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Design, Synthesis, and Three-Dimensional Structural Characterization of a Constrained Ω-Loop Excised from Interleukin-1α

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Abstract

The cyclic peptide **1**, containing a 2,7-disubstituted naphthalene spacer, was designed to mimic an exposed Ω-loop present in interleukin-1α, an important mediator of immune and inflammatory responses. The synthesis of this cyclic peptide was accomplished via solution phase fragment condensation methodology. The three dimensional characterization using 2D-NMR techniques revealed it to be an excellent mimic for the Ω-loop sequence 41-48 in interleukin -1α.

Introduction

The interleukin-1 proteins (IL-1α and IL-1β) are secreted by activated macrophages and bind cell membrane receptors on the surface of T- and B-lymphocytes to trigger inflammatory and immunological responses.[1] Two major types of IL-1 receptors have been identified to date. The type I receptor found on T-cells, fibroblasts, synovial cells, and hepatocytes mediates inflammatory responses while the type II receptor found on B-cells and macrophages is implicated in immune responses. IL-1 (IL-1α and IL-1β) molecules found in serum are relatively small proteins of approximately 17,500 daltons and both proteins are synthesized as larger precursor molecules. Despite their marked differences in amino acid sequences (less than 30 % homology), both IL-1α and IL-1β have similar three-dimensional structures[2,3] and biological activities. In addition to IL-1α and IL-1β, a third member of the interleukin-1 family of regulatory polypeptides has been identified and characterized. This form of IL-1, termed IL-1ra, appears to be a pure IL-1 receptor antagonist, showing high binding affinity for the type I receptor on T-cells similar to that possessed by the two agonist IL-1s (IL-1α and IL-1β), but having no capacity to induce receptor-mediated biological responses.[4] The ability of IL-1ra to block IL-1 induced inflammatory responses in animal models suggests that an IL-1 antagonist could be a useful therapeutic. However, IL-1ra has no significant oral activity, as is the case with the most proteins.

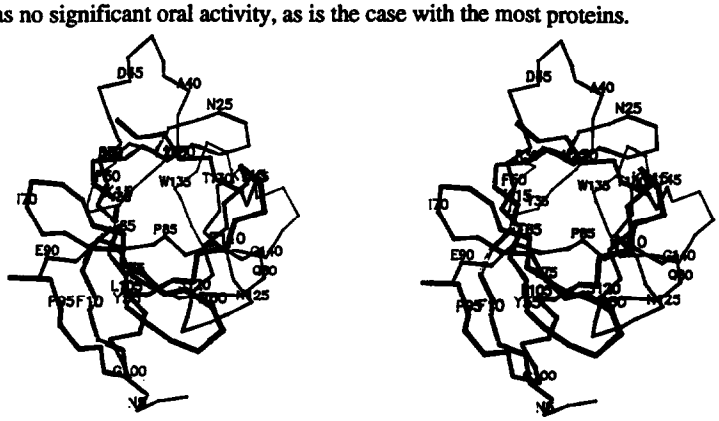


Fig. 1. Stereo view of interleukin-1α

Design of the Loop Mimetic

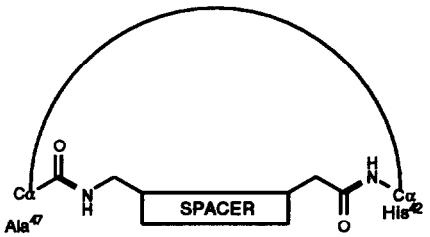
One major goal of modern medicinal chemistry is to find rational first principles for systematically transforming the information provided in natural peptide ligands into low molecular weight non-peptide molecules that bind to the target receptor. These non-peptide molecules are expected to possess improved pharmacokinetic and pharmacodynamic properties relative to natural peptides. As a first step towards this goal and also due to the lack of small molecule IL-1 receptor antagonists from random screening, we turned to a *de novo* design approach based on the structure of the IL-1 receptor ligands.

The three-dimensional structures of IL-1α and IL-1β have recently been reported.[2,3] The core of these structures is a six-stranded β-barrel which is closed at one end by another six β-strands to form a bowl-like structure. Figure 1 (of IL-1α) is a view of this structure down the barrel axis. A striking feature of the structural core is that it has nearly exact internal three-fold axis of symmetry.

The 12 β-strands are interconnected with loops and among these is a large, prominent, exposed loop spanning the sequence 41-48 in IL-1α. According to Leszczynski and Rose,[5] loops of this nature are classified as Ω-loops based on i) the segment length (6-16 residues), ii) absence of regular secondary structure, iii) the distance between segment termini (approx. 4 -11 Å), and iv) their resemblence to Greek alphabet omega (Ω). A systematic study of 67 proteins of known structure revealed the occurance of 270 omega loops and that these Ω-loops are almost invariably situated at the protein surface where they are poised to assume important roles in molecular function and biological recognition. In fact, for the soybean and Erythrina trypsin inhibitors, which have the same tertiary structure as IL-1 proteins, one of the Ω-loops binds to trypsin in the inhibitory complex.[6]

