Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3331–3337

BIOORGANIC & MEDICINAL CHEMISTRY

Incorporation of (2*S*,3*S*) and (2*S*,3*R*) β-Methyl Aspartic Acid into RGD-Containing Peptides

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Received 18 December 2001; accepted 16 April 2002

Abstract—We report the synthesis and biological activity of a series of side-chain-constrained RGD peptides containing the (2*S*,3*R*) or (2*S*,3*S*) β-methyl aspartic acid within the RGD sequence. These compounds have been assayed for binding to the integrin receptors $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ and the results demonstrate the importance of the side-chain orientation of this particular residue within the RGD sequence. Based on our findings, the (2*S*,3*S*) β-methylated analogues of our RGD sequences maintain their binding potency to the integrin receptors while the (2*S*,3*R*) β-methylated analogues exhibit a drastically reduced binding affinity. Our studies demonstrate that the three-dimensional orientation of the aspartyl side chain is a very important parameter for integrin binding and that small changes that affect the side-chain orientations give rise to drastic changes in binding affinity. These results provide important information for the design of more potent RGD mimetics.

Introduction

Integrins are a family of heterodimeric glycoprotein[1,2] complexes that regulate cell–cell and cell–matrix interaction.[3] These transmembrane receptors mediate a variety of cell adhesion events and signal transduction processes and are involved in an array of pathological events such as tumor metastasis, angiogenesis, thrombosis and osteoporosis. Integrin receptors consist of an α and β subunit which are non-covalently linked and many of these receptors bind their natural ligands through an RGD sequence.[4–6]

Among the integrin receptors, two receptors that were among the first to be discovered have emerged as being of particular interest[7] and have been extensively studied, the fibrinogen receptor, $\alpha_{IIb}\beta_3$, and the vitronectin receptor, $\alpha_v\beta_3$. The $\alpha_{IIb}\beta_3$ is important for platelet aggregation and antagonists to this receptor have therapeutic potential as antithrombotic agents.[8] The vitronectin receptor is involved in a number of biological processes such as angiogenesis, and adhesion of osteoclasts to the bone matrix. Antagonists to this receptor could be useful in the treatment of osteoporosis, diabetic retinopathy and cancer.[9]

A number of potent and specific antagonists to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$[10] have been developed and the three-dimensional structure of some of these compounds in solution have been studied. From these studies the backbone conformations of $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ specific ligands have been postulated.[11] As part of our ongoing efforts to optimize integrin antagonists and develop non-peptide analogues we have synthesized a series of peptides incorporating (2*S*,3*S*) and (2*S*,3*R*) β-methylated aspartic acid residues. These studies were carried out to constrain the Asp side chain and gain information on the structural requirements for binding of this side chain. We have chosen some of our peptide pharmacophores for these studies. In particular, we have chosen the $\alpha_v\beta_3$ selective pharmacophores, Arg-Gly-Asp-Asp-Val, Arg-Gly-Asp-Asp-(tBuG) and Arg-Gly-Asp-Tyr(Me)-Arg as well as the non-selective but potent Arg-Gly-Asp-Thr-Tic pharmacophores constrained by either disulfide bridges or a head to tail cyclization through a 3-aminomethyl benzoic acid (Mamb) residue. These pharmacophores and their activities has been described earlier.[10b,12]

