Journal of Peptide Science
J. Peptide Sci. **7**: 82–91 (2001)

Synthesis, Conformational Analysis and Biological Activities of Lanthionine Analogs of a Cell Adhesion Modulator

HAITAO LI[1], XIAOHUI JIANG[2] and MURRAY GOODMAN*

Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA 92093-0343, USA

Received 9 June 2000
Accepted 29 August 2000

Abstract: Cell adhesion is critical for many biological processes, such as hemostasis, wound healing, tumor metastasis and inflammation. Integrins are important mediators of cell adhesion. The integrin $\alpha_4\beta_1$, also known as VLA-4, is a cell surface receptor involved in inflammation. A cyclic peptide, 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH, is a potent antagonist to VLA-4 with an IC_{50} of 2.4 nM. In the current study, we synthesized the lanthionine analogs of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and determined the conformations of both the parent compound and its lanthionine analog in solution by NMR and computer simulations. The lanthionine analog retains its selectivity to VLA-4 with high nanomolar potency. Both molecules adopt similar topological arrangements in their conformations, while some important differences remain in the sulfur bridge region, which may cause the difference in potency. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cell adhesion; integrin; VLA-4; lanthionine; conformational analysis

INTRODUCTION

Cell adhesion plays an important role in many biological processes, such as wound healing, tumor metastasis, immunity, and inflammation [1]. Cell surface adhesion molecules come from different families of genes, including immunoglobulins, selectins and integrins. Among them, integrins are particular important mediators of cell migration and intramolecular signaling [2]. Because many integrin–ligand interactions are potential therapeutic targets in human diseases, there has been great interest in understanding the molecular mechanisms that mediate the specific binding. The first breakthrough in this area came with the identification of a tripeptide sequence, Arg-Gly-Asp (RGD) as a key recognition motif [3]. This sequence is at the central cell-binding domain of fibronectin. Subsequent studies revealed that RGD motifs exist in a wide range of integrin ligands, as well as in other proteins that are potential ligands.

The integrin $\alpha_4\beta_1$, also known as very late antigen 4 (VLA-4), is a receptor on the surface of leukocyte cells involved in inflammation. In inflammatory reactions leukocytes adhere to the blood vessel well at sites of immunologic challenge. This is the crucial first step in triggering an effective immune response. The integrin $\alpha_4\beta_1$ serves as a receptor for

Abbreviations: EDC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimde; BOP, benzotriazole 1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate; Cbz, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Me, methyl; DMAD, dimethylazodicarboxylate; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; Fm, 9-fluorenylmethyl; DCM, dichloromethane; DMAP, 4-(dimethylamino)-pyridine; DCC, dicyclohexylcarbodimide; Fmoc, 9-fluorenylmethoxycarbonyl; 4-EM, 4-ethyl morpholine; PMC, 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl; HOAt, 1-hydroxy-7-azabenzotriazole; 1-FCA, 1-fluorenecarboxylic acid; TFA, trifluoroacetic acid; Thz, thiazolidine-4-carboxylic acid.

* Correspondence to: Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA 92093-0343, USA.
[1] Current address: Pfizer Global Research and Development, La Jolla, CA 92037, USA.
[2] Current address: Molecular Simulations Incorporated, San Diego, CA 92121, USA.

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the cytokine-inducible endothelial cell surface protein, vascular cell adhesion molecule-1 (VCAM-1) as well as the ECM protein fibronectin. Results of several *in vivo* experiments suggest that the inhibition of VLA-4-dependent cell adhesion may prevent, inhibit or alter several inflammatory and autoimmune pathologies [4–6].

A cyclic pentapeptide, H-Arg-c[Cys-Asp-Thz-Cys]-OH (where Thz denotes a thiazolidine-4-carboxylic acid) has been found to be able to inhibit VAL-4 adhesion to fibronectin [7]. This pentapeptide was developed based on the RGD tripeptide sequence. The compound modulates cell adhesion by competing with ligands containing the appropriate amino acid sequence to bind to VLA-4. As the result, the cell adhesion protein, such as fibronectin, is sufficiently inhibited from binding to receptor cells so as to reduce cell adhesion. This peptide does not inhibit adhesion to other extracellular matrix proteins, including laminin and vitronection. This specificity suggests that the compound has great potential therapeutic utility for treating inflammatory disease. A derivative of this pentapeptide, 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH **1** (Figure 1), exhibits better potency as an antagonist to VLA-4 with an IC_{50} of 2.7 nM.

In order to study the structure–activity relationship of 1-FCA-Arg-c[Cys-Asp-ThzCys]-OH, we synthesized the lanthionine analog of this molecule **2** (Figure 1) and studied their conformations in solution by NMR and computer simulations. Lanthionine is an unusual amino acid composed of two alanine-like residues bridged by a thioether linkage. This peptidomimetic of cystine is an important building block presented in a family of bioactive peptides called lantibiotics [8]. Lanthionine-containing peptide has more constrained structure and is more stable towards enzymatic degradation [9].

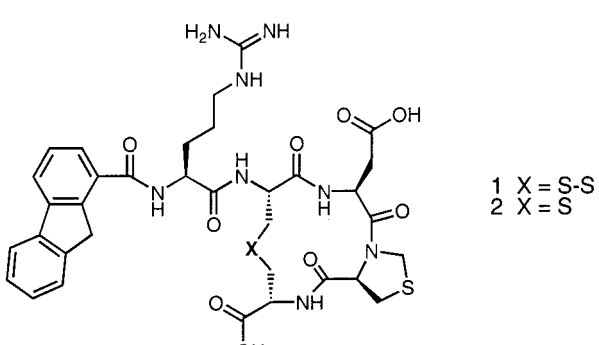

Figure 1 The structure of 1-FCA-Arg-c[Cys-Asp-Thz-cys]-OH **1** and its lanthionine analog **2**.

