Pergamon

Tetrahedron Letters 39 (1998) 6107–6110

TETRAHEDRON LETTERS

Synthesis of a Cyclic-Thioether Peptide Which Binds Anti-Cardiolipin Antibodies

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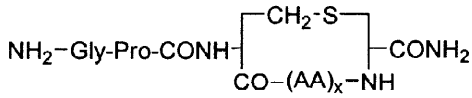
Received 1 April 1998; accepted 16 June 1998

Abstract: Thioether cyclized peptide analogue, **1**, was synthesized using solid phase FMOC chemistry on HMPB-MBHA resin. α-Methylproline was introduced as an FMOC-alanyl-α-methylproline dipeptide, and the thioether was incorporated as FMOC-protected thioether tetrapeptide intermediate, **8**. Compound **1** has been shown to bind anti-cardiolipin antibodies.

Disulfide cyclized peptides which bind anti-cardiolipin antibodies have been identified by screening phage libraries.[1,2] An example of such a compound is represented by compound **2**. Some structure activity relationships have been elucidated by comparison of cyclic disulfide analogues with respect to their ability to bind antibody,[3] and those studies led to an optimized amino acid sequence which includes the unnatural amino acid, α-methylproline.[4] The design of thioether peptide mimetic **1** incorporated the optimized sequence and replacement of the disulfide bond with a thioether. The objective in replacing the disulfide bond with a more stable thioether bond was to increase the metabolic stability of the peptide, and make it more compatible with other modifications planned for the peptide in efforts to develop a multivalent peptide conjugate (a Toleragen™)[5,6] designed to cross link surface antibody receptors on B-cells. What was not known was whether the thioether analogue would retain ACA binding activity.



1 (LJP 685), $(AA)_x$ = Ile-Leu-Leu-Ala-(α-Me)Pro-Asp-Arg
2, $(AA)_x$ = Leu-Ile-Leu-Ala-Pro-Asp-Arg

Cyclic peptides have been prepared by a variety of methods, including N-terminal to C-terminal amide cyclizations, with side chain groups suitably protected, either on resin[7] or in solution.[8] Cyclic thioether peptides have been prepared by cyclic thioalkylation reactions[9,10] or by N-terminal to C-terminal amide cylization of a peptide which was synthesized with the thioether already in place.[11] We chose to use a variation of the latter approach in which a pre-formed thioether segment, **8**, is incorporated in solid phase

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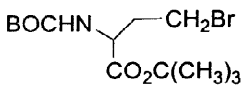
0040-4039/98/$ - see front matter
PII: S0040-4039(98)01292-1

synthesis. This approach was appealing because the thioether could be formed ahead of time allowing the use of conventional solid phase synthesis on the super acid labile HMPB-MBHA resin.

Starting materials were prepared as follows. Compound **3**, tert-butyl-N-BOC-4-bromo-2-aminobutyrate, was prepared from appropriately protected glutamic acid by the method of Barton.[12] The dipeptide, N-FMOC-L-alanyl-L-2-methylproline **4**, was prepared because the amino acid coupling to α-methylproline on the solid phase proved to be problematic. N-FMOC-L-alanine pentafluorophenyl ester (1.4 eq) was added to a solution of α-methylproline[13] (1 eq), $NaHCO_3$ (10 eq), and HOBT[14] (0.05 eq) in DMF. After 18 hours at room temperature, the product was extracted into EtOAc, washed with 1N HCl, and purified by silica gel chromatography (45/55/1 EtOAc/hexane/HOAc) to provide **4** as a white solid (86%).[15] Compound **5** was prepared by treating N-BOC-glycylproline (1eq) with 4-nitrophenol (1.2 eq) and DCC (1.4 eq) in THF at 0°C. Purification by silica gel chromatography (2.5/97.5/1 EtOAc/CH_2Cl_2/HOAc) followed by trituration with 3/1 hexane/Et_2O gave **5** as a white solid (90%).[16]



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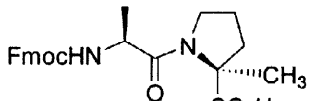

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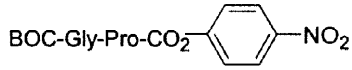