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PII: S0968-0896(02)00206-7

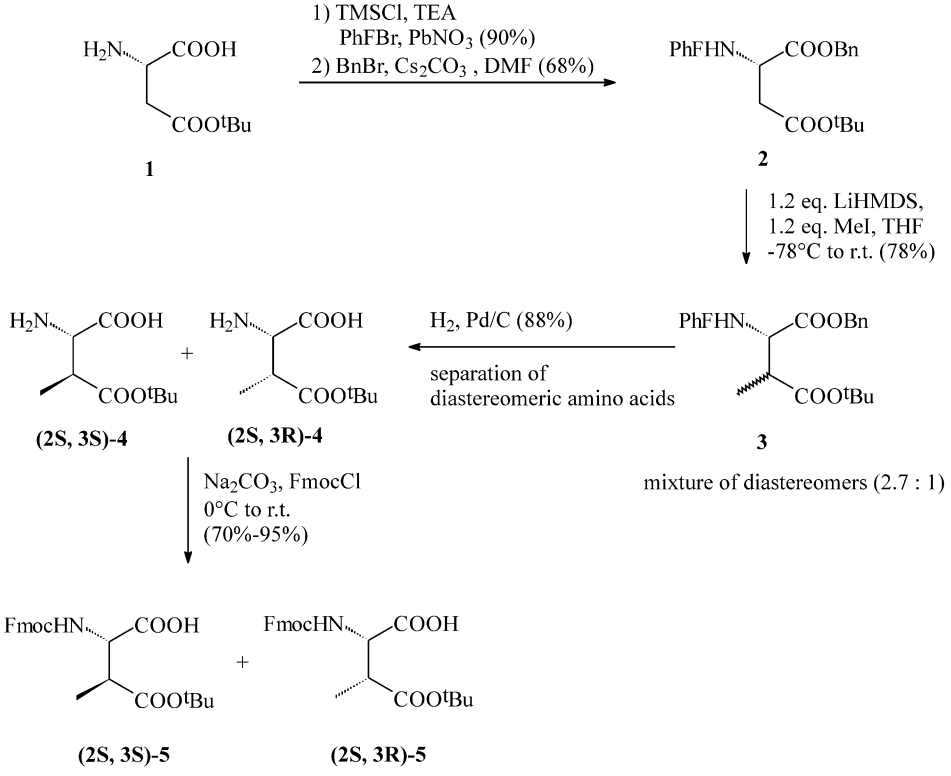
We now report the syntheses and binding results of these cyclic RGD peptide analogues containing enantiomeric pure β-methylated (2*S*,3*S*) and (2*S*,3*R*) aspartic acid building blocks.

Synthesis

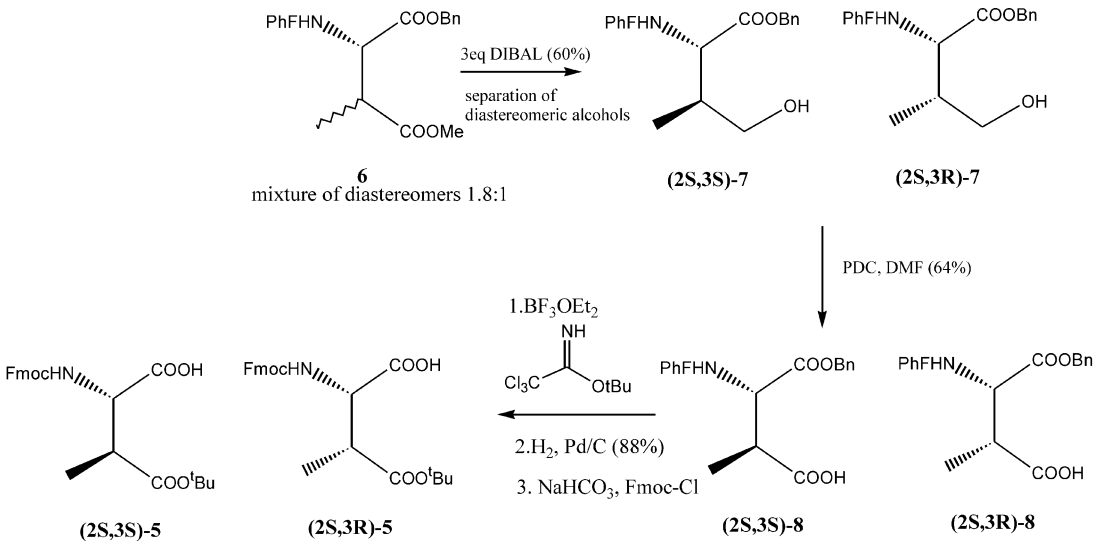
Both β-(2*S*,3*S*)- and β-(2*S*,3*R*)-methylated N-Fmoc protected aspartic acid-β *tert*-butyl ester building blocks **5** were synthesized as shown in Scheme 1 and incorporated into cyclic RGD peptides. The *tert*-butyl group as side-chain protection for the β-carboxyl group of aspartic acid has proven to be useful in solid-phase peptide synthesis using the Fmoc strategy. The synthesis of **5** commences from L-aspartic acid β *tert*-butyl ester **1**, which establishes the desired *S* configuration of the α-carbon. Introduction of the bulky 9-phenylfluorene (PhF) protecting group[13] attached to the nitrogen is achieved by addition of chlorotrimethylsilane, which protects the α-carboxyl group in situ, and following reaction with 9-bromo-9-phenylfluorene assisted by triethylamine and lead nitrate. The TMS-ester is easily cleaved by aqueous work up. The α-carboxyl group can then be converted to α-benzyl ester to yield the fully protected amino acid **2**.