Previous studies have shown that the opening of disulfide is one of the pathways leading to the degradation of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH. Therefore, the lanthionine analog may have better pharmacological properties if it can maintain the potency as an antagonist to VLA-4.

MATERIAL AND METHODS

General

The protected amino acids were purchased either from Bachem Bioscience (Torrance, CA) or Novabiochem (San Diego, CA). EDC, DCC, BOP and TFA were purchased from Chem-Impex International (Wood Dale, IL). Fmoc-Asp(O*t*Bu)-NCA, Fmoc-Arg(PMC)-NCA were obtained from Bioresearch Incorporated (San Diego, CA). The new coupling reagent, 3-(diethoxyphosphoryloxy)-1,2,3-benzotrazin-4(3H)-one (DEPBT) [10,11], was a kind gift from Professor Y.H. Ye in Peking University, China. The parent compound, L-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH **1**, was a kind gift from Dr J.W. Tilley at Hoffmann-LaRoche Incorporated (Nutley, NJ). Thin layer chromatography (TLC) was carried out on silica gel 60 (F_{254}) plates (E.M. Science).

Cbz-Ser-β-lactone 3

To triphenylphosphine (8.42 g, 32 mmol) in THF (220 ml) at -78°C DMAD (3.53 ml, 32 mmol) was added dropwise. White slurry was obtained after 10 min. To this dispersion, Cbz-Ser-OH (7.66 g, 32 mmol) in THF (48 ml) was added dropwise. The slurry obtained was stirred at -78°C for 40 min, then at room temperature for 4 h. The solvent was removed under reduced pressure to give a light yellow oil. Flash column chromatography (EtOAc:hexanes = 1:2) afforded product **3** as a solid (3.42 g, 49%). FAB-MS m/z 222 $(M+H)^+$, 244 $(M+Na)^+$. ^{1}H-NMR (360 MHz, $CDCl_3$) δ 4.43 (m, 2H, 20), 5.12 (m, 3H, α and methylene from Cbz), 5.49 (d, $J = 7$ Hz, 1H, amide), 7.36 (m, 5H, aromatics).

Boc-Cys-OMe 4

To 40% KOH (25 ml) and diethyl ether (100 ml) in an ice bath, MNNG (3.6 g, 25 mmol) was slowly added. The solution turned yellow immediately. After stirring for 10 min, the ether layer was transferred to a solution of Boc-Cys-OH (2.2 g, 10 mmol) in MeOH (10 ml). The reaction mixture was stirred at 0°C for 30 min and the solvent was removed

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under reduced pressure. The residue was dissolved in EtOAc (250 ml). The organic layer was washed with 10% Na_2CO_3 (3 × 10 ml), brine (3 × 10 ml), 5% $NaHSO_4$ (3 × 10 ml) and dried over Na_2SO_4. The solvent was taken to dryness to give the desired product **4** as a colorless oil (2.31 g, 98%). $R_f = 0.61$ (EtOAc:hexanes = 1:1). ^{1}H-NMR (360 MHz, $CDCl_3$) δ 1.43 (s, 9H, Boc), 2.94 (m, 2H, β), 3.75 (s, 3H, OMe), 4.58 (m, 1H, α), 5.38 (br, 1H, amide).

The Dipeptide Lanthionine Building Block 5

Cbz-Ala$_L$-OH
|
S
|
Boc-Ala$_L$-OMe

To Boc-Cys-OMe (3.9 g, 15.9 mmol) in DMF (25 ml) Cbz-Ser-β-lactone (2.34 g, 10.5 mmol) and Cs_2CO_3 (1.76 g, 5.2 mmol) were added. The reaction was stirred at room temperature for 5 h. The solvent was removed under reduced pressure. The crude product was dissolved in EtOAc (250 ml), washed with brine (3 × 10 ml), 5% $NaHSO_4$ (3 × 10 ml), dried over Na_2SO_4 and evaporated to dryness. Column chromatography ($CHCl_3$:MeOH = 90:10) gave the desired lanthionine **5** as a foam (1.44 g, 30%). $R_f = 0.17$ ($CHCl_3$:MeOH = 9:1). ^{1}H-NMR (360 MHz, $CDCl_3$) δ 1.42 (s, 9H, Boc), 2.78–3.15 (m, 4H, β of lanthionine), 3.73 (s, 3H, OMe), 4.35–5.00 (m, 2H, α), 5.14 (s, 2H, methylene from Cbz), 5.38 (d, $J = 7$ Hz, 1H, amide), 5.84 (br, 1H, amide), 7.11–7.39 (m, 5H, aromatics).

The Fully Protected Dipeptide Lanthionine Building Block 6

Cbz-Ala$_L$-OFm
|
S
|
Boc-Ala$_L$-OMe

To the foam of dipeptide lanthionine **5** (1.44 g, 3.15 mmol) in DCM (30 ml) 9-fluorenemethanol (0.62 g, 3.15 mmol) was added. The mixture was chilled to 0°C before DMAP (3.8 mg, 0.0315 mmol) and DCC (0.71 3.47 mmol) were added. A precipitate quickly appeared. The reaction mixture was stirred at 0°C for 1 h and the stirring was continued at room temperature for 3 h. After removal of the precipitate by filtration, the filtrate was taken to dryness. The residue was purified by column chromatography (EtOAc:petroleum ether = 1:3) to give the fully protected lanthionine **6** as a foam (1.54 g, 77%). R_f = 0.45 (EtOAc:petroleum ether = 1:3). FAB-MS m/z 767 $(M + Cs)^+$. ^{1}H-NMR (360 MHz, $CDCl_3$) δ 1.41 (s, 9H, Boc), 2.75–3.00 (m, 4H, β of lanthionine), 3.69 (s, 3H, OMe), 4.15–4.65 (m, 5H, 2α and 3H from OFm), 5.12 (s, 2H, methylene from Cbz), 5.19 (d, $J = 7$ Hz, 1H, amide), 5.65 (d, $J = 7.2$ Hz, 1H, amide), 7.17–7.80 (m, 13H, aromatics).

