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Synthesis and Evaluation of a Library of Peptidomimetics Based Upon the β-Turn

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Abstract: A diverse library of 1152 compounds based on a β-turn mimetic scaffold was synthesized. A subset of the library was characterized by ES-MS. Several compounds from the library with modest affinity to a cloned *N*-formyl-Met-Leu-Phe (fMLF) receptor were identified.

INTRODUCTION

The β-turn is one of the three major motifs of peptide and protein secondary structure.[1,2] β-Turns play a key role in many biological molecular recognition events including interactions between antigens and antibodies, peptide hormones and their receptors, and regulatory enzymes and their corresponding substrates. In order to attain high-affinity and selective binding to a targeted receptor, a β-turn mimetic must reproduce both the functionality and the orientation of the sidechains of the receptor-bound peptide ligand.[3] The inherent diversity in β-turn structure[4] compounded with difficulties in identifying the key residues responsible for binding makes the design of β-turn mimetics quite challenging. Nonetheless, significant effort has been devoted towards the construction of β-turn mimetics that possess biological activity and several reviews on the subject have appeared.[5-7] The aforementioned difficulties could be circumvented by the synthesis and biological evaluation of a library of β-turn mimetics that incorporate numerous sidechain combinations as well as multiple different sidechain orientations.

Turn Mimetic Synthesis

Earlier, we reported the general and expedient solid-phase synthesis of mimetics **1** from readily accessible precursors.[8] An α-bromo acid and a Fmoc-protected α-amino acid provide the functionalized i+1 and i+2 sidechains, respectively, and an aminoalkylthiol serves as a constraining backbone component. Bromoacetic acid, which is coupled to Rink amide resin,[9] serves as the initial starting material for the solid-phase synthesis sequence. The flexibility and orientation of the sidechain functionality can be altered by introducing different backbone aminoalkylthiols, such as to provide either 9- or 10-membered rings, or by preparing different combinations of the absolute configurations at each of the stereocenters introduced by either the i+1 or i+2 sidechains.

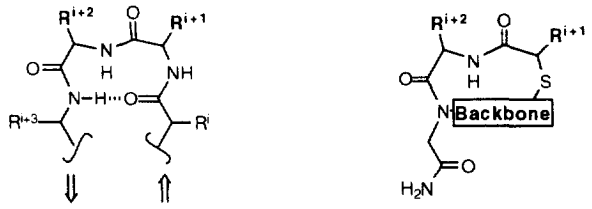
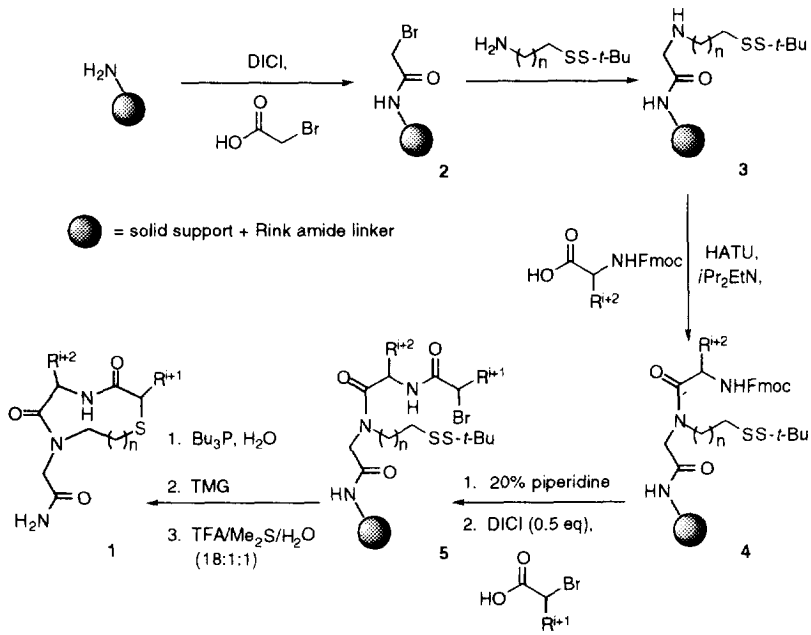


Figure 1. General structure of a β-turn and β-turn mimetic **1**.