Rapoport and co-workers[13a] reported the application of the PhF group and others have recently utilized this bulky protecting group for its unique properties.[13b–f] Placed on the nitrogen of aspartic acid it shields the proton on the α-carbon from abstraction by strong bases. Treatment of **2** with LiHMDS results in the formation of the enolate at β-carbonyl carbon which allows methylation of the β-position with methyl iodide without alkylation or epimerization of the α-carbon. A mixture of both β-methylated diastereomers (2*S*,3*S*)-**3** and (2*S*,3*R*)-**3** in a ratio 2.7:1, *syn*/*anti* was formed. At this point, it was not possible to separate these diastereomers by column chromatography on silica gel. However, the benzyl group and the PhF protecting group can be removed in one step by hydrogenation under palladium catalysis to yield the diastereomeric amino acids **4**. At this point, the diastereomers can be separated on silica gel using a mixture of ethyl acetate, isopropanol and water (8:2:1) as eluent. Finally, Fmoc-protection of the amino group was achieved by treatment of (2*S*,3*S*)-β-methyl aspartic acid *tert* butyl ester **[(2*S*,3*S*)-4]** and (2*S*,3*R*)-β-methyl aspartic acid *tert*-butyl ester **[(2*S*,3*R*)-4]** with Fmoc chloride at 0 °C in dioxane/water using sodium carbonate as base.

Since the coupling constants between the α- and β-substituted protons, which indicate the stereochemistry, cannot be differentiated in compounds **4** or in the protected derivatives **3** or **5** we had to establish the stereochemistry by synthesizing the corresponding alcohols **7** as shown in Scheme 2. To determine the configuration of the diastereomers the β-methylated diastereomeric alcohols **7** were synthesized (Scheme 2) starting from a mixture of diastereomeric aspartic acid methyl ester **6**. The methyl esters **6** were synthesized by reacting Asp(OMe)OBn with PhF bromide followed by treatment with LiHMDS and methylation of the β-carbon. The desired compounds were obtained by reduction of the methyl ester using DIBAL. The diastereomers of **7** were separated and the stereochemistry determined based on the coupling constants [J(2*S*,3*S*), syn = broad s; J(2*S*,3*R*), *anti* = 9.6 Hz) described by Rapoport et al.[14]



Scheme 1. Synthesis of (2*S*,3*R*) and (2*S*,3*S*) β-methylated aspartic acid building blocks.



Scheme 2. Reduction of (2*S*,3*R*) and (2*S*,3*S*) β-methylated aspartic acid building blocks to alcohols to establish the stereochemistry of the β-carbon.

Reoxidation of the separated alcohols (2*S*,3*S*)-**7** and (2*S*,3*R*)-**7** using PDC in DMF lead to the corresponding acids **8**, which can be converted to the β-*tert*-butyl ester by treatment with *tert*-butyltrichloroacetimidate under acidic conditions. Cleavage of the α-benzyl ester and PhF group and subsequent Fmoc-protection of the amino group yields the desired β-methylated aspartic acid building blocks **5**.

Peptide synthesis

The peptides were synthesized by stepwise coupling of Fmoc-amino acid derivatives by using standard coupling procedures on solid-phase Rink amide MBHA resin for the disulfides or chlorotrityl resin for the head-to-tail cyclic peptides. The disulfide bonds were formed on-resin by oxidation of the protected peptides with an iodine solution in DMF. During this process, the deprotection of the Acm groups from cysteine and formation of the disulfide bond occurred simultaneously. Finally, cleavage of the cyclic peptides from the resin as well as deprotection of all side-chain protecting groups were accomplished by treatment with a trifluoroacetic acid cocktail. For the head-to-tail cyclic peptides the peptides were cleaved from the resin with acetic acid/trifluoroethanol/dichloromethane 1:1:5 and the peptides were cyclized in 2.5 mM concentration in DMF using DPPA. The side chain protecting groups were removed using trifluoroacetic acid cocktail. Peptide purification was performed by preparative RP-HPLC on a Vydac C18 column using a gradient of acetonitrile in water with 0.1% TFA.

Binding data

The peptides were assayed for binding to the $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ receptor using an ELISA assay format as described in ref 10b. The data as shown in Table 1 demonstrated clearly that the (2*S*,3*R*) β-methyl Asp analogues **9**, **11**, and **14** have a considerably lower binding affinity compared to the (2*S*,3*S*) β-methyl Asp analogues **10**, **12**, and **15**. The binding affinities of the (2*S*,3*S*) β-methyl Asp analogues **12** and **15** to the $\alpha_v\beta_3$ receptor are very similar to the affinities of the parent compounds **13** and **16**. This is consistent with findings that were reported by Jackson et al.[15] We have carried out NMR studies and molecular modeling on these peptides which show that there is no significant difference between the backbone

