2.81/2.49			
(−)-β-Acc	8.17	2.35	3.19	2.35		
Val	8.04	3.94	2.02	$\gamma^{1/2}$ 0.91		

Table 2: ^{1}H NMR shifts of c-(-Arg-Gly-Asp-(−)-β-Acc-Val-) in DMSO-D_6.

Residue	δ [ppm] H^N	δ [ppm] H^α	δ [ppm] H^β	δ [ppm] H^γ	δ [ppm] H^δ	δ [ppm] H^ϵ
Arg	8.00	4.11	$\beta^{2/3}$ 1.87/1.61	$\gamma^{2/3}$ 1.46/1.42	$\delta^{2/3}$ 3.10	7.59
Gly	7.88	$\alpha^{2/3}$ 4.02/3.43				
Asp	8.02	4.63	$\beta^{2/3}$ 2.74/2.40			
(−)-β-Acc	7.70	2.75	3.60	2.20		
Val	8.49	3.87	2.13	$\gamma^{1/2}$ 1.02		

with a mixing time of 360 ms and for c-(-Arg-Gly-Asp-(−)-β-Acc-Val-), the NOE spectrum with a mixing time of 350 ms was chosen for the determination of the hydrogen-hydrogen distances. These were obtained after referencing the intensities to the glycine H^{α}. NMR data was acquired and processed using Topsin and assigned with Sparky [6]. Proton shifts are shown in Tables 1 and 2 and carbon shifts in Tables 3 and 4.

Table 3: ^{13}C NMR shifts of c-(-Arg-Gly-Asp-(+)-β-Acc-Val-) in DMSO-D_6.

Residue	δ [ppm] C	δ [ppm] C^{α}	δ [ppm] C^{β}	δ [ppm] C^{γ}	δ [ppm] C^{δ}
Arg					
Gly	169.5	52.4			
Asp	172.7	49.3	35.7	172.3	
(−)-β-Acc	169				171.4
Val	171.8	60.6	29.9	$\gamma^{1/2}$ 19.7	

Table 4: ^{13}C NMR shifts of c-(-Arg-Gly-Asp-(−)-β-Acc-Val-) in DMSO-D_6.

Residue	δ [ppm] C	δ [ppm] C^{α}	δ [ppm] C^{β}	δ [ppm] C^{γ}	δ [ppm] C^{δ}
Arg	172.2		28.1		41.2
Gly	169.3	43.1			
Asp	171.1	48.9	40.6	172.2	
(−)-β-Acc	169.5	27.3	52.6	26.9	
Val	171.3	61.0	30.0	$\gamma^{1/2}$ 19.7/18.5	

Table 5: Distances from NOESY correlations for c-(-Arg-Gly-Asp-(+)-β-Acc-Val-) in DMSO-D_6 (*exp*: experimental value, *max*: maximum tolerance, *min*: minimum tolerance, *rmd*: value according to restrained molecular dynamics, for not-assigned diastereotopic H^{β}- and H^{γ}-atoms pseudoatoms (Q) were used, minimum restraints were set (with *max*=2400 and *min*=400).

Atom 1	Atom 2	d [pm]			
		exp	max	min	rmd
Arg Q^{β}	Arg Q^{δ}	269	296	242	273
Arg Q^{β}	Arg H^{N}	300	330	270	287
Arg Q^{β}	Arg H^{α}	254	279	229	255
Arg Q^{δ}	Arg H^{α}	264	290	238	305
Arg Q^{γ}	Arg H^{α}	225	248	202	261
Arg Q^{γ}	Arg H^{N}	359	395	223	340