Fmoc-Asp(O*t*Bu)-Thz-OH 7

To a solution of $NaHCO_3$ (4.18 g, 49 mmol) in water (60 ml) H-Thz-OH (3.31 g, 25 mmol) was added. Once the solution became clear, THF (40 ml) was added, followed by addition of Fmoc-Asp(O*t*Bu)-NCA (2.18 g, 4.98 mmol) within 2 min. The reaction mixture was stirred at room temperature for 30 min. The solution was acidified with $NaHSO_4$ until a pH 4 and extracted with EtOAc five times (5 × 100 ml). The combined EtOAc extracts were washed with brine, dried over $MgSO_4$ and taken to dryness. The crude product was subjected to column chromatography (EtOAc:HOAc = 100:1). The pure dipeptide **7** was obtained as a foam (1.34 50%). $R_f = 0.29$ (toluene:HOAc = 85:15). FAB-MS m/z 527 $(M + H)^+$. ^{1}H-NMR (360 MHz, $CDCl_3$) δ 1.42 (s, 9H, *t*Bu), 2.52–2.83 (dd, 2H, β of Asp), 3.17–3.30 (m, 2H, β of Thz), 4.00–5.14 (m, 7H, 2α, 3H from Fmoc, 2 δ H from Thz), 5.75–5.93 (d, $J = 8.6$ Hz, 1H, amide), 7.17–7.19 (m, 8H, aromatics).

The Lanthionine-containing Tetrapeptide 8

Cbz-Ala$_L$-OFm
|
S
|
Fmoc-Asp(OtBu)-Thz-Ala$_L$-OMe

To the fully protected dipeptide lanthionine **6** (1.32 g, 2.08 mmol) 50% TFA/DCM (25 ml) was added. It was stirred at room temperature for 30 min. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (150 ml) and washed with 5% $NaHCO_3$ and brine. The organic phase was dried over Mg_2SO_4 and evaporated to dryness. To this deprotected dipeptide in DMF (20 ml) Fmoc-Asp(O*t*Bu)Thz-OH **7** (1.31 g, 2.49 mmol) was added. The flask was chilled to 0°C before BOP (1.1 g, 2.49 mmol) and 4-EM (0.53 µl, 4.14 mmol) were added. The solution was stirred at 0°C for 30 min and gradually warmed to room temperature and stirred overnight. The solvent was removed under reduced pressure. The crude product was dissolved in EtOAc (250 ml), washed with 5% $NaHCO_3$ (3 × 5 ml), brine (3 × 5 ml), 5% $NaHSO_4$ (3 × 5 ml), dried over Na_2SO_4 and evaporated to dryness. Flash column chromatography (EtOAc:petroleum ether = 2.5:1) gave the desired tetrapeptide **8** as a foam (1.63 g, 76%). $R_f = 0.62$ (EtOAc:petroleum ether = 2.5:1). FAB-MS m/z 1065 $(M + Na^+)$. ^{1}H-NMR (360

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MHz, $CDCl_3$) δ 1.36 (s, 9H, *t*Bu), 2.51–3.16 (m, 6H, β of lanthionine, β of Asp), 3.40–3.49 (m, 2H, β from Thz), 3.68 (s, 3H, OMe), 4.09–4.82 (m, 12H, 4α, 2Hδ from Thz, 6H from OFm and Fmoc), 5.10 (s, 2H, methylene from Cbz), 5.43 (b, 2H, amides), 5.96 (br, 1H, amide), 7.23–7.74 (m, 21H, aromatics).

The Lanthionine-containing Cyclized Tetrapeptide 9

Cbz-Ala_L-Asp(OtBu)-Thz-Ala_L-OMe
(S)

Deprotection. To the linear tetrapeptide (229 mg, 0.219 mmol) in DMF (8 ml) piperidine (2 ml) was added. After stiffing at room temperature for 2 h, the solvent was removed under reduced pressure. The crude product was purified by column chromatography ($CHCl_3$:MeOH:NH_4OH = 85:15:3). The deprotected peptide was obtained as a foam (80 mg, 61%). FAB-MS m/z 643 $(M + H)^+$.

Cyclization. To the deprotected peptide (150 mg, 0.233 mmol) in DMF (150 ml) DEPBT (140 mg, 0.468 mmol) and 4-EM (29.7 µl, 0.234 mmol) were added. The reaction mixture became yellow. It was stirred at room temperature for 24 h before another batch of DEPBT (70 mg, 0.234 nimol) was added. After stirring at room temperature for another 24 h, the solvent was removed under reduced pressure. The crude product was dissolved in EtOAc (250 ml), washed with 5% $NaHCO_3$ (3 × 2 ml), brine (3 × 2 ml), 5% $NaHSO_4$ (3 × 2 ml), dried over Na_2SO_4 and evaporated to dryness. Column chromatography ($CHCl_3$:MeOH = 100:1) gave the desired tetrapeptide **9** as a white foam (67 mg, 46%). FAB-MS m/z 625 $(M + H)^+$. ^{1}H-NMR (360 MHz, $CDCl_3$) δ 1.36 (s, 9H, *t*Bu), 2.65–3.00 (m, 6H, β of lanthionine, β of Asp), 3.25–3.44 (m, 2H, β from Thz), 3.73 (s, 3H, OMe), 4.43–4.82 (m, 6H, 4α, 2Hδ from Thz), 5.03 (m, 3H, methylene from Cbz and an amide), 5.22 (b, 1H, amide), 5.91 (br, 1H, amide), 7.11–7.40(m, 5H, aromatics).