The synthesis of **1** is illustrated in Scheme 1. Mimetic **1** is assembled in eight steps on Rink amide-derivatized Rapp Tentagel™. The support-bound Rink amine is acylated with bromoacetic acid using diisopropylcarbodiimide (DICI). The backbone element is then introduced by treatment of α-bromo amide **2** with either 2-aminoethanethiol *t*-butyl disulfide or 3-aminopropanethiol *t*-butyl disulfide in DMSO. The secondary amine **3** is subsequently coupled with the appropriate Fmoc-protected amino acid employing O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) to incorporate the i+2 sidechain. The Fmoc protecting group is removed from **4** and the resulting free amine is acylated with the α-bromo acid to incorporate the i+1 sidechain. Treatment of the mixed *t*-butyl disulfide **5** with tributylphosphine and tetramethylguanidine (TMG) results in disulfide reduction followed by cyclization to afford the 9- or 10-membered ring mimetic **1**. It was necessary to employ a DMF/PrOH/H_2O comixture and Rapp Tentagel™ resin, which swells in aqueous solvents, in order to achieve clean reduction of the disulfide. Cleavage from support and sidechain deprotection is then accomplished by treatment with 1:1:18 water/dimethyl sulfide/trifluoroacetic acid.



Scheme 1.

Eleven mimetics **1a-1k** were prepared to demonstrate the generality of the synthesis sequence. Before initiating the construction of each derivative, *p*-nitrophenylalanine was coupled to the Rink amide linker to provide a convenient UV tag for accurate determination of the overall purity at the completion of the synthesis. Any sideproducts, such as truncation sequences, acyclic compounds, or cyclic dimers, would also contain the *p*-nitrophenylalanine chromophore and thus would be readily apparent by HPLC with UV monitoring. The derivatives that were synthesized incorporated both 9- and 10-membered rings, different combinations of absolute stereochemistry at the i+1 and i+2 positions, and the sidechain functionality of aspartic acid, lysine, serine, and tyrosine.

Table 1. Derivatives of **1** prepared (Scheme 1).

	derivative[a]			purity (%)[b]	
entry	R^{i+1}	R^{i+2}	Backbone (n)	PEG-PS	Pins
a	CH_3	CH_2Ph	2	90	79
b	CH_3[c]	CH_2Ph	2	59	90
c	$CH(CH_3)_2$	CH_2Ph	2	81	86
d	CH_2CO_2H	CH_2Ph	2	65	79
e	CH_3	$(CH_2)_4NH_2$	2	72	87
f	CH_3	CH_2CO_2H	2	63	86
g	H	CH_2Ph	2	85	91
h	H	CH_2OH	2	82	93
i	CH_3	CH_2Ph-4-OH	2	74	88
j	CH_3	CH_2Ph	1	77	75
k	CH_2Ph-4-OH	CH_3	1	81	90

(a) The stereochemical configuration at the i+1 site is (*R*) and at the i+2 site is (*S*) unless otherwise specified. (b) Percent purity is determined as the ratio of the peak area of the desired product to the total peak area of all of the products as evaluated by HPLC analysis employing a C18 reverse-phase column with a gradient of 20 to 100% methanol in 0.1% trifluoracetic acid in H_2O monitoring at 270 nm. (c) The stereocenter has the (*S*) configuration.

Preliminary Conformational Analysis

Comprehensive determinations of the minimum energy conformations of different turn mimetics have yet to be performed. It is expected that ring size, sidechain structure, and sidechain stereochemistry will all play a role in defining the mimumum energy conformations. Initial conformational studies performed upon mimetic **6** indicate that, at least for this mimetic, a turn structure is observed in aqueous solution. The coupling constant between the central amide N-H and the Cα-H of the i+2 residue of derivative **6** was measured in a solution of 10% DMSO-d_6 in water. The coupling constant, 9.5 Hz, was then used to constrain the backbone dihedral angle (ϕ_{i+2}) in a conformational search analysis. The lowest energy conformation (20 kJ more stable than the next lower conformer) was found to best fit (rms = 0.29Å) a type II' β-turn. Further conformational studies are being performed on bioactive mimetics where solution conformation can be correlated with bioactivity.