Table 1. Binding affinities of the (2*S*,3*R*) and (2*S*,3*S*) β-methylated peptides to the α,β$_3$ and $\alpha_{IIb}\beta_3$ receptors

	Peptide sequence	$\alpha_v\beta_3$ IC_{50} (μM)	$\alpha_{IIb}\beta_3$ IC_{50}(μM)
9	Ac-c[Cys-Arg-Gly-(*R*)β-MeAsp-Tyr(Me)-Arg-Cys]-NH_2	2.1	4.5
10	Ac-c[Cys-Arg-Gly-(*S*)β-MeAsp-Tyr(Me)-Arg-Cys]-NH_2	0.03	0.15
11	Ac-c[Cys-Arg-Gly-(*R*)β-MeAsp-Thr-Tic-Cys]-NH_2	1.6	>10
12	Ac-c[Cys-Arg-Gly-(*S*)β-MeAsp-Thr-Tic-Cys]-NH_2	0.005	0.186
13	Ac-c[Cys-Arg-Gly-Asp-Thr-Tic-Cys]-NH_2	0.005	n.t.
14	Ac-c[Cys-Arg-Gly-β(*R*)MeAsp-Asp-Val-Cys]NH_2	4.1	>10
15	Ac-c[Cys-Arg-Gly-β(*S*)MeAsp-Asp-Val-Cys]NH_2	0.304	0228
16	Ac-c[Cys-Arg-Gly-Asp-Asp-Val-Cys]NH_2	0.181	n.t.
17	Ac-c[Cys-Arg-Gly-β(*S*)MeAsp-Asp-(t-BuG)-Cys]-NH_2	0.077	0.167
18	Ac-c[Cys-Arg-Gly-β(*S*)MeAsp-β(*S*)Me Asp-(*t*-BuG)-Cys]-NH_2	0.185	0.075
19	c[Arg-Gly-β(*S*)MeAsp-Asp-(*t*-BuG)-Mamb]	0.002	0.122
20	c[Arg-Gly-β(*S*)MeAsp-β(S)MeAsp-(*t*-BuG)-Mamb]	0.004	0.75
21	c[Arg-Gly-Asp-Asp-(*t*-BuG)-Mamb]	0.002	0.140

conformation of the (2*S*,3*R*) β-methylated and (2*S*,3*S*) β-methyl Asp containing peptides in a given sequence. The results of these studies will be reported elsewhere.

The differences between the compounds containing the (2*S*,3*R*) β-methylated and (2*S*,3*S*) β-methylated Asp residues can only be attributed to the side-chain orientation of the Asp side chain. Based on these results it can be assumed that the topochemical array accessible to the (2*S*,3*S*) β-methyl Asp residue is acceptable for binding to the receptors whereas the (2*S*,3*R*) analogues cannot adopt the Asp side-chain orientation required for binding.

Compounds **17**–**21** contain two Asp residue in the sequence and we have incorporated the (2*S*,3*S*) β-methyl Asp residue in one or both of the residues. The results of this series of compounds demonstrate that the selectivity of peptides containing the sequence RGDD can be modified by incorporating a second (2*S*,3*S*) β-methyl Asp residue following the RGD sequence. When comparing compounds **19**, **20**, and **21** it is obvious that compound **19**, which contains only one (2*S*,3*S*) β-methyl Asp residue is very similar in potency and specificity to the parent compound **21**. Both compounds are $\alpha_v\beta_3$ specific and their binding affinity to the $\alpha_v\beta_3$ receptor is a factor of 50–70 higher than that to the $\alpha_{IIb}\beta_3$. The incorporation of a second (*S*)β-methyl Asp residue into the this sequence gives rise to a compound with significantly decreased binding affinity to the $\alpha_{IIb}\beta_3$. Compound **20** essentially maintains the binding affinity to the $\alpha_v\beta_3$ receptor. As a result the compound is considerably more $\alpha_v\beta_3$ specific than the parent compound or compound **19**. The loss of binding affinity to the $\alpha_{IIb}\beta_3$ receptor in compound **20** is equivalent to increased $\alpha_v\beta_3$ selectivity compared to compound **19** and **21**.

These findings will be useful in the design of peptidomimetics with an appropriate side chain orientation and increased $\alpha_v\beta_3$ selectivity.