table 5 continued

Atom 1	Atom 2	d [pm]			
		exp	max	min	rmd
Arg H^N	Asp H^α	400	2400	400	603
Arg H^N	(+)-β-Acc H^α	400	2400	400	445
Arg H^N	(+)-β-Acc H^β	400	2400	400	591
Arg H^α	Asp H^α	400	2400	400	768
Arg H^α	(+)-β-Acc H^β	400	2400	400	727
Arg H^α	(+)-β-Acc H^α	400	2400	400	701
Gly Q^α	Gly H^N	238	262	214	194
Gly Q^α	Asp H^N	210	231	189	204
Gly H^N	Asp H^N	229	252	206	377
Gly H^N	Asp H^α	400	2400	400	560
Gly H^N	(+)-β-Acc H^β	400	2400	400	641
Gly Q^α	(+)-β-Acc H^β	400	2400	400	511
Gly Q^α	(+)-β-Acc H^α	400	2400	400	810
Gly Q^α	Asp H^α	400	2400	400	407
Asp H^α	(+)-β-Acc H^N	250	275	225	299
Asp Q^β	Asp H^N	190	209	171	292
Asp H^N	(+)-β-Acc H^β	400	2400	400	383
Asp H^N	(+)-β-Acc H^α	400	2400	400	716
Asp H^α	(+)-β-Acc H^β	400	2400	400	482
Asp H^α	(+)-β-Acc H^α	400	2400	400	667
(+)-β-Acc H^α	(+)-β-Acc H^N	298	358	238	472
(+)-β-Acc H^γ	(+)-β-Acc H^N	298	358	238	204
(+)-β-Acc H^α	Val H^N	254	305	203	423
(+)-β-Acc H^γ	Val H^N	254	305	203	287
(+)-β-Acc H^N	Asp H^N	233	256	210	257
Val H^β	Arg H^N	319	351	287	270
Val H^β	Val H^α	270	297	243	333
Val H^β	Val H^N	267	294	240	157
Val Q^γ	Arg H^N	318	350	286	274
Val Q^γ	Val H^α	263	289	237	352
Val Q^γ	Val H^N	255	281	229	232
Val H^N	Asp H^α	400	2400	400	668
Val H^N	(+)-β-Acc H^β	400	2400	400	426

table 5 continued

Atom 1	Atom 2	d [pm]			
		exp	max	min	rmd
Val H^{α}	Asp H^{α}	400	2400	400	609
Val H^{α}	(+)-β-Acc H^{α}	400	2400	400	296
Val H^{α}	(+)-β-Acc H^{β}	400	2400	400	435

Table 6: Distances from NOESY correlations for c-(-Arg-Gly-Asp-(−)-β-Acc-Val-) in DMSO-D_6 (*exp*: experimental value, *max*: maximum tolerance, *rmd*: value according to restrained molecular dynamics, for not-assigned diastereotopic H^{β}- and H^{γ}-atoms pseudoatoms (Q) were used.

Atom 1	Atom 2	d [pm]			
		exp	max	min	rmd
Arg H^{α}	Arg H^{N}	239	263	215	268
Arg Q^{β}	Arg Q^{γ}	318	382	254	293
Arg Q^{β}	Arg H^{α}	247	272	222	256
Arg Q^{β}	Arg H^{ϵ}	366	403	329	355
Arg Q^{β}	Gly H^{N}	345	379	311	316
Arg Q^{γ}	Arg H^{α}	344	413	275	288
Arg Q^{γ}	Arg H^{ϵ}	335	402	268	185
Arg Q^{γ}	Arg H^{N}	425	510	340	315
Arg H^{ϵ}	Arg H^{N}	356	392	320	404
Arg Q^{γ}	Arg Q^{β}	258	310	206	227
Arg Q^{γ}	Arg Q^{γ}	284	341	227	235
Arg Q^{γ}	Arg H^{ϵ}	400	480	320	257
Arg Q^{γ}	Arg H^{N}	314	377	251	283
Arg Q^{γ}	Gly H^{N}	385	462	308	476
Arg Q^{γ}	Val H^{β}	375	450	300	425
Arg Q^{γ}	Arg H^{α}	299	329	269	280
Arg H^{N}	Val H^{N}	266	293	239	247
Gly Q^{α}	Gly H^{N}	231	254	208	256
Gly Q^{α}	Asp H^{N}	270	297	243	282
Gly Q^{α}	(−)-β-Acc H^{N}	393	432	354	438
Gly H^{N}	Val H^{N}	409	450	368	406
Asp H^{α}	Asp H^{N}	263	289	237	284