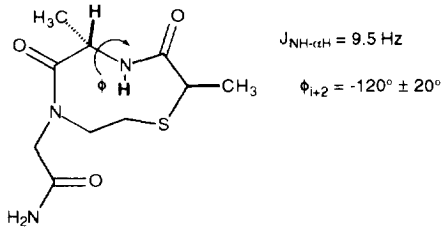


Figure 2. Conformational analysis of a β-turn mimetic **6**.

LIBRARY SYNTHESIS AND EVALUATION

Library Synthesis

We elected to employ the Chiron Mimotopes pin apparatus in order to rapidly and expediently prepare a library of mimetics **1**.[10] The Mimotopes apparatus is configured such that 96 polyethylene pins are attached to a supporting block so that each pin fits into a well of a 96-well microtiter plate. The pins are derivatized with

aminoalkyl groups providing sites for substrate attachment. Each synthesis step can rapidly and efficiently be performed on multiple pins simultaneously by employing existing microtiter plate based instrumentation. Before preparing the mimetic library, we used the Mimotopes apparatus to prepare the 11 turn mimetics that had been previously synthesized on Tentagel™ resin. As shown in Table 1, this experiment established that the reaction sequence that had been optimized on Tentagel™ resin performed equally well on the Chiron Mimotopes pins.[11]

A library of 1152 compounds comprising all combinations of two aminoalkylthiol backbone components, 32 α-amino acids, and 18 α-bromo acids was prepared in less than two weeks (Figure 3). In order to reduce