Experimental

Abbreviations

LiHMDS: lithium hexamethyldisilazide; PhF: phenylfluorenyl; Fmoc: fluorenylmethyl oxycarbonyl; DIBAL: di-isobutylaluminum hydride, PDC: pyridinium dichromate; HOBt: hydroxybenzotriazole; DMF: *N*,*N*-dimethylformamide; DIEA: diisopropylethyl amine: HATU: *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*,*N*-tetramethyluronium hexafluorophosphate; HBTU: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*,*N*-tetramethyluronium; DIC: diisopropylcarbodiimide; TMSCl: trimethylchloro-silane; TFA: trifluoroacetic acid; MeCN: acetonitrile, PE: petroleum ether.

Binding assays

Each well of a microtiter plate (Nunc MaxiSorp) was coated with 120 μL of purified receptor (0.5 μg/mL in assay buffer (2 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM Tris, 150 mM NaCl at pH 7.4) with 4 mM octyl glucoside overnight at room temperature with shaking. The receptor solution was removed, and each well was washed with 200 μL of 0.5% bovine serum albumin in assay buffer for 10 min. This step was repeated for a total of three washes. Fifty microliters of 10-fold dilutions (from 0.0002 to 200 μg/mL) of the inhibitory compounds in assay buffer was added to the wells. Fifty μL of biotinylated ligand (fibrinogen for $\alpha_{IIb}\beta_3$ and vitronectin for for $\alpha_v\beta_3$) in assay buffer was added to the wells. The plates were sealed and incubated overnight at room temperature with shaking.

The ligand/competitor solution was removed, then each well was washed with 250 μL wash buffer (0.05% Tween 20, 50 mM Tris, 150 mM $NaCl_2$, pH 7.4) for five min. This step was repeated for a total of three washes. One hundred microliters of an Avidin Biotin Peroxidase Complex (Pierce Chemical ABC kit 32050) in wash buffer was added to each well. The plates were incubated for 30 min at room temperature with shaking. The ABC solution was removed, then each well was washed with 250 μL wash buffer for 5 min. This step was repeated for a total of three washes. One hundred microliters of a peroxidase substrate (3,3,5,5 tetramethylbenzidine, Pierce Chemical TMB substrate kit 34021) was added to each well.

The conversion of the substrate was monitored kinetically in a microtiter plate reader (Molecular Devices) at 650 nm. Optical density readings were made of each well at 12-s intervals for 10 min. The software for the plate reader was used to calculate the concentration at which 50% of the binding of the ligand to the receptor was inhibited (IC_{50}). The maximal velocity of the enzymatic conversion (V_{max}) was calculated for each well and expressed in milli-optical density units per min (mOD/min). The V_{max} values were plotted as a function of inhibitor concentration, and a four parameter logistic curve was fitted to the data. The inflection point of this curve is the IC_{50}.

Synthesis

***N*-(9-Phenylfluorenyl)-*S*-aspartic acid α-benzyl β-*tert*-butyl ester (2).** To a solution of 2.27 g (12 mMol) L-Asp(O*t*Bu)OH in 25 mL of dry dichloromethane was added 1.62 mL (1.06 equiv) TMSCl at room temperature and the mixture was stirred for 2 h. Dry triethylamine (3.56 mL, 2.13 equiv) and, after 15 min, lead nitrate (3.97 g, 1.0 equiv) and 9-bromo-9-phenylfluorene (3.85 g, 1.0 equiv) dissolved in 20 mL of dry DCM were added. Stirring was continued for at least 3 days at rt. Then methanol (6 mL) was added and after 10 min the mixture was filtered and evaporated. Aqueous citric acid and ethyl acetate were added to the residue. After extraction of the aqueous phase with ethyl acetate the combined organic layer was washed with brine, dried and concentrated to give 4.66 g of the PhF-protected product N-PhF-Asp(O^{t}Bu)OH (90% yield): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.42 (s, 9H, ^{t}Bu), 1.76 (dd, J = 17.4, 4.8, 1H, CH_2), 2.70 (dd, J = 17.4, 3.3, 1H, CH_2), 2.80 (dd, J = 4.8, 3.3, 1H, CHN), 7.1–7.45 (m, 11H, arom. H), 7.7–7.8 (m, 2H, arom H).

The crude N-PhF-Asp(OtBu)OH (2.01 g) was dissolved in 20 mL DMF and 1.78 g (1.05 equiv) Cs_2CO_3 was added at room temperature. After 30 min, benzyl bromide (0.74 mL, 1.2 equiv) was added dropwise and the mixture was stirred for 3 h. Then the suspension was filtered through Celite and concentrated under reduced pressure. Ethyl acetate and satd $NaHCO_3$ solution were added, the aqueous phase was extracted several times and the organic phase was washed with brine, dried and concentrated. The crude product was purified by column chromatography using PE/EA 10:1 to yield 1.85 g (68%) of **2** as colorless solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.44 (s, 9H, *t*Bu), 2.19 (dd, J = 15.0, 5.7, 1H, CH_2), 2.50 (dd, J = 15.0, 5.0, 1H, CH_2), 2.98, 3.34 (each m, 2H, CHN, NH), 4.76, 4.88 (each d, J = 12.3, 2H, CH_2Ph), 7.1–7.4 (m, 16H, arom H), 7.65 (m, 2H, arom H).