In an earlier study, several linear peptides with overlapping sequences of IL-1 proteins were prepared as a part of a general strategy to elucidate the functional regions. All of these linear peptides were found to lack binding to the IL-1 receptor.[7] Next we turned to the preparation of constrained cyclic peptides that would mimic various loops present in the protein. Standard methods of constraining loops involve, for example, introduction of cysteine residues into the sequence to form disulfide bridges, side-chain to side-chain cyclizations involving glutamic acid or aspartic acid with lysine residues, or end-to-end cyclizations. The cyclic peptides obtained when this approach is applied to large Ω loops, such as the one present in IL-1α, are highly flexible, and cannot necessarily be expected to adopt a conformation analogous to that in the protein. Because of these implications and also the possibility that the 41-48 Ω-loop in IL-1α may be involved in receptor recognition, we chose to design a spacer molecule that would span the intrachain distance and also constrain it enough to maintain the excised loop in a conformation which is similar to the one observed in IL-1α. *This paper describes the design, synthesis, three-dimensional structural characterization of this loop mimetic and the comparison of its structure with the corresponding loop in IL-1α.*

A closer examination of the 3-D structure of the loop sequence indicated that the most appropriate place to introduce the constraint with the spacer molecule would be at positions Leu 41 and Val 48 because the side chains of these residues project into the loop and are not expected to be involved in receptor recognition. The X-ray crystallographic coordinates of IL-1α corresponding to the Ω-loop sequence 41 - 48 were used as a template to evaluate several spacer molecules to bridge this sequence. The structural parameters used in screening of spacer molecules are summarized in Figure 2.

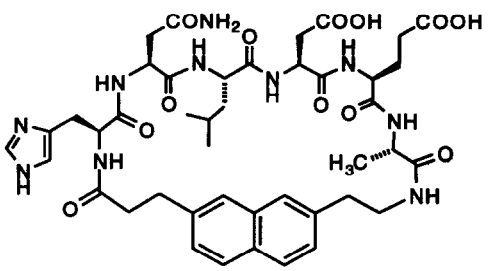


Distance between Leu ^{41} CO and Val ^{48} NH ------ 10.944 Å

Torsion Angle between the highlighted bonds ------ 125.9°

Fig 2. Structural parameters of the Ω-loop

After careful screening of several spacers (not shown) for distance and torsional complementarity to the Ω loop sequence peptide using SYBYL molecular modeling software[8], 7-aminoethyl-2-naphthalenepropanoic acid was chosen to bridge the Ω-loop sequence in IL-1α. Thus the cyclic peptide **1** was designed by incorporating the spacer molecule and the 42 - 47 sequence of IL-1α was then energy minimized using MAXIMIN routine in SYBYL.[9] After energy minimization, **1** had a total energy of 36 kcal/mol and the backbone was found to fit the Ω-loop sequence with an RMS deviation of only 0.288 Å (Figure 3). Based on these modeling studies the cyclic peptide **1** was chosen as a synthetic target to mimic the Ω loop structure in IL-1α.



1

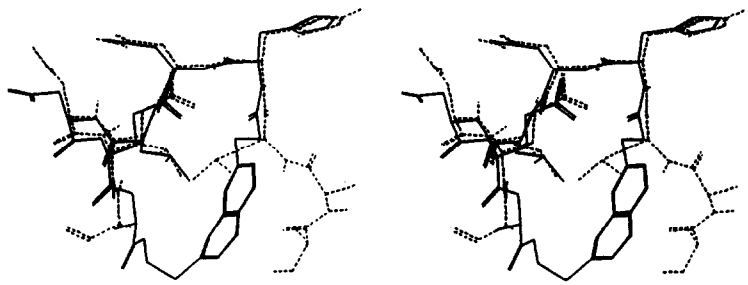
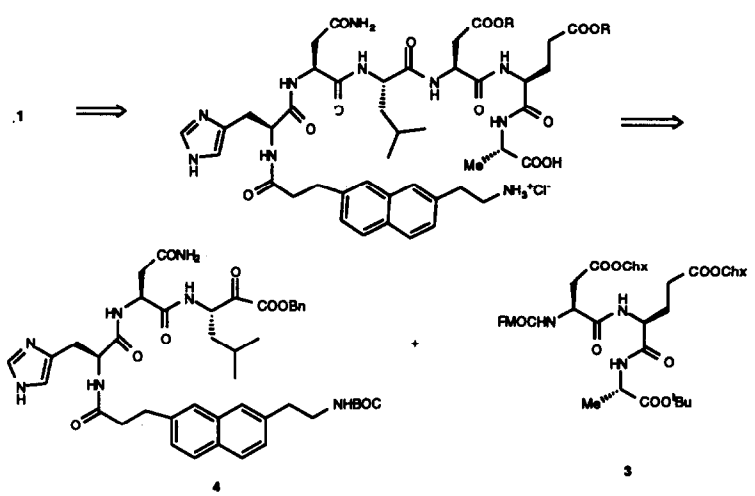


Fig. 3. Stereo view of the superposition of modeled IL-1α loop on the cyclic peptide **1**