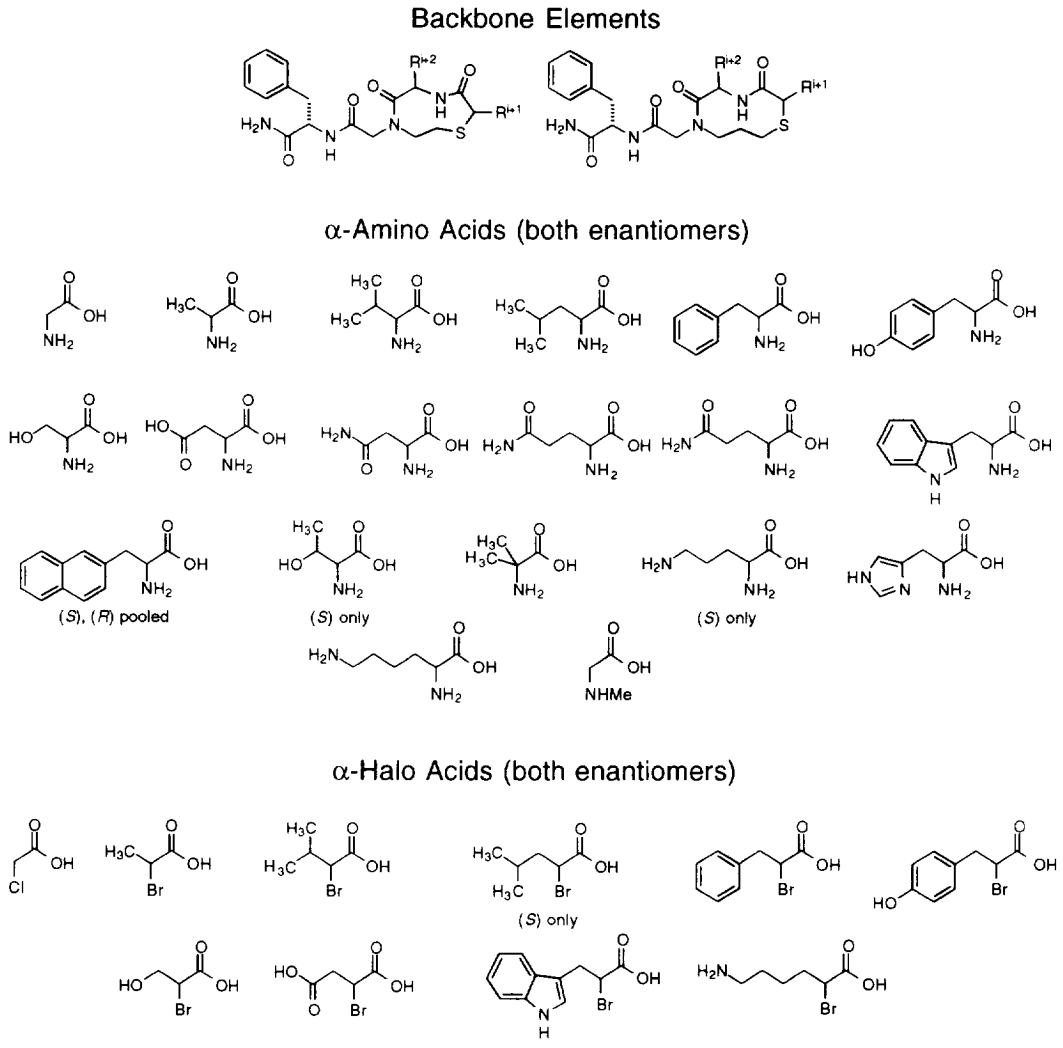


Figure 3. Library of 1152 peptidomimetics.

the number of pins handled while also keeping the work required for deconvolution of active wells to a minimum, the two aminoalkylthiol backbone components were pooled so that each well contained an equimolar mixture of both the 9- and 10-membered rings. The enantiomers of the α-amino acids and α-bromo acids were

kept separate to facilitate the acquisition of structure versus activity relationships. Furthermore, a phenylalanine residue was inserted between the Rink amide linkage and the bromoacetic acid-derived anchor in all derivatives to provide an additional hydrophobic interaction since the library would be screened primarily against membrane-bound receptors.

The library was synthesized according to the previously described reaction sequence on polyethylene-poly *N*,*N*-dimethylacrylamide/methacrylic acid graft copolymer pins (5.92 μmol per pin) that had been pre-derivatized with the acid-cleavable Rink linker. All pins were derivatized as one batch until the i+2 sidechains were introduced. At this point the pins were affixed to pin holders such that each pin fit into a corresponding well of a 96-well microtiter plate. The remaining steps in the synthesis sequence were performed in microtiter plates. Different Fmoc-amino acids were used in each column of the microtiter plates to introduce the different i+2 sidechains, and, in the next step, different α-bromo acids were employed across each row of the microtiter plates to introduce the i+1 sidechains, thereby providing all combinations of the sidechains at the i+1 and i+2 positions. Upon completion of the synthesis, isolation of the spatially separate mimetics in microtiter plates was accomplished by cleavage of the mimetics off of the pins with concomitant sidechain deprotection, followed by concentration *in vacuo*.

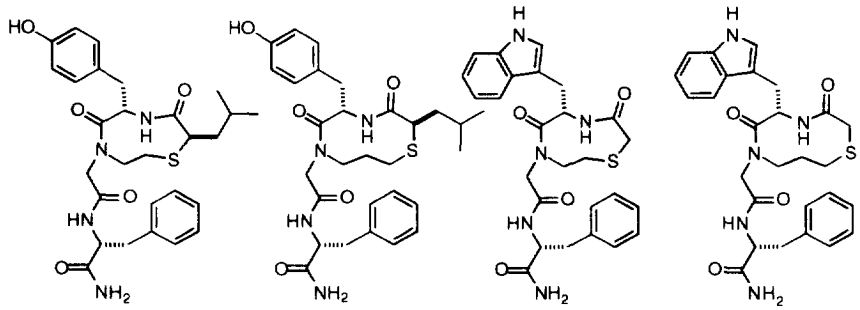
Analytical Characterization

The library was characterized by mass spectrometry using electrospray ionization. Thirty wells (7% of the library) were selected at random for analysis. For twenty nine wells, the expected molecular ions corresponding to both the 9- and 10-membered ring mimetics were observed. Furthermore, little else aside from the ammonium adducts were observed in the mass spectra suggesting a high degree of purity for all of the compounds analyzed. The expected molecular ions were not observed for the turn mimetics prepared with *N*-Fmoc-*N*-methyl-Gly-OH (i+2 sidechain) and *(S)*-2-bromoisovaleric acid (i+1 sidechain), but rather the mass spectral peaks that were observed were 18 mass units greater than expected. It is possible that hydrolysis of the hindered tertiary amide bond may have occurred during cleavage from support. The lability of some hindered tertiary amide bonds to trifluoroacetic acid cleavage conditions has been reported by other researchers.[12]