The Fully Protected Cyclic Pentapeptide 10

Fmoc-Arg(PMC)-Ala_L-Asp(OtBu)-Thz-Ala_L-OMe
(S)

To the cyclized tetrapeptide **9** (67 mg, 0.107 mmol) in CH_3OH (10 ml) and HOAc (0.5 ml) 30% Pd/C (70 mg) was added. The reaction mixture was placed under hydrogenation apparatus for 24 h. It was filtered through celite and the filtrate was taken to dryness. To this deprotected material in THF (2 ml) 4-EM (13.6 µl, 0.107 mmol) and Fmoc-Arg(PMC)-NCA (81 mg, 0.118 mmol) were added. The reaction mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure. The resulting crude product was subjected to column chromatography ($CHCl_3$:MeOH = 100:1.5). The desired pentapeptide **10** was obtained as a solid (82 mg, 68%). FAB-MS m/z 1135 $(M + H)^+$.

The Fully Protected Target Molecule 11

1-FCA-Arg(PMC)-Ala_L-Asp(OtBu)-Thz-Ala_L-OMe
(S)

To pentapeptide **10** (32 mg, 0.028 mmol) in DMF (6 ml) piperidine (1.2 ml) was added. After stirring at room temperature for 2 h, the solvent was removed under reduced pressure. The deprotected peptide was purified by HPLC (30% to 65% CH_3CN/H_2O, 0.1% TFA in 30 min). The deprotected product was obtained as a powder. R_t = 20 min. FAB-MS m/z 913 $(M + H)^+$, 935 $(M + Na)^+$. To a solution of fluorene-1-carboxylic acid (9.4 mg, 0.044 mmol) in DMF (1 ml) HOAt (6 mg, 0.044 mmol) and EDC (8.4 mg, 0.044 mmol) were added. The reaction mixture was stirred at room temperature for 40 min before being transferred to the solution of the above deprotected material in DMF (1 ml). To this reaction mixture 4-EM (6 µl, 0.044 mmol) was added at 0°C. The solution was stirred at 0°C for 30 min and gradually warmed to room temperature and stirred overnight. The solvent was removed under reduced pressure. The crude product was dissolved in $CHCl_3$ (60 ml), washed with 5% $NaHCO_3$ (2 × 1 ml), brine (2 × 1 ml), 5% $NaHSO_4$ (2 × 1 ml), dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by HPLC (50% to 80% CH_3CN/H_2O, 0.1% TFA in 30 min). The pure pentapeptide **11** was obtained as a powder (20 mg, 83%). R_t = 18.7 min.

The Lanthionine Analog of the VLA-4 Antagonist Methyl Ester

1-FCA-Arg-Ala_L-Asp-Thz-Ala_L-OMe
(S)

To the fully protected peptide **11** (20 mg, 0.018 mmol) the cleavage mixture (0.25 ml H_2O, 0.25 ml anisole and 4.5 ml TFA) was added. After stirring at room temperature for 1 h, the solvent was removed under reduced pressure. The crude product was purified by HPLC (29% CH_3CN/H_2O, 0.1% TFA, isocratic). The title compound was obtained as a powder (8 mg, 58%). HR-MS (FAB): calcd.: 783.2594 $(M + H)^+$; found 783.2575.

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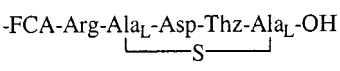
The Lanthionine Analog of the VLA-4 Antagonist 2



To the fully protected peptide **11** (20 mg, 0.018 mmol) in MeOH (6 ml) 2N LiOH solution (94 μl, 0.054 mmol) was added. After stirring at room temperature for 10 h, the reaction was quenched with HOAc. The solvent was removed under reduced pressure. The crude product was dissolved in $CHCl_3$ (60 ml), washed with brine (2×1 ml), 5% $NaHSO_4$ (2×1 ml), dried over Na_2SO_4 and evaporated to dryness. To this saponified peptide the cleavage mixture (0.25 ml H_2O, 0.25 ml anisole and 4.5 ml TFA) was added. After stirring at room temperature for 1 h, the solvent was removed under reduced pressure. The crude product was purified by HPLC (24% CH_3CN/H_2O, 0.1% TFA, isocratic). Compound **2** was obtained as a white powder (12 mg, 85%). HR-MS (FAB): calcd. 769.2421 $(M + H)^+$; found 769.2437.

NMR Experiments

The samples were dissolved in DMSO-d_6 at concentrations of approximately 3–6 mM. The ^{1}H-NMR spectra were measured on a Bruker AMX 500 spectrometer operating at 500 MHz. The one-dimensional (1D) spectra contain 16384 data points with a spectral width of 6000–6500 Hz. The 1D spectra acquired at temperatures of between 300 and 320 K were used to measure the amide proton temperature coefficients. The two-dimensional (2D) spectra were acquired at 300 K for assignments and NOE measurements, 300–320 K for the total correlation spectroscopy (TOCSY) to measure the amide proton temperature coefficients that are overlapped in the 1D spectra. The TOCSY experiments were performed using the MLEV-17 sequence with the time proportional phase increment (TPPI). A mixing time of 75 ms with a spin-locking field of 10 kHz was employed. The rotating frame nuclear Overhauser (ROESY) experiments were carried out with a mixing time around 150 ms. A spin-locking field of 2.5 kHz was used. The TOCSY and ROESY experiments were obtained using 2048 data points in the f2 domain and 256–512 data points in the f1 domain.

The spectra were processed by using the program FELIX (Molecular Simulations Incorporated, San Diego, CA, USA). Zero filling was applied in the f1 and f2 domains to obtain a matrix of 2048×2048 data points. Multiplication with a phase-shifted sine-bell function was employed to enhance the spectra. Chemical shifts were measured using DMSO-d_6 ($\delta = 2.49$ ppm) as an internal standard.