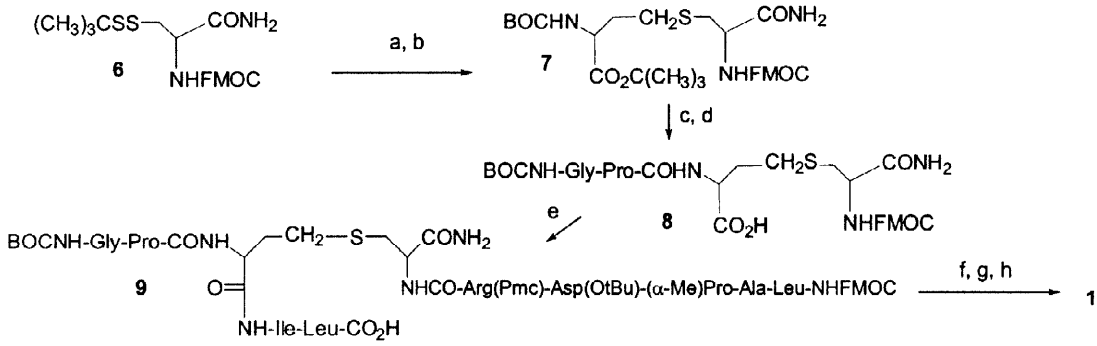
5

FMOC-S-t-butylthiocysteineamide, **6**, was prepared from FMOC-S-t-butylthiocysteine (1 eq), NHS (1 eq), and DCC (1.5 eq) (THF, 0°C, 1 hour; then aqueous $(NH_4)HCO_3$ 4.5 hours, 0°C to room temperature). Most of the THF was removed under vacuum and the resulting aqueous phase with white solid was stirred with CH_2Cl_2 to dissolve solids. The CH_2Cl_2 phase was washed successively with 1 N HCl and saturated $NaHCO_3$ solution, dried (Na_2SO_4), and filtered. The filtrate was crystallized (CH_2Cl_2/hexane) to provide **6** (87%) as a white solid.[17]

The synthesis of **1** is described in the following scheme. Reduction of the disulfide of compound **6** followed by alkylation of the resulting thiol with compound **3** provided **7** as a white solid.[18] Removal of t-butyl protective groups and acylation with **5** provided **8**.[19] Peptide **9** was prepared by standard FMOC synthesis on N-FMOC-L-leucinyl-HMPB-MBHA resin[20] using FMOC-amino acid, HOBT, and DIC (3 eq. each) for each coupling step with the exception of the coupling of **8** which used 2 eq. of **8** and HOBT and DIC (3 eq. each). After the final coupling step, cleavage from the resin was accomplished by multiple 2 minute treatments (1% trifluoroacetic acid/CH_2Cl_2 filtered into 1/9 pyridine/MeOH). The filtrates were concentrated and purified by HPLC (21.4 mm x 250 mm C_{18} column, gradient, 60/40/0.1 CH_3CN/H_2O/TFA to 90/10/0.1 CH_3CN/H_2O/TFA over 40 minutes, 12 mL/min, 230 nm detection, retention time 36-38 minutes) to give peptide **9**.[21]

Peptide **9** was converted to **1** by removal of the FMOC group, cyclization, and removal of side chain protecting groups. Thus treatment of **9** with a 1% solution of DBU in CH_3CN gave a white solid after concentration and trituration with Et_2O. The solid was dissolved in CH_3CN and treated with pyridine (6 eq, 1.0 M solution in CH_3CN) and DPPA (6 eq, 0.1 M solution in CH_3CN). The mixture was stirred for 20 hours and concentrated under vacuum. The residue was triturated with Et_2O, and the white opaque residue was treated with 92/3/2/3 TFA/anisole/EDT/Me_2S for 1 hour. The product was precipitated (Et_2O) and purified by

HPLC (21.4 mm x 250 mm C_{18} column, gradient, 10/90/0.1 CH_3CN/H_2O/TFA to 35/65/0.1 CH_3CN/H_2O/TFA over 40 minutes, 12 mL/min, 230 nm detection, retention time 32-35 minutes) to give **1** .[22]