***N*-(9-Phenylfluorenyl)-3-methyl-S-aspartic acid α-benzyl β-*tert*-butyl diester (3).** A solution of 1.84 g of **2** in 25 mL of dry THF (5 mL/mmol) was cooled to −78 °C under nitrogen and 4.6 mL (1.2 equiv) LiHMDS (1 M solution in THF) was added. After 1 h, 0.29 mL methyl iodide (1.2 equiv) was added dropwise at −78 °C and then the mixture was warmed up to room temperature overnight for completion of the reaction. The solution was quenched with satd NH_4Cl solution. After extraction with ethyl ether, the combined organic phase was washed with brine, dried and concentrated under reduced pressure. The residue was chromatographed on silica gel (PE/EA 10:1) to give 1.48 g of **3** as a solid in 78% yield and a 2.7:1 ratio of *syn*/*anti** diastereomers according to ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.87* (d, J = 6.9, 3H, Me*), 1.16 (d, J = 7.2, 3H, Me), 1.32, 1.41* (each s, 9H, *t*Bu), 2.49 (m, each 1H, CHMe), 2.82, 2.98* (each m, 1H, CHN), 3.10 (m, each 1H, NH), 4.49*, 4.52, 4.68*, 4.70 (each d, J = 12.3, OCH_2Ph), 7.05–7.4 (m, each 16H, arom H), 7.65 (m, each 2H, arom H).

3(2*S*,3*S*)- and 3(2*S*,3*R*)-Methyl-*S*-aspartic acid β-*tert*-butyl ester (4). A mixture of **3** (2.27 g) and 10% Pd/C in 40 mL of methanol was degassed under reduced pressure and placed under hydrogen (1 atm). After 24 h, the catalyst was removed by filtration through Celite and the filtrate was concentrated. Purification and separation of the diastereomers was achieved on silica gel using a mixture of ethyl acetate/isopropanol/water 8:2:1 as eluent to yield 410 mg of (2*S*,3*S*)-**4** and 120 mg (2*S*,3*R*)-**4** (88%). TLC in EA/*n*BuOH/THF/water 2:2:1:1 R_f 0.27; **(2*S*,3*S*)-4**: $[\alpha]_D^{23\,^\circ C} = -7.0$ (*c* 1, dioxane/water 1:1); ^{1}H NMR (D_2O, 400 MHz) δ 1.22 (d, J = 7.6, 3H, Me), 1.46 (s, 9H, *t*Bu), 3.05 (dq, J = 7.6, 4.4, 1H, CHMe), 3.97 (d, J = 4.4, 1H, $CHNH_2$). **(2*S*,3*R*)-4**: $[\alpha]_D^{23\,^\circ C} = +13.0$ (*c* 1, dioxane/water 1:1); ^{1}H NMR (D_2O, 400 MHz) δ 1.26 (d, J = 7.6, 3H, Me), 1.44 (s, 9H, *t*Bu), 3.13 (dq, J = 7.6, 4.4, 1H, CHMe), 3.89 (d, J = 4.4, 1H, $CHNH_2$).

***N*-(9-Fluorenylmethoxycarbonyl)-3(*S*/*R*)-methyl-L aspartic acid β-*tert*-butyl ester (5).** At 0 °C, 535 mg (2.5 equiv) of sodium carbonate were added to a solution of 410 mg of (2*S*,3*S*)-**4** or in 40 mL dioxane/water (2:1). After 15 min 680 mg of Fmoc chloride (1.3 equiv) was added and stirring was continued for 2 h at 0 °C. Then icebath was removed and the mixture was stirred at room temperature for 1 h to drive the reaction to completion. Afterwards the solvent was removed. The residue was dissolved in ethyl acetate, washed with 0.5 M hydrochloric acid and brine, dried and concentrated. The crude product was chromatographed on silica gel using PE/EA 1:1 as eluent to yield 832 mg (97%) of **(2*S*,3*S*)-5**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.25 (d, J = 6.9, 3H), 1.44 (s, 9H), 2.97 (m, 1H), 4.23 (t, J = 6.9, 1H), 4.40 (d, J = 6.9, 2H), 4.70 (m, 1H), 5.60 (d, J = 8.7, 1H), 7.31 (td, J = 7.5, 1.2, 2H), 7.40 (t, J = 7.2, 2H), 7.59 (m, 2H), 7.76 (d, J = 7.5, 2H). MS (ES) *m*/*e* M^+_{Na} 448.