Biological Evaluation

The library of turn mimetics has been evaluated in a number of assays, as exemplified by a collaboration with researchers at Berlex where ligands with low μM IC_{50} values to the fMLF receptor have been identified. The exogenous tripeptide fMLF, first identified as a bacterial isolate,[13] is bound by receptors in the plasma-membrane of neutrophils[14] where it initiates a range of responses including chemotaxis, directing the cells to areas of infection and inflammation, the production of superoxide radicals, and the release of proteases. Antagonists of the fMLF receptor would possess potential as therapeutic agents in the treatment of inflammatory and infectious diseases. For this reason, the requirements for agonist and antagonist binding have been explored by the synthesis and screening of a variety of natural, unnatural, and modified peptides.[15-17] Notably, no evidence reported to date suggests that a β-turn is present or essential for binding to fMLF.

The library of turn mimetics was screened in a radioligand binding assay against a cloned fMLF receptor. Several active wells were observed when the library was screened such that the total concentration of β-turn mimetics in each well was 10 μM (9- and 10-membered ring were each 5 μM). Four compounds **7**-**10**, corresponding to the contents from two of the most active wells were prepared on a larger scale and purified. In both instances, the inhibitory activity was confirmed and shown to reside primarily in one of the two compounds from each well. The IC_{50} values of compounds **7** and **10** were 10 μM and 13 μM, respectively.

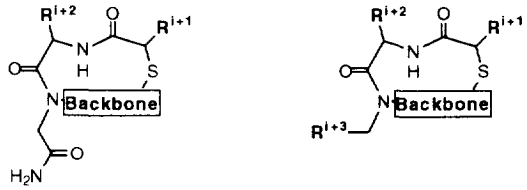


7 8 9 10

Figure 4. Contents of the most active wells when screened against the fMLF receptor.

DISCUSSION

The design of peptidomimetics as receptor ligands has been hampered by difficulties in identifying the key residues and the bioactive conformation of these residues that is necessary for high-affinity and specific binding. Herein, we have reported the preparation of a library of mimetics **1** that incorporate a range of sidechain functionality at two positions as well as multiple different orientations of that functionality. Recently, we have developed a second generation of β-turn mimetics **11** that is designed to possess improved binding affinity, solubility, and perhaps bioavailability,[18] by incorporating the i + 3 sidechain and by eliminating the primary amide functionality.[19] The library synthesis and characterization protocol described in this report are currently being applied to mimetic **11**.



1 11

EXPERIMENTAL

General

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. When used as a reaction solvent CH_2Cl_2 was distilled from CaH_2, and ether and tetrahydrofuran (THF) were distilled under N_2 from sodium benzophenone ketyl, all immediately prior to use. Polyethylene glycol-polystyrene graft copolymer (Rapp Tentagel™) was purchased from Rapp Polymere (Tubingen, Germany). Rink amide-derivatized pins with a loading level of 5.92 μmol/pin were supplied by Chiron Mimotopes (Victoria, Australia). Amino acids and *N*-Fmoc-protected amino acids were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA) or Bachem Bioscience Inc. (King of Prussia, PA). α-Bromo acids were prepared in one step in optically pure form from the corresponding α-amino acids according to a modification of the procedure described by Rapoport.[20] The sidechains of Orn, Lys and Trp were protected with Boc groups, the sidechains of Asp, Glu, Ser, Thr, and Tyr were protected with *t*-Bu groups, and the Asn and Gln sidechains were protected with trityl groups. Backbone aminoalkylthiols were synthesized as described

previously. All other reagents and solvents were purchased from Aldrich (Milwaukee, WI). Chemically resistant 1-mL polypropylene microtiter plates were obtained from Beckman (Fullerton, CA), catalog #267006.

^{3}H-fMLF (71.5 Ci/mmol) was purchased from DuPont NEN (Boston, MA). GF/B Unifilter plates were obtained from Packard (Meriden, CT). All other reagents used for the biological evaluation of the library of compounds were purchased from Sigma (St. Louis, MI).