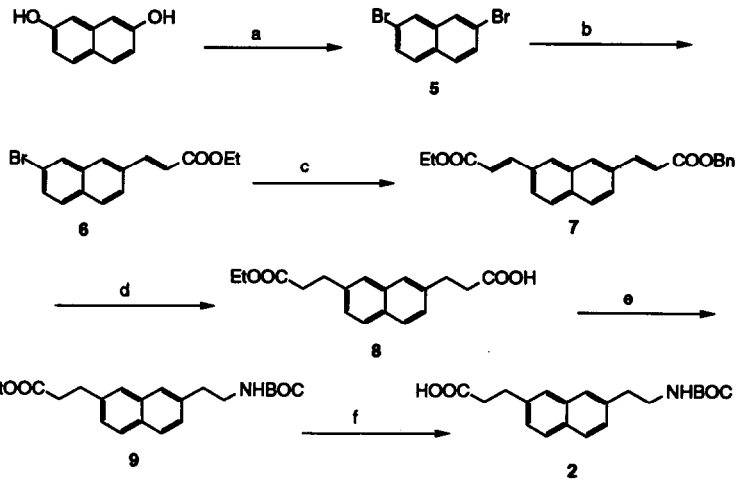
Synthesis of the Cyclic Peptide

The synthesis of cyclic peptide **1** was carried out using the strategy shown in Scheme 1. This strategy is based on fragment condensation methods and takes into consideration the problems posed by Glu-Asp sequences.[10] Cyclohexyl esters were used as side-chain protecting groups for Asp and Glu residues to avoid their loss while the C-terminal (t-butyl ester) and N-terminal (BOC-group) protecting groups of the linear peptide were being de blocked. These protecting groups also prevent the formation of imides corresponding to Glu and Asp residues. The spacer molecule **2** was prepared from readily available 2,7-dihydroxynaphthalene in six steps (Scheme 2). First, 2,7-dihydroxynaphthalene was converted to the corresponding dibromo derivative **5** using a known reaction procedure.[11] Compound **5** was transformed to the differentially protected bis(acrylate) ester **7** via two sequential Pd^0-catalysed acrylate couplings. Hydrogenation of **7** yielded mono ester **8**, which was converted to the BOC-protected amino ester **9**, via Curtius rearrangement. This material **9**, was hydrolyzed to give the NHBOC amino acid **2**, which was incorporated into the peptide sequence.

Scheme 1. Synthetic Strategy

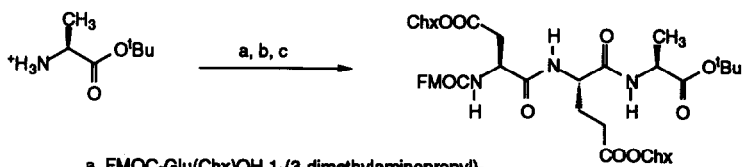


Scheme 2. Synthesis of the Spacer



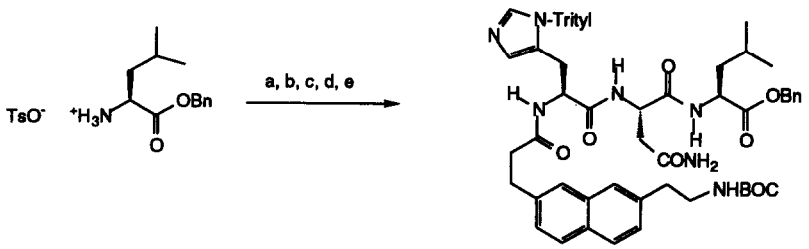
a. Ph_3P, Br_2, 77% b. Ethylacrylate, Et_3N, $Pd(OAc)_2$, $Pd(PPh_3)_4$,DMF, 30%
c. Benzyl acrylate, Same as b, 40% d. H_2, 10% Pd-C, 86% e. DPPA, Et_3N, tBuOH, 85°C, 65%f. KOH, 96%.

Scheme 3. Synthesis of Peptide Fragments



a. FMOC-Glu(Chx)OH,1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/HOBT 73% b. 4-Aminomethylpiperidine, 95% c. FMOC-Asp(Chx)OH,1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/HOBT, 67%

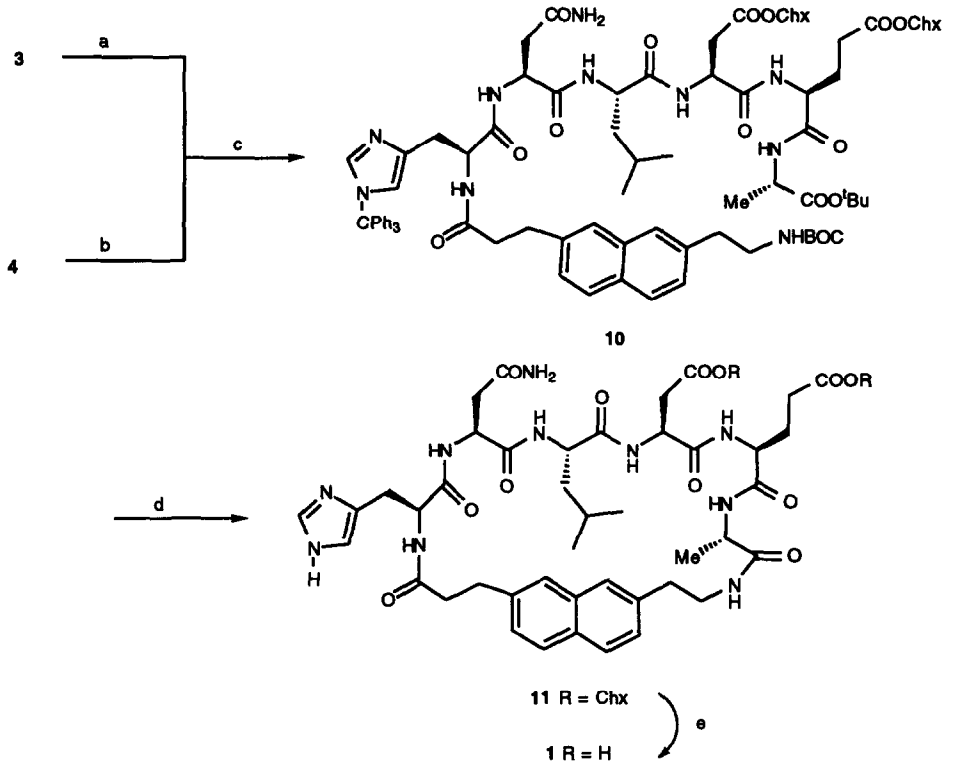
3



4

a. FMOC-AsnOH,1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/HOBT 76% b. 4-Aminomethylpiperidine, 95% c. FMOC-His(Trityl)OH,1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/HOBT, 32% d. 4-Aminomethylpiperidine, 95% e. 2,1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/HOBT, 68%