The same procedure was used to synthesize **(2*S*,3*R*)-5** in 85% yield: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.24 (d, J = 7.2, 3H), 1.47 (s, 9H), 3.18 (m, 1H), 4.24 (t, J = 6.9, 1H), 4.40 (m, 2H), 4.57 (m, 1H), 5.86 (d, J = 8.7, 1H), 7.31 (td, J = 7.5, 1.2, 2H), 7.40 (t, J = 7.2, 2H), 7.60 (m, 2H), 7.76 (d, J = 7.5, 2H); MS (ES) *m*/*e* M^+_H 426, M^+_{Na} 448, $(M-H)^-$ 424.

N*-(9-Phenylfluorenyl)-3(*S*/*R*)-methyl-*S*-homoserine benzyl ester (7)**. A mixture of two diastereomers of **6 (1.8:1) was dissolved in dry DCM (10 mL/mmol) and cooled to −78 °C under nitrogen. A 1 M solution of DIBAL in hexane (3 equiv) was dropwise added. After 2 h the reaction was quenched with methanol and warmed up to room temperature. The mixture was treated with ethyl acetate and sat. K/Na-tartrate solution, the aqueous phase was extracted several times with EA and the combined organic layer was washed with brine, dried and concentrated. Column chromatography on silica gel using PE/EA 8:1 as eluent leads to (2*S*,3*S*)-**7** and (2*S*,3*R*)-**7** in 60% yield.

(2*S*,3*S*)-7: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.87 (d, J = 7.2, 3H, Me), 1.67 (m, 1H, CHMe), 2.64, 3.24 (each br. s, 1H, NH, OH), 2.83 (br. s, 1H, CHN), 3.37 (dd, J = 11.1, 3.3, 1H, CH_2OH), 3.49 (m, 1H, CH_2OH), 4.61, 4.78 (each d, J = 12.3, 1H, OCH_2Ph), 7.05–7.46 (m, 16H, arom H), 7.67–7.7 (m, 2H, arom H).

(2*S*,3*R*)-7: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.48 (d, J = 6.9, 3H, Me), 1.91 (m, 1H, CHMe), 2.53 (d, J = 9.6, 1H, CHN), 3.37 (dd, J = 10.8, 8.7, 1H, CH_2OH), 3.56 (dd, J = 11.1, 3.0, 1H, CH_2OH), 4.33, 4.77 (each d, J = 12.3, 1H, OCH_2Ph), 7.07–7.47 (m, 16H, arom H), 7.70 (d, J = 7.5, 2H, arom H).

***N*-(9-Phenylfluorenyl)-3(*S*/*R*)-methyl-*S*-aspartic acid benzyl ester (8).** To a stirred solution of alcohol (2*S*,3*S*)-**7** or (2*S*,3*R*)-**7** in DMF (10 mL/mmol) were added 6 equiv PDC at room temperature. After 24 h, the mixture was diluted with diethylether and washed with water and brine. The organic layer was dried and concentrated under reduced pressure. Purification of the crude product was achieved by column chromatography on silica gel (PE/EA 3:1).

(2*S*,3*S*)-8. 55% yield, starting from (2*S*,3*S*)-**7**; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.09 (d, J = 7.5, 3H, Me), 2.45 (dq, J = 7.5, 4.5, 1H, CHMe), 3.07 (d, J = 4.5, 1H, CHN), 4.64, 4.83 (each d, J = 12.3, each 1H, OCH_2Ph), 7.07–7.38 (m, 16H, arom H), 7.5–7.7 (m, 2H, arom H).

(2*S*,3*R*)-8. 64% yield, starting from (2*S*,3*R*)-**7**; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.82 (d, $J=6.9$, 3H, Me), 2.53 (dq, $J=10.8$, 6.9, 1H, CHMe), 2.83 (d, $J=10.8$, 1H, CHN), 4.30, 4.77 (each d, $J=12.1$, each 1H, OCH_2Ph), 7.3–7.4 (m, 16H, arom H), 7.70 (m, 2H, arom H).