Computer Simulations

The distance geometry program DGEOM was used to generate structures consistent with the distance constraints derived from NOEs. A family of 500 initial conformations was generated. Energy minimization was carried out to remove bad contacts among the atoms. The force field CFF91 was employed. A distance-dependent dielectric constant (4*r) was used to simulate the solvent effects. The steepest descents algorithm, followed by the quasi-Newton–Raphson algorithm (BFGS), was used for minimization until the maximum derivative was less than 0.01 kcal/mol Å.

Cluster analysis was performed to divide the conformers into groups according to their backbone torsion angles. The lowest energy conformation of each group was used for further studies.

The distance constraints and ϕ angles derived from NMR measurements were compared with the structures after cluster analysis. An error of $\pm 30°$ was tolerated for the angles. The structures that violate the above criteria were discarded.

Restrained molecular dynamics were carried out on the structure with the lowest energy and satisfied the NMR data to investigate its dynamic properties. The distance constraints derived from NOEs were applied during the simulations. A quadratic force constant of 25 kcal/mol Å was employed. The simulations were performed at 298 K for 100 ps with the time step of 1 fs. Trajectories were collected every 1 ps for analysis.

RESULTS AND DISCUSSION

Chemistry

The synthesis of the lanthionine analog **2** was carried out in solution. A convergent strategy for the synthesis was planned, as shown in Figure 2. Fragment condensation of the dipeptide lanthionine building block **II** and the dipeptide **III** resulted in the linear tetrapeptide. After cyclization, the peptide was coupled to 1-FCA-Arg-OH **I** to furnish the desired pentapeptide.

The chemistry was initiated with the preparation of lanthionine building block (Figure 3). A previously published procedure from our laboratories was followed to prepare the lanthionine building block **5** [12]. The free carboxyl in **5** was then

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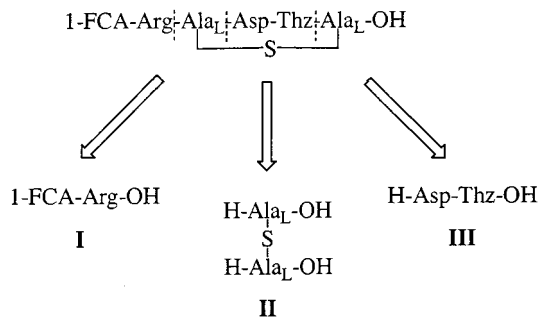

Figure 2 Retroanalysis of the synthesis of the lanthionine analog of the cell adhesion modulator.

protected as 9-fluorenylmethyl (Fm) ester to prepare the fully protected lanthionine **6**.

The dipeptide Fmoc-Asp(O*t*Bu)-Thz-OH **7** was obtained by treatment of Fmoc-Asp(O*t*Bu)-NCA with unprotected Thz in basic solution. Since the NCA (the α-amino acid *N*-carboxy anhydride) is an activated form of an amino acid, in this case an aspartic acid, no other coupling reagent was needed. More interestingly, it was not necessary to protect the carboxyl terminal of the amino acid. An unprotected amino acid, such as H-Thz-OH, could be directly used for coupling.

After treatment with TFA, the *N*-deprotected lanthionine was readily coupled with dipeptide Fmoc-Asp(O*t*Bu)-Thz-OH **7**, which resulted in tetrapaptide **8** in a 76% yield. Treatment of the linear tetrapeptide **8** with piperidine simultaneously removed Fmoc protection on Asp and –OFm from one of the carboxyls of the lanthionine. The peptide was cyclized with the new coupling reagent DEPBT in 55% yield [10,11].

The original plan was to couple the cyclized peptide **9** with 1-FCA-Arg-OH. However, a small-scale reaction revealed substantial racemization at the arginine during this particular coupling. The *N*-terminal 1-fluorenecarboxylic acid (1-FCA) contributed to the racemization, since it greatly facilitated 5(4H)-oxazolone formation.