Scheme 4. Synthesis of the Cyclic Peptide



a. 4-(Aminomethyl) piperidine, $CHCl_3$, 99% b. 10%Pd/C- H_2 99%
c. 1-(3-dimethylaminopropyl)-3-ethylcarbodimide/HOBT, 77%
d. Diphenylphosphorylazide, Et_3N, 42.1% e. HF, Thioanisole, 40%

The tri- and tetrapeptide fragments (**3** and **4**)were assembled via solution phase sequential coupling of FMOC amino acids using the water soluble diimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodimide and HOBT, and 4-(aminomethyl)piperidine as a reagents for peptide coupling and FMOC deblocking, respectively (Scheme 3).[12] These two peptide fragments were condensed to form the protected linear hexapeptide **10**. The macrocyclization of the hexapeptide **10**, after C-terminal and N-terminal deprotection to close the 29 membered ring was accomplished using diphenylphosphorylazide and triethylamine under high dilution conditions (42.1% isolated yield) (Scheme 4). The cyclic peptide **11**, was treated finally with HF to cleave the cyclohexyl ester protecting groups[13] to yield compound **1**.

Three-Dimensional Structure Determination

The three-dimensional structure of **1** was determined using two-dimensional NMR techniques in combination with molecular dynamics and energy minimization procedures.

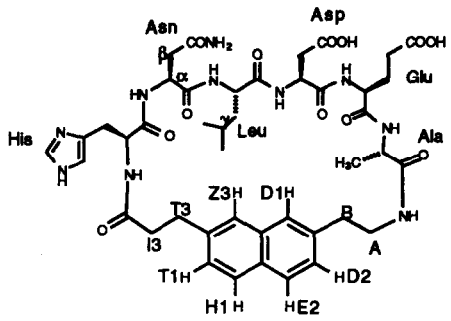


Table 1. Proton NMR Assignments[a] for the Cyclic Peptide 1

Residue	NH	CαH	CβH	CγH	CδH	Other
His	8.41	4.35	3.01, 2.89			C4H: 6.90 C2H: 7.62
Asn	8.15	4.48	2.63			$CONH_2$: 7.68, 7.07
Leu	8.35	4.25	1.56	1.67	0.86, 0.82	
Asp	8.39	4.56	2.83, 2.59			
Glu	7.54	4.26	1.99, 1.85	2.33		
Ala	8.10	4.18	1.16			
Nap	7.93	I3 : 2.54	T3 : 3.04, 2.98			D1H : 7.57, Z3H : 7.66
		A:3.12, 3.60	B:2.94, 2.86			T1H : 7.31, H1H : 7.77 E2H : 7.79, D2H : 7.33

a) Chemical shifts in δ units

The structure of the cyclic peptide **1** was determined in DMSO-d_6 at room temperature. The ^1H resonances of the compound **1** were completely assigned from COSY and NOESY experiments (Table 1). The NOEs were quantified and converted into distance constraints using assumptions and a classification scheme described previously (Table 2).[14] These distances were used to calculate a penalty function in a structural optimization scheme using molecular dynamics and energy minimization in the CHARMM program package,[15] as has been described using 0.01 - 3.0 for the weighting factor (WNOE) and 2 or 4 for the exponent (ENOE) in the energy term: $E = WNOE(kT/2)[(R-R_0)/\partial R]^{ENOE}$ wherein R, R_0, and ∂R are the distance between the protons, the distance between protons from nOe, and the estimated error in the distances, respectively. For the cyclic peptide **1**, the optimization yielded 30 low-energy structures that are consistent with the experimental data. In a typical fit to the data, 66% of the distances in the models fit the experimental ones within the estimated error, and the remaining 33% show an additional RMS deviation of 0.15Å (Fig. 4). As can be seen from the overlay of 30 conformations, the structure of the peptide backbone appears to be similar for all the conformations. The major difference among the conformations is the relative position of the naphthalene spacer with respect to the peptide backbone.

Table 2. Intraresidue and Sequential NOE's for Cyclic Peptide 1[a]

Entry	Size	Entry	Size	Entry	Size
His HN - His Hα	SM	Glu HN - Glu Hβ	S	Nap HI3 - Nap HT3	M/M
His HN - His Hβ	S/SM	Glu Hα - Glu Hβ	M/S	His Hα - Asn HN	ML
His Hα - His Hβ	ML/M	Glu Hα - Glu Hγ	SM	Asn Hα - Leu HN	M
His Hδ2 - His Hβ	ML/M	Ala HN - Ala Hα	SM	Leu Hα - Asp HN	M
Asn HN - Asn Hα	ML	Ala HN - Ala Hβ	ML	Asp Hα - Glu HN	ML
Asn HN - Asn Hβ	L	Ala Hα - Ala Hβ	L	Glu Hα - Ala HN	ML
Asn Hα - Asn Hβ	L	Nap HN - Nap HA	ML/ML	Ala Hα - Nap HN	L
Asn Hβ - Asn Hδ	LL/L	Nap HN - Nap HB	SM/SM	His HN - Asn HN	SM
Leu HN - Leu Hα	SM	Nap HN - Nap HD1	SM	Asp HN - Glu HN	S
Leu HN - Leu Hβ	M	Nap HA - Nap HB	M/SM	Ala HN - Nap HN	S
Leu HN - Leu Hγ	S	Nap HD1 - Nap HA	M/M	Asn HN - His Hβ	SM/SM
Leu Hα - Leu Hβ	L	Nap HD2 - Nap HA	M/M	Leu HN - Asn Hβ	M
Leu Hα - Leu Hγ	SS	Nap HD1 - Nap HB	LL/L	Asp HN - Leu Hβ	S
Leu Hα - Leu Hδ	ML	Nap HD2 - Nap HB	ML	Ala HN - Glu Hβ	S/S
Asp HN - Asp Hα	M	Nap HZ3 - Nap HT3	L/L	Nap HN - Ala Hβ	M
Asp HN - Asp Hβ	SM/SM	Nap HT1 - Nap HT3	M/ML	Nap HI3 - His HN	ML
Asp Hα - Asp Hβ	ML/M	Nap HZ3 - Nap HI3	LL	Nap HI3 - Asn HN	S
Glu HN - Glu Hα	SM	Nap HT1 - Nap HI3	ML		