Library Synthesis and Analysis

Batchwise Synthesis The Fmoc protecting groups on 117.4 g (~640) Rink amide pins were removed by treatment with 20% piperidine in DMF for 0.5 h. The pins were rinsed with DMF (2x), MeOH, and allowed to air-dry for 15 minutes. The pins were placed in a 500 mL Erlenmeyer and a solution of 30.9 g (55.8 mmol) of Fmoc-Phe-OPfp and 7.54 g (55.8 mmol) of HOBt in 280 mL of DMF was added. After 6 h, the pins were transferred to a Buchner funnel and rinsed with DMF and MeOH (2x). The Fmoc protecting groups were removed and the pins were rinsed as before. A solution of 8.34 g (60.0 mmol) of bromoacetic acid and 10.3 mL (66.0 mmol) of DICI in 300 mL of DMF was added to a 600 mL beaker containing the pins. After 11 h, the pins were collected in a Büchner funnel and rinsed with DMF and MeOH (2x), then dried *in vacuo*. To half of the pins in a 250 mL Erlenmeyer flask was added 150 mL of a DMSO solution containing 4.06 g (18.8 mmol) of 3-aminopropanethiol *t*-butyl disulfide hydrochloride, 3.79 g (18.8 mmol) of 2-aminoethanethiol *t*-butyl disulfide hydrochloride, and 5.21 g (41.3 mmol) of TMG. The flask was sealed with parafilm and allowed to stand for 19 h. At this point, the DMSO solution was decanted into another 250 mL Erlenmeyer flask containing the remainder of the pins and reacted as before. The pins were rinsed with DMF (2x), MeOH, 1% AcOH in MeOH (v/v), and allowed to air-dry. The pins were stored as the AcOH salts of the secondary amines to minimize amine oxidation and formation of carbamic acids.

Synthesis in Microtiter Plates. At this point, 576 pins were transferred to six 96-well microtiter plate pin holders. The pins were neutralized by soaking in 20% piperidine in DMF for 5 minutes and then rinsed with DMF, MeOH (3x), and allowed to air-dry. Coupling of Fmoc amino acids was accomplished by immersion of the pins in the wells of a microtiter plate. Each well contained 450 μL of a DMF solution of the appropriate Fmoc amino acid (0.2 M), HATU (0.2 M), and *i*-Pr_2EtN (0.4 M). After 16 h, the pins were removed from the microtiter wells and washed with DMF, MeOH (2x), and allowed to air-dry. The pins were deprotected with 20% piperidine in DMF and then rinsed with DMF, MeOH (3x), and allowed to air-dry. Each pin was then immersed in a microtiter plate wherein each well contained 450 μL of a 0.1 M solution of the appropriate anhydride (pre-formed by treatment of the corresponding α-bromo acid in DMF with an equivalent of DICI for 0.5 h, then filtration into an equal volume of CH_2Cl_2) in a 1:1 comixture of DMF and CH_2Cl_2. After 22 h, the pins were removed from the microtiter wells and rinsed with DMF, MeOH (2x), and air-dried. Disulfide reduction was achieved by soaking the pins in 2.5% (v/v) Bu_3P in a 5:3:2 comixture of PrOH/DMF/H_2O that had been purged with Ar (20 min) for 4 h under an atmosphere of Ar maintained in a glove bag. The pins were rinsed with MeOH (2x) and air-dried. The pins were then immersed in a 2.5% (v/v) solution of TMG in THF. After 17 h, the pins were rinsed with 1% AcOH in DMF, DMF, MeOH, CH_2Cl_2, and air-dried. The β-turn mimetics were cleaved by immersing each pin in ~500 μL of 88:5:5:2 trifluoroacetic acid/dimethylsulfide/water/1,2-ethanedithiol for 3 h. The cleavage cocktail was then removed with a Jouan RC10.10 concentrator equipped with a microtiter-plate rotor to provide the β-turn mimetics, spatially separate in the individual wells of six microtiter plates.

Analysis by Mass Spectrometry. Five wells were selected at random from each of the six microtiter plates containing the β-turn mimetics. Approximately 300 nmol (quantity based on the initial pin loading level of 5.92 μmol and assuming a quantitative yield) of the mixture of two β-turn mimetics contained in each well was diluted to a volume of 50 μL with 0.1% trifluoroacetic acid in acetonitrile/water (3:2 v/v) and submitted for

analysis. Electrospray mass spectral analyses of each sample were recorded on a Sciex API III Biomolecular Analyser in positive ion mode at Chiron Mimotopes (Clayton, Australia).