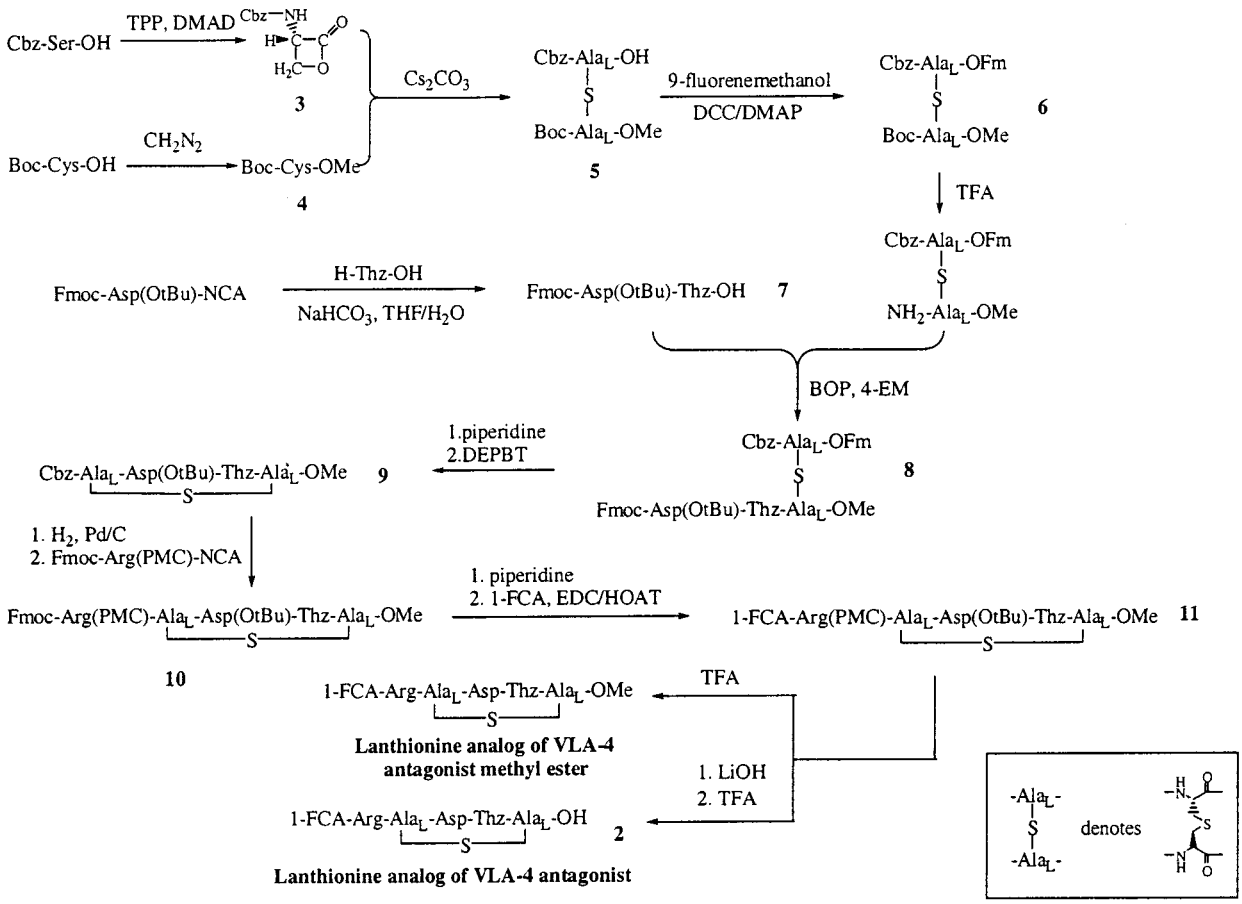

Figure 3 The synthesis of the lanthionine analog of VLA-4 antagonist **2**.

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To avoid this problem of racemization, the resulting cyclized peptide **9** was hydrogenated, followed by a coupling with Fmoc-Arg (PMC)-NCA to afford the pentapeptide **10**. The peptide **10** was treated with piperidine to remove the Fmoc protection and coupled to 1-FCA with the carbodiimide method. The fully protected peptide **11** was then ready for deprotection.

One batch of compound **11** was treated with TFA to afford the methyl ester analog. The other batch was saponified followed by TFA deprotection to furnish the free acid analog **2**. We determined the effect of the two different *C*-terminal groups on biological activity.

Biological Activities

Both lanthionine analogs show antagonist activity towards VLA-4 integrin. The IC_{50} of the parent compound is 2.7 nM. The IC_{50} of the lanthionine analog of the parent compound and its methyl ester are 557 and 209 nM, respectively. The lanthionine analog is less potent than the parent compound although the structural difference between the two analogs is only a sulfur atom.

The parent compound and the lanthionine analog show no affinities to other integrin receptors. In the platelet aggregation tests, the parent molecule, 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH, and its lanthionine analog do not inhibit platelet aggregation (Table 1). In the other test, the binding affinities of the parent compound and its lanthionine analog to $\alpha_v\beta_3$ were measured using JY cell based assay. None of the compounds resulted in inhibition of JY cell binding to vitronectin. The IC_{50} of all three compounds to $\alpha_v\beta_3$ are greater than 100 μg/ml. Although the lanthionine analog is less potent than the parent disulfide compound, the selectivity towards different integrin receptors remains unchanged. All of the compounds exhibit the same selectivity toward the $\alpha_v\beta_1$ (VLA-4) integrin.

Table 1 The Inhibition of Platelet Aggregation in Citrate by 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and its Lanthionine Analogs

Compounds	Concentration (μM)	Inhibition of aggregation (%)
GRGDSP peptide	135	50
Parent disulfide compound	142	0
Lanthionine analog of parent compound	120	0
Lanthionine analog of parent compound methyl ester	139	0

NMR Spectroscopy Studies

The NMR studies were carried out to determine the conformations of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH **1** and its lanthionine analog **2** in DMSO-d_6 solution at 300 K. The ^{1}H chemical shifts of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]OH and its lanthionine analog are listed in Table 2. The $J_{NH\text{-}\alpha}$ coupling constants are used to calculate the ϕ angles, and the results are listed in Table 3. The temperature coefficients of the amide protons are shown in Table

Table 2 The Chemical Shifts of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and its Lanthionine Analog

Residue	(δ in ppm)	Parent compound	Lanthionine analog
Arg[1]	NH	8.62	8.42
	α	4.47	4.50
	β	1.85, 1.70	1.75, 1.68
	γ, δ	1.60, 3.13	1.55, 3.12
Cys[2,a]	NH	7.98	8.39
	α	4.58	4.53
	β	3.07	2.65
Asp[3]	NH	8.48	8.67
	α	4.66	4.93
	β	2.70	2.74, 2.54
Thz[4]	α	4.97	5.05
	β	3.38, 3.24	3.43, 3.05
	δ	4.88, 4.31	5.08, 4.15
Cys[5,a]	NH	8.66	7.91
	α	4.50	4.36
	β	3.27, 2.88	3.07, 2.84
1-FCA[b]	2H, 3H	7.63, 7.50	8.05,7.49
	4H, 5H	8.06, 7.95	7.63, 7.93
	6H, 7H	7.40, 7.33	7.39, 7.34
	8H, 9H	7.60, 4.13	7.61, 4.10

[a] This residue is lanthionine in the analog.
[b] The numbering of the 1-FCA residue is following:

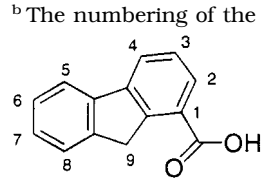

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Table 3 The Coupling Constants $J_{NH\text{-}\alpha}$ of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and its Lanthionine Analog

Residue	Parent compound		Lanthionine analog	
	$J_{NH\text{-}\alpha}$ (Hz)	Calculated ϕ (°)	$J_{NH\text{-}\alpha}$ (Hz)	Calculated ϕ (°)
Arg[1]	8.02	−91, −148	8.03	−91, −148
Cys[2,a]	5.87	34, 85, −73, −166	8.04	−91, −148
Asp[3]	6.60	43, 73, −79, −160	7.34	−84, −155
Cys[5,a]	7.34	−85, −154	7.33	−84, −155

[a] This residue is lanthionine in the analog.