a) NOEs were classified as follows (with centering values Å): LL (2.0); L (2.3); ML (2.55); M (2.8); SM (3.05); S (3.3); and SS (3.6)[14]

A fit of the lowest energy conformer of the 30 structures determined from molecular dynamics with the IL-1α loop sequence 41-48 showed that three of six C-α atoms of the backbone fitted with an RMS deviation of < 0.5Å and an overall RMS deviation of 1.3Å (Fig. 5).

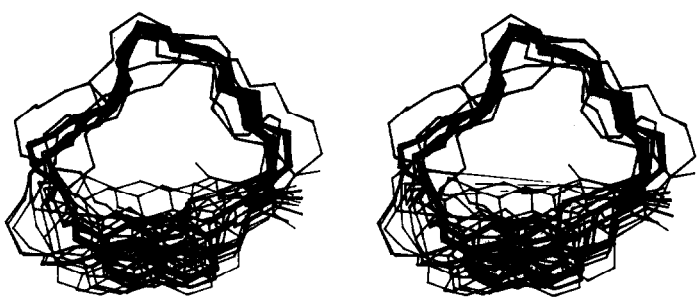


Fig. 4 Stereo view of the overlay of 30 possible conformations for cyclic peptide 1

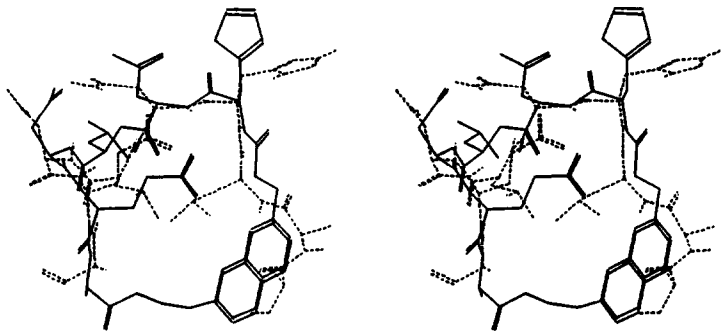


Fig. 5 Superposition of IL-1α 41-48 sequence over the lowest energy conformer of **1**

A search for the best fit conformer of the 30 structures revealed a conformer whose C-α atom RMS deviation from IL-1α loop was only 0.86 Å and was higher in energy by 4.3 kcals/mole from the lowest energy conformer.

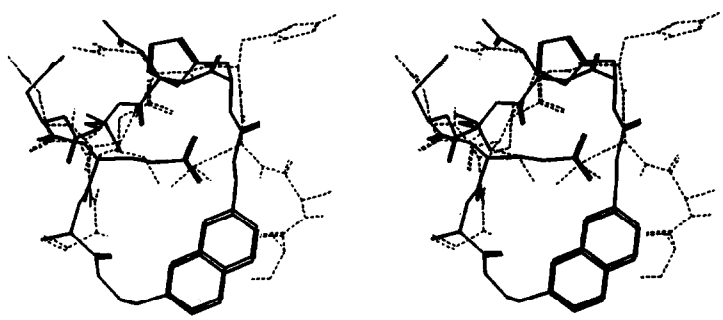


Fig. 6 Superposition of IL-1α 41-48 loop sequence over the best fit conformer of **1**.

The cyclic peptide **1** was assayed for its ability to compete with ^{125}I-labelled IL-1α in binding to type I IL-1 receptors on mouse EL-4 cell membranes.[16] These assays indicated that compound **1** did not inhibit IL-1 binding to its receptor to a concentration of 2 mM. This observation indicates that the constrained Ω loop mimetic is not recognized by the receptor. This negative result would suggest that either the loop is not involved in receptor binding, or the compound **1** does not present a sufficient number of interactions to bind, or that the loop conformation adopted by **1** is different from the receptor-bound conformation. Recently, Ju, et al. have proposed a potential binding epitope of IL-1α and IL-1β based on site-directed mutagenesis.[17] These studies indicate that the binding epitopes of IL-1 molecules consist of 7-9 clustered, noncontiguous residues, none of which are present in the 41-48 sequence of IL-1α.

The studies reported here demonstrate the possibility of designing molecules that mimic exposed loop structures in proteins by bridging sequences from a protein loop with a properly designed spacer molecule. The chief design criteria are the geometrical complementarity of the spacer and its effect on overall energy of the loop sequence. Once these two requirements are met, the protein sequence, at least in the case of exposed Ω loops, should adopt conformations similar to those observed in the proteins. This strategy may be generally useful in designing other Ω-loop mimics which are known to be involved in molecular recognition, such as soybean and

Erythrina trypsin inhibitors[6]. In those cases it may be possible to assess the validity of the strategy via a biological assay.