table 6 continued

Atom 1	Atom 2	d [pm]			
		exp	max	min	rmd
Asp H^{α}	(−)-β-Acc H^{N}	263	289	237	253
Asp Q^{β}	Asp H^{α}	254	279	229	255
Asp Q^{β}	(−)-β-Acc H^{N}	394	433	355	426
Asp Q^{β}	Asp H^{N}	274	301	247	270
(−)-β-Acc H^{α}	Gly H^{N}	472	519	425	482
(−)-β-Acc H^{α}	(−)-β-Acc H^{β}	216	238	194	293
(−)-β-Acc H^{α}	(−)-β-Acc H^{N}	324	356	292	351
(−)-β-Acc H^{α}	Val H^{α}	362	398	326	384
(−)-β-Acc H^{α}	Val H^{N}	209	230	188	185
(−)-β-Acc H^{β}	Asp H^{α}	399	439	359	419
(−)-β-Acc H^{β}	Asp H^{N}	390	429	351	425
(−)-β-Acc H^{β}	(−)-β-Acc H^{N}	263	289	237	262
(−)-β-Acc H^{β}	Val H^{N}	359	395	323	412
(−)-β-Acc H^{γ}	Asp H^{α}	415	457	373	456
(−)-β-Acc H^{γ}	(−)-β-Acc H^{α}	282	310	254	318
(−)-β-Acc H^{γ}	(−)-β-Acc H^{β}	293	322	264	317
(−)-β-Acc H^{γ}	(−)-β-Acc H^{N}	266	293	239	309
(−)-β-Acc H^{γ}	Val H^{N}	387	426	348	450
(−)-β-Acc H^{N}	Gly H^{N}	273	300	246	306
(−)-β-Acc H^{N}	Asp H^{N}	252	277	227	288
(−)-β-Acc H^{N}	Val H^{N}	400	440	360	381
Val H^{α}	Arg H^{N}	259	285	233	340
Val H^{α}	(−)-β-Acc H^{N}	431	474	388	448
Val H^{α}	Val H^{N}	277	305	249	274
Val H^{β}	Arg H^{N}	302	332	272	260
Val H^{β}	Val H^{α}	230	253	207	265
Val H^{β}	Val H^{N}	270	297	243	244
Val Q^{γ}	Arg H^{N}	399	479	319	380
Val Q^{γ}	(−)-β-Acc H^{α}	438	526	350	476
Val Q^{γ}	Val H^{α}	345	414	276	265
Val Q^{γ}	Val H^{β}	335	402	185	182
Val Q^{γ}	Val H^{N}	321	385	257	327

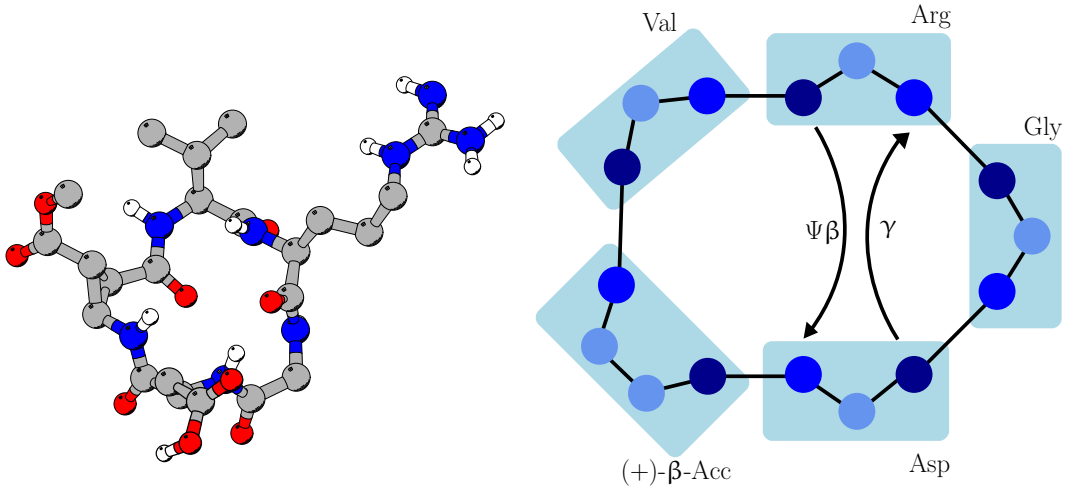

Figure 6: Stereoview and schematic representation of the central structure of c-(-Arg-Gly-Asp-(+)-β-Acc-Val-).