4. The important NOEs obtained from ROESY experiments are compared in Table 5. The intensities of ROESY cross-peaks were calibrated against the cross-peaks of the Hβ and Hδ protons in Thz and were qualitatively classified as strong (s), medium (m) or weak (w). The interproton distance was assigned as 2.5 Å or less for a strong NOE, 2.5–3.0 Å for a medium NOE and 3.0–4.0 Å for a weak NOE.

For most of the residues in the two compounds, the NOEs of Hα and NH between residues i and $i+1$ are strong, while the NOEs between NH and Hα in the same residue are in the medium range. This observation indicates most of the residues are in the extended β conformation. This is consistent with the $J_{NH\text{-}\alpha}$ coupling constants. In addition, the measurements of the temperature coefficients (-ppb/K) of the amide protons suggest that no hydrogen bonds exist inside the molecule. In both molecules, the NOE of Asp[3]-Hα-Thz[4]-Hα is strong. This NOE is a characteristic indication of a *cis* amide bond between Asp[3] and Thz[4]. The difference between the two molecules arises from the Thz[4]-Cys[5] region. The NOE of Cys[5]-NH-Thz[4]-Hα is strong in the parent compound while it is weak in the lanthionine analog. On the other hand, the NOE of Cys[5]-NH-Thz[4]-Hδ is strong in the lanthionine analog while it does not exist in the parent molecule. In addition, a weak NOE of Asp[3]-NH-Cys[5]-NH was observed for the lanthionine analog.

Table 4 The Temperature Coefficients (-ppb/K) of the Amide Protons of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and its Lanthionine Analog

Residue	Parent compound	Lanthionine analog
Arg[1]	4.7	4.4
Cys[2,a]	5.0	6.6
Asp[3]	5.1	2.6
Cys[5,a]	2.0	4.1

[a] This residue is lanthionine in the analog.

Computer Simulations

Distance constraints derived from NOE intensity were employed in distance geometry calculations to generate conformations of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH **1** and its lanthionine analog **2**. Cluster analysis and energy minimiation were use to select conformers that are consistent with NMR results. For each compound there was only one cluster satisfied with the NNR data. The selected conformations with the lowest energy of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and its lanthionine analog are shown in Plate 1. Both molecules have similar topological placements, exocyclic 1-FCA-Arg, a *cis* amide bond between Asp[3]-Thz[4] and a disulfide or a monosulfide to close the ring structure. The differences of the two molecules exist at the Thz[4]-Cys[5] region. As shown in Plate 1, the amide proton of Cys[5] is at the same side as the Hα of Thz in the parent compound, while in the lanthionine analog, the amide proton of residue **5** is at the opposite side of the Hα of Thz[4] and it is close to the Hδ of Thz[4]. Therefore, the amide proton of residue **5** is also at the same side of the rincr plane as the amide proton of Asp[3] in the lanthionine analog. These structural differences are consistent with the NMR observations.

The superposition of the backbone of the parent compound and its lanthionine analog is presented in Plate 2. As discussed above, the placements of side chain functional groups are very similar in the two molecules. However, the ring size of the lanthionine analog is smaller by one sulfur atom than the parent compound.

The constrained molecular dynamics simulations (100 ps) reveal the dynamic properties of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and its lanthionine

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Table 5 The Important NOEs of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH and its Lanthionine Analog

NOEs	Parent compound	Lanthionine analog
Arg^1-NH-Arg^1-Hα	m	m
Cys^2-NH-Arg^1-Hα	s	s
Cys^2-NH-Cys^2-Hα	m	m
Cys^2-Hα-Cys^5-Hβ	w	w
Asp^3-NH–Cys^2-Hα	s	s
Asp^3-NH–Asp^3-Hα	m	m
Asp^3-NH–Cys^5-NH	–	w
Asp^3-Hα–Thz^4-Hα	s	s
Cys^5-NH–Thz^4-Hα	s	w
Cys^5-NH–Thz^4-Hδ	–	s
Cys^5-NH–Cys^5-Hα	m	m
Cys^5-Hα–Cys^2-Hβ	m	w

Note: Cys^2 and Cys^5 are in 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH. The corresponding residue in the analog is lanthionine. The intensities of NOE signals are classified as strong (s), medium (m) or weak (w).

analog. As shown in Plates 3 and 4, from the trajectories it is clear that the ring structure is highly constrained while the exocyclic portion of the molecule, 1-FCA-Arg, is free to rotate. When the two molecules are compared, the lanthionine analog is even more constrained than the parent compound in the cyclic region. The standard deviation of the RMSD of the heavy atoms in the ring structure for the lanthionine analog is 0.49 Å, while the value for the parent compound is 0.60 Å.