The contents of the analyzed wells are denoted by the one-letter code for the Fmoc amino acid (i+2) followed by the α-bromo acid (i+1) that the corresponding pins were exposed to in the synthesis sequence. Capital letters correspond to the (*S*) stereochemistry about the sidechain a-carbon while lowercase letters correspond to the (*R*) stereochemistry. O designates ornithine and Z stands for sarcosine. The molecular ion (MH^+) is reported first for the nine-membered ring, and then, for the ten-membered ring.

Table 2. Low-resolution Mass Spectral (ES-MS) Analysis of the Library of Mimetics.

Contents of Well	Calcd (MH^+)	Found
fv	511.2, 525.2	511.0, 525.0
Ll	491.3, 505.3	491.2, 505.2
Va	435.2, 449.2	435.0, 449.0
VY	527.2, 541.2	527.0, 541.0
ay	499.2, 512.2	499.0, 513.0
fk	540.3, 554.3	540.2, 554.2
dV	479.2, 493.2	479.0, 493.0
LK	506.3, 520.3	506.2, 520.2
vd	479.2, 493.2	479.0, 493.0
yA	499.2, 513.2	499.0, 513.0
Ow	565.3, 579.3	565.2, 579.2
Ok	507.3, 521.3	508.2, 521.2
dK	508.2, 522.2	508.0, 522.0
Sw	538.3, 552.3	538.0, 552.0
YF	575.2, 589.2	575.0, 589.0
wf	598.2, 612.3	598.0, 612.0
qV	492.2, 506.2	492.0, 506.0
Qf	540.2, 554.2	540.0, 554.0
na	450.2, 463.2	450.0, 464.0
nv	478.2, 492.2	478.0, 492.0
Wk	579.3, 593.3	579.2, 592.8
ZV	435.2, 449.2	453.0, 467.0
ed	509.2, 522.2	509.0, 523.0
EK	522.2, 536.3	522.0, 536.2
kG	450.2, 464.2	450.0, 464.0
Gy	485.2, 499.2	485.0, 499.0
hd	517.2, 531.2	517.0, 531.0
hW	588.2, 602.3	588.0, 602.2
kf	540.3, 554.3	540.2, 554.2
Kk	521.3, 535.3	521.2, 535.2

Compounds Resynthesized on Larger Scale for Biological Evaluation.

β-turn mimetics **7-10** were prepared as described previously using 500 mg of Rapp Tentagel™.[8] A portion of each compound was rigorously purified by preparative HPLC (Rainin 2 cm x 25 cm 10 μ C_{18} column with UV detection at 220 nm) prior to analytical characterization and biological evaluation. For all of the β-turn

mimetics **7-10**, ^{1}H NMR spectra (300 MHz) were obtained with d_6-DMSO solutions that were heated to 75 °C to ensure the coalescence of NMR signals that corresponded to different turn rotamers. NMR chemical shifts are expressed in ppm relative to internal solvent peaks. J values are in hertz.

β-turn Mimetic 7. ^{1}H NMR: δ 0.74 (d, 3, J = 6.6), 0.79 (d, 3, J = 6.6), 1.02-1.10 (m, 1), 1.32-1.45 (m, 1), 1.59-1.68 (m, 1), 2.58 (dd, 1, J = 9.5, 14.3), 2.77-2.85 (m, 3), 2.98-3.14 (m, 3), 3.28 (dd, 1, J = 5.5, 9.8), 3.45 (dd, 1, J = 4.3, 9.8), 3.64 (d, 1, J = 15.8), 3.97 (d, 1, J = 15.8), 4.41-4.49 (m, 1), 4.81-4.89 (m, 1), 6.61 (d, 2, J = 8.5), 6.96 (br s, 2), 6.97 (d, 2, J = 8.5), 7.14-7.27 (m, 5), 7.74 (d, 1, J = 8.5), 8.60 (d, 1, J = 9.9), 8.79 (s, 1). LRMS (MALDI-TOF): mass calcd for $C_{28}H_{37}N_4O_5S$ (MH^+) 541.2, found 541.1.