Peptide synthesis

The peptides were synthesized by stepwise coupling of Fmoc-amino acid derivatives by using standard coupling procedures on solid-phase Rink amide MBHA resin for the disulfides or chlorotrityl resin for the homodet cyclic peptides. Usually, HBTU and a 0.5 M HOBt-solution in DMF were used as activation reagents for peptide bond formation. The reactions were carried out in the presence of 2 equiv of DIEA. For the more hindered coupling to the secondary amino group in tetrahydroisoquinoline carboxylic acid (Tic) the more active HATU was utilized instead of HBTU. The sensitive amino acid cysteine was coupled using a combination of DIC and 0.5 M HOBt solution without any base to minimize the risk of racemization. The side-chain protecting groups were acetamidomethyl (Acm) for cysteine, the *tert*-butyl group for aspartic acid and threonine, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine. The cyclic disulfide constraints in the molecules were formed on-resin by oxidation of the protected peptides with an iodine solution in DMF. During this process the deprotection of the Acm groups from cysteine and formation of the disulfide bond occurred simultaneously. Finally, cleavage of the cyclic peptides from the resin as well as deprotection of all side-chain protecting groups were accomplished by treatment with a trifluoroacetic acid cocktail, which contains triisopropylsilane, phenol and water as scavengers. For the head to tail cyclic peptides the peptides were cleaved from the resin with acetic acid/trifluoroethanol/dichloromethane 1:1:5 for 30 min to 1 h. The peptides were cyclized in 2.5 mM concentration in DMF using 10 equiv of $NaHCO_3$ and DPPA at room temperature. The side chain protecting groups were removed using trifluoroacetic acid in the presence of triisopropylsilane (2%), anisole (5%), and water (5%). Peptide purification was performed by preparative RP-HPLC on a Vydac C18 column using a gradient of acetonitrile in water with 0.1% TFA.

(Ac-c[Cys-Arg-Gly-β(2*S*,3*S*)Me-Asp-Tyr(Me)-Arg-Cys]-NH_2. RP-HPLC: 18.5 min (10–30% $MeCN/H_2O$, 0.1% TFA, 30 min, 10 mL/min); $C_{37}H_{58}N_{14}O_{11}S_2$ (938 g/mol); MS (ES) *m*/*e* M_H^+ 939, M_{Na}^+ 961, $(M-H)^-$ 937, $(M-TFA)^-$ 1051; ^{1}H NMR.

Ac-c[Cys-Arg-Gly-β(2*S*,3*R*)Me-Asp-Tyr(Me)-Arg-Cys]-NH_2. RP-HPLC: 19.7 min (10–30% $MeCN/H_2O$, 0.1% TFA, 30 min, 10 mL/min); $C_{37}H_{58}N_{14}O_{11}S_2$ (938 g/mol); MS (ES) *m*/*e* M_H^+ 939, M_{Na}^+ 961, $(M-H)^-$ 937; ^{1}H NMR.

Ac-c[Cys-Arg-Gly-β(2*S*,3*S*)Me-Asp-Asp-Val-Cys]-NH_2. RP-HPLC: 12.0 min (10–30% $MeCN/H_2O$, 0.1% TFA, 30 min, 10 mL/min); $C_{30}H_{49}N_{11}O_{12}S_2$ (819 g/mol); MS (ES) *m*/*e* M_H^+ 820, $(M-H)^-$ 818; ^{1}H NMR.

Ac-c[Cys-Arg-Gly-β(2*S*,3*R*)Me-Asp-Asp-Val-Cys]-NH_2. RP-HPLC: 12.3 min (10–30% $MeCN/H_2O$, 0.1% TFA, 30 min, 10 mL/min); $C_{30}H_{49}N_{11}O_{12}S_2$ (819 g/mol); MS (ES) *m*/*e* M_H^+ 820, M_{Na}^+ 842, $(M-H)^-$ 818; ^{1}H NMR.

Ac-c[Cys-Arg-Gly-β(2*S*,3*S*)Me-Asp-Thr-Tic-Cys]-NH_2. RP-HPLC: 16.2 min (10–50% $MeCN/H_2O$, 0.1% TFA, 30 min, 10 mL/min); $C_{35}H_{51}N_{11}O_{11}S_2$ (865 g/mol); MS (ES) *m*/*e* M_H^+ 866, $(M-H)^-$ 864; ^{1}H NMR.

Ac-c[Cys-Arg-Gly-β(2*S*,3*R*)Me-Asp-Thr-Tic-Cys]-NH_2. RP-HPLC: 17.1 min (10–50% $MeCN/H_2O$, 0.1% TFA, 30 min, 10 mL/min); $C_{35}H_{51}N_{11}O_{11}S_2$ (865 g/mol); MS (ES) *m*/*e* M_H^+ 866, $(M-H)^-$ 864.

Acknowledgements

We gratefully acknowledge financial support from the National Institutes of Health (DK51938-03).

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