CONCLUSION

We designed and synthesized the lanthionine analog of a cell adhesion modulator, 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH. We also studied their structure–activity relationships by determining their preferred conformations in solution. Because the molecules in the current study are highly constrained, their conformations in DMSO solution can be used as a probe to investigate the interaction with the receptor. Although the molecules adopt similar topological arrangements, some important differences remain. These differences may contribute to their different binding potency. One difference is the orientations of the amide bond between residues **4** and **5** are opposite in the two molecules. If this bond is involved in any hydrogen bonding with the receptor, the lanthionine analog can not offer the same interaction as the parent compound. The other difference arises from the reduction in the size of ring structure from 14 to 13 atoms, which alters residue positions of the monosulfide as compared with the disulfide. If the disulfide serves as a hydrophobic group for the binding, the different position and with only one sulfur atom in the lanthionine analog will certainly reduce the activity. It is important to note that the lanthionine molecule retains high nanomolar potency as a VLA-4 antagonist. Thus, the lanthionine analog is still recognized by the $\alpha_v\beta_4$ integrin. In addition, it does not bind to other integrins and the selectivity remains unchanged.

Acknowledgements

This research was supported by the National Institute of Health (DK 15410). We would like to thank Dr J.W. Tilley at Hoffmann-LaRoche Incorporated and Dr D.G. Mullen at Telios Pharmaceuticals, Incorporated for biological assays.

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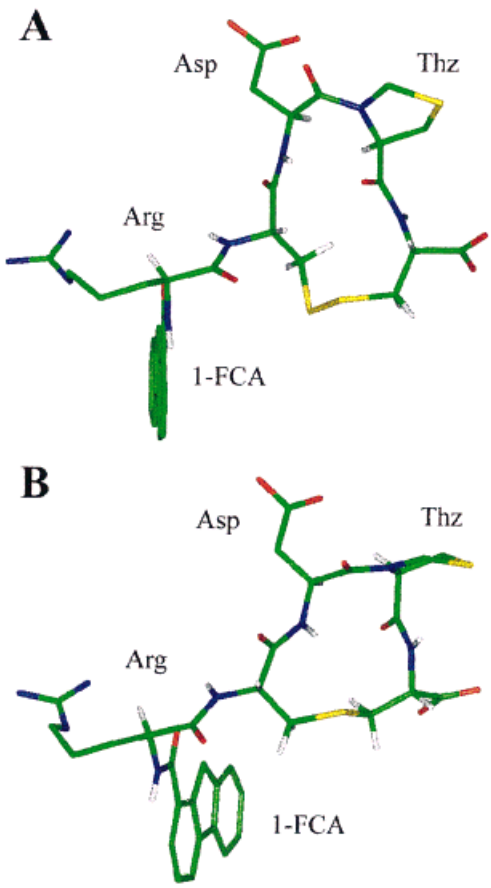

Plate 1 The preferred conformations of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH (A) and its lanthionine analog (B) in DMSO solution.

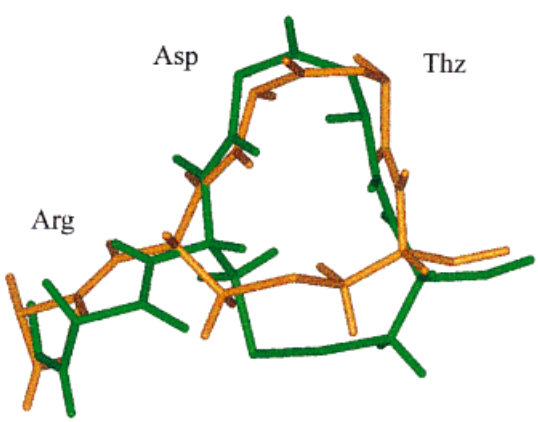

Plate 2 The superposition of the backbone of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH (green) and its lanthionine analog (brown).

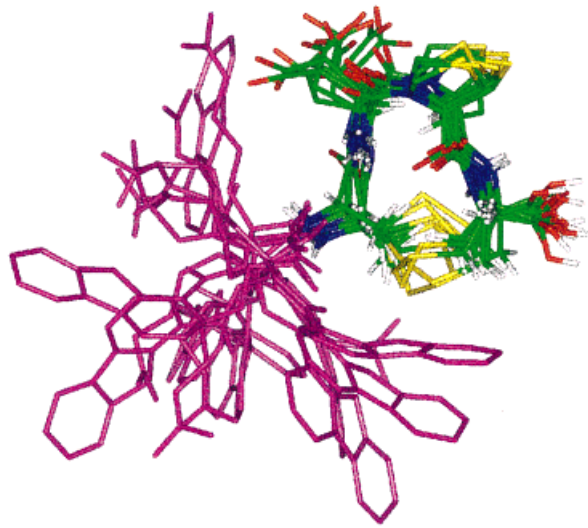

Plate 3 The trajectories (every 10 ps) from a constrained molecular dynamics simulation (100 ps) of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH.

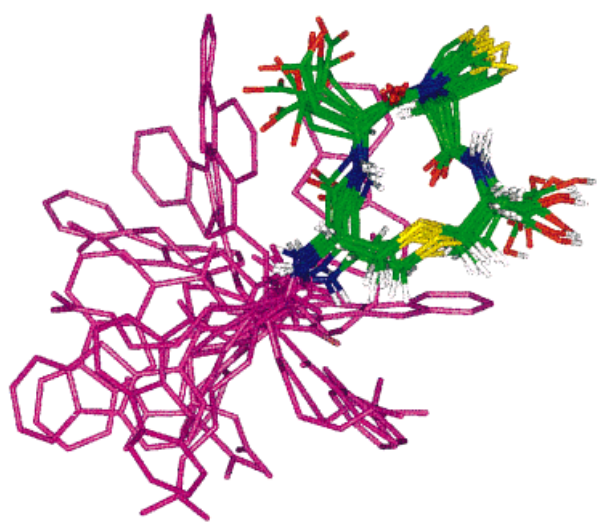

Plate 4 The trajectories (every 10 ps) from a constrained molecular dynamics simulation (100 ps) of the lanthionine analog of 1-FCA-Arg-c[Cys-Asp-Thz-Cys]-OH.

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