β-turn Mimetic 8. ^{1}H NMR: δ 0.74 (d, 3, J = 6.5), 0.78 (d, 3, J = 6.5), 0.97-1.06 (m, 1), 1.20-1.32 (m, 2), 1.51-1.60 (m, 1), 1.89-2.01 (m, 1), 2.59-2.72 (m, 3), 2.82 (dd, 1, J = 9.3, 14.1), 2.93 (d, 1, J = 15.1), 3.03 (dd, 1, J = 5.4, 14.2), 3.08-3.14 (m, 3), 3.35 (dd, 1, J = 4.6, 10.3), 3.76 (d, 1, J = 15.8), 3.83 (d, 1, J = 15.8), 4.41-4.49 (m, 1), 4.76-4.82 (m, 1), 6.59 (d, 2, J = 8.5), 6.96 (br s, 2), 6.97 (d, 2, J = 8.5), 7.15-7.27 (m, 5), 7.76 (d, 1, J = 8.4), 8.48 (d, 1, J = 9.6), 8.80 (s, 1). LRMS (MALDI-TOF): mass calcd for $C_{29}H_{39}N_4O_5S$ (MH^+) 555.3, found 555.4.

β-turn Mimetic 9. ^{1}H NMR: δ 2.70-2.95 (m, 6), 3.12-3.29 (m, 4), 3.66 (d, 1, J = 15.5), 3.98 (d, 1, J = 15.5), 4.42-4.50 (m, 1), 4.90-5.0 (m, 1), 6.94-7.06 (m, 4), 7.16-7.26 (m, 6), 7.32 (d, 1, J = 7.8), 7.56 (d, 1, J = 7.8), 7.72 (m, 1), 8.61 (m, 1), 10.53 (s, 1). LRMS (MALDI-TOF): mass calcd for $C_{26}H_{30}N_5O_4S$ (MH^+) 508.2, found 507.7.

β-turn Mimetic 10. ^{1}H NMR: δ 1.32-1.42 (m, 1), 1.85-1.95 (m, 1), 2.55-2.65 (m, 3), 2.75-2.85 (m, 2), 2.96-2.99 (m, 1), 3.09-3.21 (m, 3), 3.30 (dd, 1, J = 6.2, 14.8), 3.74 (d, 1, J = 15.9), 3.83 (d, 1, J = 15.9), 4.42-4.49 (m, 1), 4.76-4.82 (m, 1), 6.94-7.06 (m, 4), 7.15-7.27 (m, 6), 7.31 (d, 1, J = 7.8), 7.56 (d, 1, J = 7.8), 7.70 (d, 1, J = 8.5), 8.53 (d, 1, J = 8.9), 10.56 (s, 1). LRMS (MALDI-TOF): mass calcd for $C_{27}H_{32}N_5O_4S$ (MH^+) 522.2, found 522.2.

Biological Evaluation of the Library of Mimetics

Cell Culture. HL-60 cells were maintained as a suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. The fMLF receptors were induced by incubating the culture for 72 h in the presence of 0.5 mM N6-2U-O-dibutyryl adenosine 3U:5U-cyclic monophosphate (dbcAMP) as described by Perez, et al.[21] The cells were harvested by centrifugation and the cell pellets were stored at -70 °C.

Cell Membrane Preparation. The cell pellets were pooled and homogenized in 50 mM Tris buffer (pH 7.4 at 25 °C) by ten passes of a motor-driven glass-Teflon homogenizer (TRI-R Instruments Inc., Rockville Center, NY). The homogenate was centrifuged at 45,000 x g for 45 min at 4 °C. The resultant pellets were suspended in 50 mM Tris buffer at a protein concentration of 4 mg/ml and stored at -70 °C.

Binding Assay. The binding assay was performed in 96-well plates in a buffer consisting of 50 mM Tris, pH7.4, 0.1% BSA, 1 mM $MnCl_2$, and 30 μM bacitracin. Each well contained 2 nM 3H-fMLF, 15 μM membrane protein, and 5 μM of each test compound in 0.1 mL of buffer. The nonspecific binding was determined by addtion of 10 μM unlabeled fMLF. After incubation at room temperature (20°C) for 1 h, the samples were filtered through GF/B Unifilter plates using a 96-well plate harvester (Filtermate-196, Packard) and washed four times with 0.2 mL of ice cold 50 mM Tris buffer (pH 7.4). Radioactivity retained on the filter was measured in a TopCount (Packard). The nonspecific binding was approximately 15-20% of total binding.

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