Experimental Section

All reactions were performed under argon atmosphere. ^{1}H-NMR were recorded with Varian XL-200 and XL-400 spectrometers, using tetramethylsilane (TMS) as internal standard. Electon impact (EI, 70 ev) and fast atom bombardment (FAB) mass spectra were taken on VG ZAB-1F or VG 70E-HF mass spectrometers. TLC analysis was performed on Merck silica gel 60 F-254 plates and visualized first by UV illumination and then by charring with 10% phosphomolybdic acid in 95% ethanol. Melting points are uncorrected. All solvents used in the preparation of peptides, e.g. methylene chloride, DMF, and methanol were Burdick and Jackson "distilled in glass" grade and used without additional distillation. TFA, HOBt, EDT were purchased from Aldrich Chemical Co. All protected amino acids were of the L- configuration and were obtained from Bachem California. For amino acid analyses, peptides were hydrolyzed in 6 N HCl containing phenol at 115 °C for 24 h in evacuated Racti-Therm hydrolysis tubes. Analyses were performed on a Beckman 121M amino acid analyzer. HPLC analyses were carried out on Waters Delta Prep 3000 liquid chromatograph using Delta Pak C18-100Å column (7.8 mm X 30 cm).

(E)-3-(7-Bromo-2-naphthalenyl)-2-propenoic acid ethyl ester 6: To a flame dried 3L, three neck flask equipped with a mechanical stirrer, thermometer, and an argon inlet, DMF (500 mL), 2,7-dibromonaphthalene(30 g, 0.105 mol)[11], triethylamine (30 mL, 0.215 mol), and ethylacrylate (12.5 mL, 0.115 mol) were added sequentially. The resulting solution was deoxygenated vigorously with argon for 20 min. Palladium diacetate (1.5g, 0.006 mol) followed by tetrakis-triphenylphosphine palladium (1.0 g) was added to the solution. The reaction color changed from orange to black in about 40 min. The reaction mixture was kept at 85 °C for 7h. TLC (1:1 ethyl acetate-hexane) analysis indicated formation of the desired product, $R_f = 0.5$ and a more polar blue fluorescent spot, $R_f = 0.3$, which represents bis coupled product. The DMF was removed in vacuo and the residue obtained was passed through a plug of silica gel (300 g), eluting with 1:1 hexanes-ethyl acetate to yield the crude product, 35 g. The crude product was purified by Waters Prep HPLC 500A, using 5% ethyl acetate-hexanes mixture to afford 9.0 g (30%) of **6** as a white powder. m.p. 102.5 - 103 °C; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.35 (t, 3H), 4.3 (q, 2H), 6.5 (d, J = 16 Hz, =CH, 1H), 7.7 (d,J = 16 Hz, =CH, 1H), 7.7 (t, 2H), 7.8 (m, 3H), 8.0 (s, 1H). Anal. Calcd for $C_{15}H_{13}O_2Br$: C, 59.03; H, 4.29; Br, 26.19. Found: C, 58.70; H, 4.18; Br, 26.33.

(E,E)-3,3'-(2,7-Naphthalenylene)-bis-2-propenoic acid ethyl phenylmethyl ester 7: To a 3 L, 3 neck round bottom flask, equipped with a mechanical stirrer, thermometer and an argon inlet was added a solution of **6** (18.0 g, 0.059 mol) in dry DMF (1500 mL), triethylamine (12.1 mL, 0.087 mol) and benzyl acrylate (18 mL, 0.117 mol). Palladium diacetate (0.8 g, 0.0035 mol) and tetrakis-triphenylphosphine palladium (1.0 g) were added after deoxygenating vigorously with argon for 20 min. The reaction mixture was heated gradually to an internal temperature of 85 °C. A color change from orange to black was observed at about 40 °C. The reaction was stirred for 16 h. The volatiles were removed in vacuo and the residue was passed through a plug of coarse silica gel (450 g) eluting with 1:1 ethyl acetate-hexanes to afford the crude product 30 g. The crude product was purified by Waters Prep HPLC 500A. The material was eluted with 10% ethyl acetate-hexanes mixture to yield 9.8 g of **7**. The product was further purified by recrystallization from ethanol to give 9.3 g (41%) of **7** as a white crystalline solid: mp 114 - 115 °C; ^{1}H-NMR ($CDCl_3$) δ 1.35 (t, 3H), 4.3 (q, 2H), 5.28 (s, 2H), 6.55 (d, J = 16 Hz, =CH, 1H), 6.65 (d, J = 16 Hz, =CH, 1H), 7.40 (m, 6H), 7.70 (m, 2H), 7.85 (m, 3H), 7.90 (m, 2H). Anal. Calcd for $C_{25}H_{22}O_4$: C, 77.70; H, 5.74. Found: C, 77.37; H, 5.60.

2,7-Naphthalenedipropanoic acid monoethyl ester 8: A solution of **7** (9.3 g, 0.024 mol) in of THF (300 mL) was hydrogenated over 10% palladium on carbon for 12 h. The reaction was filtered through a pad of celite and concentrated in vacuo to yield the crude product (6.6 g). The crude product was purified by recrystallization from ethyl acetate-hexanes to afford 6.2 g (86%) of **8** as a white powder: mp 122 - 123.5 °C: ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 3H), 2.69 (t, 2H), 2.76 (t, 2H), 3.1 (m, 4H), 4.13 (q, 2H), 7.29 (d, 2H), 7.58 (s, 2H), 7.73 (d, 2H). Anal. Calculated for $C_{18}H_{20}O_4$: C, 71.98; H, 6.71. Found: C, 71.61; H, 6.67.