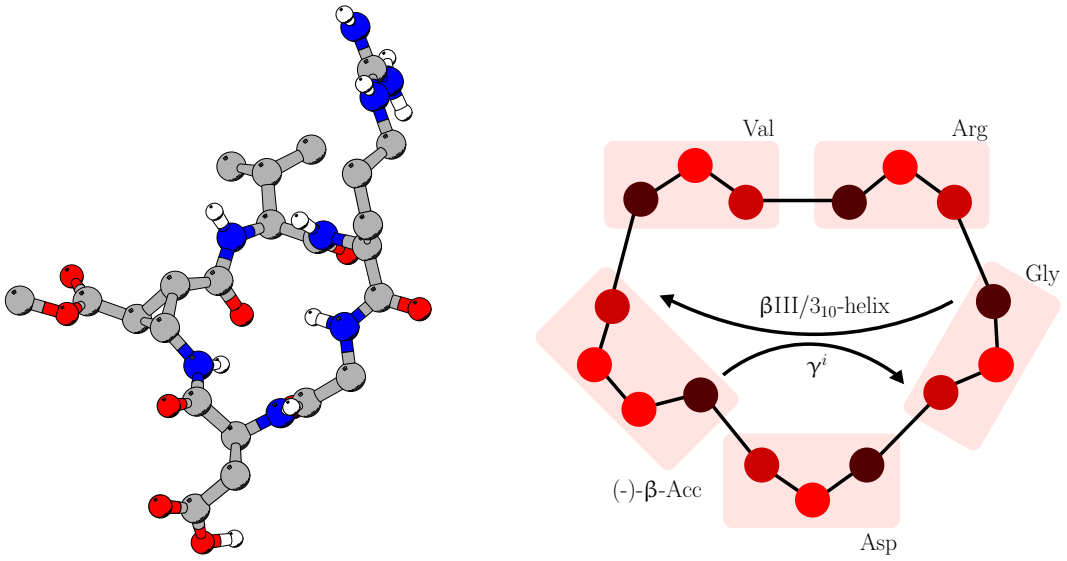

Figure 7: Stereoview and schematic representation of the central structure of c-(-Arg-Gly-Asp-(−)-β-Acc-Val-).

Molecular Dynamics Calculations

The distance restraints derived from NOE spectra were applied in distance geometry calculations followed by simulated annealing using Xplor-NIH [7]. 1000 structures fulfilling all NOE restraints were created and clustered according to torsion angles with a cutoff of $\pm 60°$.[8] The central structure of the main cluster was employed as starting structure in a 2.5 ns restrained molecular dynamics calculation.

For this, the molecule was embedded in a truncated octrahedral DMSO solvent box using the box distributed for GROMOS96 [9]. Between the external peptide atoms and the wall of the box, a minimum distance of 1.6 nm was ensured during the building of the box. The system was allowed to relax using steepist descent energy minimization before the simulation

Table 7: Torsion angles of the central structure from the restrained molecular dynamics calculations of c-(-Arg-Gly-Asp-(+)-β-Acc-Val-) and c-(-Arg-Gly-Asp-(−)-β-Acc-Val-) in DMSO-D_6.

	c-(-Arg-Gly-Asp-(+)-β-Acc-Val-)			c-(-Arg-Gly-Asp-(−)-β-Acc-Val-)		
Residue	ϕ	ψ	μ	ϕ	ψ	μ
Arg	-114°	90°		-56°	-42°	
Gly	84°	-52°		-103°	-111°	
Asp	-82°	-77°		-71°	48°	
(±)-β-Acc	-115°	-122°	10°	174°	-137°	5°
Val	-42°	-64°		-80°	-45°	

was started. The calculations were carried out with the GROMOS++ software suite [9] and applied the GROMOS 45A3 [10] united atom force field with enhanced DMSO parameters [11] on a LINUX system. The calculations were started with a weak temperature coupling of solvent and solute to an external bath of 300 K with a relaxation time of 0.1 ps. The constant pressure of 1 atm was held with a relaxation time of 0.5 ps and an isothermal compressibility of $4.575 \cdot 10^{-4}$ kJ mol^{-1} nm^{-3}. The H-H distances obtained from NOE data were used as time-averaged distance restraints in the simulation. For the constriction of the bond length, a SHAKE algorithm [12] with a geometric tolerance of 10^{-4} was applied. The equations of motion were integrated with the leapfrog algorithm and a time step of 2 fs.

A cluster analysis according to the torsion angles as described above was applied to every tenth structure of the molecular dynamics calculations. The central structures of the resulting clusters were then used as starting structure in free molecular dynamics calculations, which were carried out for 10 ns according to the same protocol as the restrained simulations.

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