Reagents: a) PBu_3 (1.05 eq), $NaHCO_3$, (2 eq), N_2 sparged 2/1 dioxane/water, 1h, room temperature, extract (2/1 CH_2Cl_2/1 N HCl); b) **3** (1.2 eq), K_2CO_3 (2.5 eq), N_2 sparged 4/1 dioxane/water, 16 h, room temperature, extract (1/9/10 MeOH/ CH_2Cl_2/1 N HCl) silica gel chromatography (1/3 CH_3CN/CH_2Cl_2), (80%); c) 20/1/1 TFA/H_2O/mercaptoethanol; d) **5** (1.6 eq), $NaHCO_3$ (2.0 eq), 2/1 dioxane/H_2O, extract (3/1 CH_2Cl_2/1 N HCl), silica gel chromatography (5/95/1 MeOH/CH_2Cl_2/HOAc), (60%); e) solid phase peptide synthesis, N-FMOC-L-leucinyl-HMPB-MBHA resin, HOBT/DIC/DMF using **4** and **8**, then 1% trifluoroacetic acid/CH_2Cl_2, then 1/9 pyridine/MeOH, preparative HPLC (CH_3CN/H_2O/0.1% TFA) (49%); f) 1% DBU/CH_3CN; g) pyridine/DPPA/CH_3CN; h) 92/3/2/3 TFA/anisole/EDT/Me_2S, preparative HPLC (CH_3CN/H_2O/0.1% TFA), (90%).

Peptides **1** and **2** were compared for their ability to compete with native antigen (β_2-GPI).[23] The IC_{50} for **1** in the competitive ELISA was 2.17 x 10^{-4} M, whereas the IC_{50} for peptide **2** was 8.70 x 10^{-4} M.

We have developed a novel synthesis of thioether cyclized peptides based on a pre-formed thioether which can be used in FMOC synthesis. The biological results show that, for this class of ACA-binding peptides, the replacement of a disulfide by a thioether bond is well tolerated in that it does not prevent antibody binding. Studies are in progress toward developing a peptide based Toleragen™ for treatment of autoimmune thrombosis.