7-[2-[[(1,1-dimethyloxy)carbonyl]amino]ethyl]-2-naphthalenepropanoic acid ethyl ester 9: A distillation apparatus with a 500 mL receiving flask was flame dried under vacuum. The substrate **8** (6.2 g, 0.021 mol), was placed in the receiving flask together with diphenylphosphoryl azide (6.6 mL, 0.03 mol) and dry triethylamine (4.1 mL, 0.03 mol). Tertiary butanol was distilled over sodium metal until the volume in the receiving flask was approximately 180 mL. The distillation apparatus was removed and the reaction flask stirred at 85 °C for 16 h under argon. TLC analysis (5:1 hexanes - ethyl acetate) indicated essentially complete reaction.

The solvent was removed in vacuo and the residue extracted into ethyl acetate (2 X 400 mL), washed with water (2 X 100 mL), brine (1 X 100 mL), and dried (Na_2SO_4). The residue obtained after concentration was purified by Waters HPLC, eluting with 10% ethyl acetate - hexanes to afford 5.2 g of **9**. This material was recrystallized from hexanes to yield 4.96 g (65%) as a white crystalline solid: mp = 122 - 123 °C; ^{1}H NMR ($CDCl_3$) δ 1.25 (t, 3H), 1.45 (s, 9H), 2.7(t, 2H), 2.9 (t, 2H), 3.1 (t, 2H), 4.1 (q, 2H), 4.5 (bs, 1H), 7.25-7.8 (m, 6H). Anal. Calcd for $C_{22}H_{29}NO_4$: C, 71.13; H, 7.87; N, 3.77. Found: C, 70.96; H, 7.84; N, 3.79.

7-[2-[[(1,1-Dimethylethoxy)carbonyl]amino]ethyl]-2-naphthalenepropanoic acid 2: A solution of **9**, (4.96 g, 0.013 mol), in ethanol (150 mL) was treated with 1M KOH (26 mL). The mixture was stirred at 40 °C for 2 h under argon. The solvent was removed in vacuo and the residue diluted with water (200 mL). The aqueous solution was acidified carefully with cold 1N HCl and extracted into ethyl acetate (3 X 200 mL), washed with water (2 X 100 mL), and dried (Na_2SO_4). The residue obtained after concentration was purified by recrystallization from ethyl acetate - hexanes to afford 4.41 g (96%) of **2** as a white powder: mp = 130 - 131 °C: ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9H), 2.77(t, 2H, J = 1.9 Hz), 2.95 (t, 2H, J = 1.6 Hz), 3.1 (t, 2H, J = 1.9 Hz), 3.47 (t, 2H, J = 1.6 Hz), 4.55 (bs, 1H), 7.3 (m , 2H), 7.58 (s, 1H), 7.6 (s, 1H), 7.75 (m, 2H). Anal. Calcd for $C_{20}H_{25}NO_4$. 0.05 mol H_2O: C, 69.77; H, 7.35; N, 4.07. Found: C, 69.49; H, 7.25; N, 3.93.

General Procedure for Peptide Couplings: A solution of amine (1.00 eq), N-protected-α-amino acid (1.5 eq), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride(1.75 eq), N-ethylmorpholine (1.2 eq), and HOBT (1.30 eq) were stirred in DMF soultion at room temperature for 4-16 h. The reaction mixture was then diluted with methylene chloride, and washed with water and brine. The organic layers were dried and concentrated. The crude product thus obtained was purified by flash chromatography using chloroform-methanol mixtures.

General Procedure for FMOC- group deprotection: The procedure followed here was developed by Carpino et al.[13] and this method involves treatment of a chloroform solution of the FMOC-protected peptide with 4-aminomethylpiperidine (3 mL per 1 mmol of the peptide in 10 mL chloroform). The solution was stirred for 10 min at room temperature. The solution was then diluted with chloroform and washed with a pH 5.75 buffer solution (prepared from 90 g of $Na_2H_2PO_4.H_2O$ and 32.7 g of Na_2HPO_4 in 500 mL water) (2 X 25 mL). The resulting organic layer was dried and concentrated to yield the peptide amine, which is used as such for the next coupling reaction.

FMOC-Asp(Chx)-Glu(Chx)-Ala-o^{t}Bu 3: FMOC-Glu(Chx)OH was coupled with alanine t-butyl ester hydrochloride following the general procedure to yield the dipeptide, FMOC-Glu(Chx)-Ala-O^{t}Bu, in 73% yield. This material was then deprotected using the general procedure described above. This dipeptide amine was coupled with FMOC-Asp(Chx)OH following the standard procedure to yield the tripeptide **3**, in 67% yield. ^{1}H NMR ($CDCl_3$) δ 1.10-1.9 (m, 32 H), 2.0 (m, 1H), 2.15 (m, 1H), 2.5 (m, 2H), 2.7(dd, J_1 = 6 Hz, J_2 = 14Hz, 1H), 3.05 (dd, J_1 = 4Hz, J_2 = 14 Hz, 1H), 4.25 (m, 2H), 4.40 (m, 3H), 4.55 (m, 1H), 4.77 (m, 2H), 5.91 (d, J = 8.5 Hz, 1H), 6.8 (d, J = 7.3 Hz, 1H), 7.35 (m, 2H), 7.4 (m, 2H), 7.6 (d, J = 5.5 Hz, 2H), 7.76 (d, J = 8.5 Hz, 2H). FAB MS: 776 (M+H), 720 (776-C_4H_8), 631 (776-Ala O^{t}Bu).