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14. Abbreviations used; β_2-GPI (β_2-glycoprotein I), NHS (N-hydroxysuccinimide), DCC (1,3-dicyclohexylcarbodiimide), HOBT (1-hydroxybenzotriazole), DIC (1,3-diisopropylcarbodiimide), DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), DPPA (diphenylphosphoryl azide).
15. Compound **4** was characterized as follows: mp 59-60°C; ^{1}H NMR ($CDCl_3$) δ 1.39 (d, 3H), 1.67 (s, 3H), 1.93 (m, 2H), 2.06 (m, 2H), 3.78 (m, 2H), 4.22 (m, 1H), 4.40 (d, 2H). 4.56 (m, 1H), 5.09 (t, 1H), 5.69 (d, 1H), 7.32 (t, 2H), 7.42 (t, 2H), 7.62 (d, 2H), 7.78 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 17.8, 21.8, 24.1, 38.5, 47.3, 48.4, 61.3, 66.1, 67.0, 120.1, 125.4, 127.2, 127.9, 141.4, 144.1, 156.0, 171.7, 175.1. HRMS (FAB) Calculated for $C_{24}H_{27}N_2O_5$ $(M+H)^+$: 423.193. Found: 423.1904.
16. Compound **5** was characterized as follows: mp 98-98.5°C; TLC R_f 0.09, 40/60/1 EtOAc/hexane/HOAc; ^{1}H NMR ($CDCl_3$) δ 1.09-2.55 (m, 4H), 1.48 (s, 9H), 3.47-3.77 (m, 2H), 4.05 (m, 2H), 4.74 (m, 1H), 5.45 (bd s, 1H), 7.35 (d, 2H), 8.30 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 25.2, 28.4, 29.7, 43.2, 46.2, 48.1, 59.3, 122.5, 125.4, 156.0, 168.0, 170.1. Analysis Calculated for $C_{18}H_{23}N_3O_7$: C, 54.96; H, 5.89; N, 10.68. Found: C, 55.18; H, 6.16; N, 10.70.
17. Compound **6** was characterized as follows: mp 127-129°C; TLC R_f 0.29, 95/5/1 CH_2Cl_2/CH_3CN/MeOH; ^{1}H NMR ($CDCl_3$) δ 1.36 (s, 9H), 3.06-3.24 (m, 2H), 4.25 (t, 1H), 4.55 (m, 3H), 5.56 (bd s, 1H), 5.70 (bd s, 1H), 6.23 (bd s, 1H), 7.38 (d of d, 2H), 7.45 (d of d, 2H), 7.63 (d, 2H), 7.80 (d, 2H); ^{13}C NMR (CDCl3) 29.8, 41.9, 47.1, 48.5, 54.2, 67.2, 120.0, 125.0, 127.1, 127.8, 141.3, 143.6, 156.1, 172.2. Analysis Calculated for $C_{22}H_{26}N_2O_3S_2$: C, 61.37; H, 6.09; N, 6.51. Found: C, 61.47; H, 6.03; N, 6.51.
18. Compound **7** was characterized as follows: TLC R_f 0.27, 1/3 CH_3CN/CH_2Cl_2. Further purification of an analytical sample was done by recrystallization from hexane/EtOAc; mp 104-106.5°C; ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H), 1.49 (s, 9H), 1.96 (m, 1H), 2.11 (m, 1H), 2.69 (m, 2H), 2.88 (m, 1H), 3.03 (m, 1H), 4.29 (t, 1H), 4.36 (m, 2H), 4.50 (m, 2H), 5.21 (m, 1H), 5.49 (m, 1H), 5.81 (m, 1H), 6.54 (m, 1H), 7.34 (t, 2H), 7.42 (t, 2H), 7.61 (d, 2H), 7.80 (d, 2H); ^{13}C NMR (CD_3OD) δ 26.1, 26.7, 28.2, 28.7, 34.8, 54.8, 55.7, 68.1, 80.5, 82.7, 120.9, 126.3, 128.2, 128.8, 142.6, 145.2, 160.0, 162.7, 173.4, 175.8. Analysis Calculated for $C_{31}H_{41}N_3O_7S$: C, 62.08; H, 6.89; N, 7.01. Found: C, 62.23; H, 7.12; N, 7.39.
19. Compound **8** was characterized as follows: mp 111-113°C; TLC R_f 0.59, 10/90/1 MeOH/CH_2Cl_2/HOAc; ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 2.00 (m, 2H), 2.18 (m, 2H) 2.56 (m, 1H), 2.69 (m, 1H), 2.85 (m, 2H), 3.49 (m, 2H). 3.62 (m, 2H), 3.85 (m, 2H), 4.08 (m, 1H), 4.12 (m, 1H), 4.22 (t, 1H), 4.38 (m, 1H), 4.49 (m, 2H), 4.61 (m, 2H), 5.98 (bd s, 1H), 6.12 (bs s, 1H), 6.43 (bd s, 1H), 7.35 (d, 2H), 7.40 (d, 2H), 7.61 (d, 2H), 7.78 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 25.3, 27.8, 28.6, 29.4, 32.6, 35.1, 44.1, 46.9, 47.3, 52.2, 53.9, 61.2, 67.8, 80.8, 120.8, 125.7, 128.2, 129.0, 142.0, 144.5, 157.6, 157.8, 170.6, 171.2, 172.9, 173.1. Analysis Calculated for $C_{34}H_{43}N_5O_9S$: C, 58.52; H, 6.21; N, 10.03. Found: C, 58.38; H, 6.17; N, 10.20.
20. A solution of N-FMOC-L-leucine in CH_2Cl_2 and a few drops of DMF was prepared and cooled to 0°C and treated with DIC for 20 minutes at 0°C. The mixture was concentrated and dissolved in DMF, and the resulting solution was added to pre-swelled HMPB-MBHA resin (Novabiochem) followed by DMAP dissolved in DMF (1 eq of per eq of resin substitution). After 1 hour the resin was washed (2 X DMF, 2 X MeOH, 2 X DMF, 2 X MeOH) and dried. The substitution was determined to be 0.540 mmol/g.
21. MS (electrospray): m/e 1814 $(M+H)^+$.
22. MS (electrospray): m/e 1151 $(M+H)^+$.
23. ACA ELISA data were obtained as follows. Polystyrene microtitration plates were coated with cardiolipin (50 ug/well) in ethanol. After evaporation of solvent, wells were blocked (5% fish gelatin/PBS, pH 7.2), washed (TBS, pH 7.4), and coated with β_2-GPI (2.3% IgG-depleted normal human serum). Wells were incubated with a mixture of peptide and ACA serum, diluted 1:400 with 3% fish gelatin in 1: 1 TBS/PBS, then washed (TBS) and treated with goat anti-human IgG alkaline phosphatase conjugate. After washing with TBS, the wells were treated with colorimetric substrate to determine the amount of ACA bound. The IC_{50} is the concentration of peptide that inhibited ACA binding by 50%.