BOC-Nap-His(trityl)-Asn-Leu-OBn 4: FMOC-Asn-OH was coupled with leucine benzyl ester following the general procedure described above to yield the dipeptide, BnO-Leu-Asn-FMOC, in 76% yield. This material was then treated with 4-aminomethyl piperidine and the dipeptide amine thus obtained was then coupled with FMOC-His(trityl)OH to yield the tripeptide, FMOC-His(trityl)-Asn-Leu-OBn in 32% yield. The tripeptide was then converted to its amine and was then coupled with the spacer amino acid **2** (BOC-Nap) to yield the desired tetrapeptide fragment **4** in 68% yield. ^{1}H NMR ($CDCl_3$) δ 0.84 (d, J = 4.3 Hz, 6H), 1.43 (s, 9H), 1.6 (m, 3H), 2.34 (dd, J_1 = 5 Hz, J_2 = 15.5 Hz, 1H), 2.47 (m , 1H), 2.51 (m, 1H), 2.95 (m, 2H), 3.05 (m, 5H), 3.45 (m, 2H), 4.56 (m, 3H), 4.80 (m, 1H), 4.96 (d, J = 12.5 Hz, 1H), 5.06 (d, J = 12.5 Hz, 1H), 5.27 (bs, 1H), 5.73 (bs, 1H), 6.52 (s, 1H), 7.06 (m, 6H), 7.30 (m, 18H), 7.54 (s, 2H), 7.71 (m, 3H), 8.93 (d, J = 8.6 Hz, 1H). FAB MS: 1040 (M + H).

BOC-Nap-His(trityl)-Asn-Leu-Asp(Chx)-Glu(Chx)-Ala-O^{t}Bu 10: The peptide fragments **3** and **4** were deprotected using 4-(aminomethyl)piperidine and H_2, 10% Pd/C respectively in nearly quantitative yields (99%). The resulting amine and carboxylic acid were coupled using the standard conditions to yield the heptapeptide **10** in 77% yield. ^{1}H NMR ($CDCl_3$) δ 0.82 (d, 6H), 1.2-1.5 (m, 23H), 1.60- 1.80 (m, 11H), 2.15 (m, 1H), 2.32 (m, 1H), 2.40 (m, 2H), 2.50 (m, 1H), 2.60 (m, 1H), 2.75 (m, 2H), 2.95 (m, 4H), 3.15 (m, 2H), 3.45 (m, 2H), 4.08 (m, 1H), 4.32 (m, 2H), 4.45 (m, 2H), 4.60 (m, 2H), 4.75 (m, 2H), 5.40 (bs, 1H), 6.66 (s, 1H), 6.72 (bs, 1H), 6.95-7.85 (m, aromatic H, 22H). MS FAB: M+H 1485.

Cyclo[Nap-His-Asn-Leu-Asp(Chx)-Glu(Chx)-Ala] 11: The heptapeptide **10** (100.4 mg, 0.067mmol) was treated with trifluoroacetic acid (5 mL) for 2 h at room temperature and then the solution was concentrated and diluted with THF (10 mL). The THF solution was then saturated with HCl gas. This solution was concentrated and azeotroped with benzene (2 X 50 mL). The product thus obtained was dissolved in DMF (5 mL) and cooled to 0 °C and diphenylphosphoryl azide (DPPA) (100 μL, 0.464 mmol) was added. This solution was stirred for 1.5 h at 0 °C and then was added to a triethylamine (500 μL, 3.59 mmol) solution in DMF (500 mL) over a period of 2 h with a syringe pump. After the addition was complete, the solution was left in a refrigerator for 16 h. The solvent was removed in vacuo and the crude product obtained was purified using reverse phase HPLC (Waters Delta Pak 7.8 X 300 mm C_{18} column, 6 ml/min flow rate, 5-95% CH_3CN-water (0.1% TFA) gradient over 50 min, and 215 nm UV detection). The uncyclized linear peptide (37.5% gradinet, 15.8 min retention time) was isolated (20.2 mg, 24.9%) in addition to the desired product, which was eluted at 62% gradient (24 min retention time). The product was obtained after lyopholization (33.7 mg, 42.1 % yield; 55.3% based on recovered starting material) as a white foamy solid. FAB MS (M+H) calculated for $C_{55}H_{76}N_{10}O_{12}$: 1069.0, Found, 1069.0. Amino acid analysis : Alanine 1.0; Glutamic acid 1.0; Aspartic acid 2.0; Leucine 1.0; Histidine 1.0.

Cyclo[Nap-His-Asn-Leu-Asp-Glu-Ala] 1: *Caution! This reaction should be carried out in a special hood and teflon apparatus designed for HF cleavage reactions.* A solution of the cyclic peptide **11** (17.7 mg, 0.015 mmol) in thioanisole (2 mL) and ethane-1,2-dithiol (1 mL) was treated with HF (10 mL) at -78 °C. The mixture was slowly warmed to 0 °C and kept there for 2 h. The HF was evaporated and the mixture was lyopholized in acetic acid. The crude product thus obtained weighed 44.6 mg. This material was purified under identical reverse phase HPLC conditions described above. The cyclic peptide **1** was elulted at 23% acetonitrile-water gradient (11 min retention time). The product weighed 6.0 mg (39.4 % yield) after lyopholization. ^1H NMR (DMSO-d_6) See the text : FAB MS (M+H) calculated for $C_{43}H_{56}N_{10}O_{12}$: 905.0, Found 905.0. Amino acid analysis. Alanine 0.9; Glutamic acid 1.0; Histidine 1.0; Leucine 1.0; Aspartic acid 1.9.

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