Rediscovering an Endothelin Antagonist (BQ-123): A Self-Deconvoluting Cyclic Pentapeptide Library

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A "self-deconvoluting" cyclic pentapeptide library, designed to produce 82 944 head-to-tail-linked peptides in 48 vials, has been prepared. The mixture included amino acids found in a recently optimized endothelin antagonist, BQ-123, originally isolated from microbial sources by Banyu investigators. Using a positional scan approach, the most potent of 12 residues at each of the four variable positions uniquely rediscovered the BQ-123 sequence or cyclo(L-Pro-D-Val-L-Leu-D-Trp-D-Asp). Resynthesis of the four most potent amino acid combinations gave the following values of relative potency: cyclo(L-Pro-D-Val-L-Leu-D-Trp-D-Asp) or BQ-123 = 1.0, cyclo(L-Pro-D-Pro-L-Leu-D-Trp-D-Asp) = 0.0, cyclo(L-Pro-D-Pro-L-Trp-D-Trp-D-Asp) = 0.0, and cyclo(L-Pro-D-Val-L-Trp-D-Trp-D-Asp) = 0.1. This study reflects the first time that the positional scan approach has been applied to cyclic peptide libraries using a known target. Although no analogs more potent than BQ-123 were discovered, our results provide verification of our synthetic methods for preparing head-to-tail cyclic peptide libraries and also lend support to the use of carefully designed sublibraries for the rapid elucidation of potential leads within a relatively constrained set of peptide macrocycles.

Introduction

A variety of peptide and peptide-related structures have been prepared as combinatorial mixtures for drug lead discovery.[1−3] Most of these have been linear structures, although there is increasing interest in nonpeptide organics as more rigid functional group templates.[4−7]

Cyclic peptide mixtures represent a middle ground between constrained systems and linear peptide libraries.[8] Naturally occurring cyclic peptide "libraries" in the form of disulfide-bridged peptide toxins have been found in many of the 500 species of *Conus*, each of which may contain 100 or more constrained peptides.[9] The first examples of synthetic disulfide-bridged libraries were prepared by De Grado and co-workers, who oxidized phage display libraries containing pairs of cysteines to form their cyclic counterparts.[10] But these compounds still retain free amine and carboxyl termini and are thus susceptible to proteolytic attack.

Head-to-tail cyclization confers partial enzyme resistance (toward exopeptidases) but preserves the rich diversity of stereochemically defined side chains characteristic of amino acid-derived analogs. A particularly facile method for preparing head-to-tail cyclic peptides using aspartic acid linked to a polystyrene resin was recently reported.[11] A variety of other methods for preparing cyclic peptides has also been described.[12,13] We have previously described the utility of side chain attachment and resin-bound cyclization for the preparation of relatively clean cyclic peptide mixtures.[14,15] We now wish to report the preparation and bioassay of a cyclic pentapeptide library designed to validate the efficiency of moderately large mixtures combined with a positional scan approach as applied to cyclic peptides and related compounds.

Results and Discussion

The starting point for our synthesis was a single D-aspartic acid linked to a solid phase support through its side chain β-carboxylic acid function. Boc-D-Asp-OFm (**1**) was initially attached to a *p*-hydroxymethyl poly(styrene) resin with a functionalization of 0.8 mequiv/g. The substitution yield was evaluated by cleavage and UV quantification of fluorenylmethanol on a known amount of acylated resin[16] (**2**) and was found to be 0.52 mmol/g. Synthesis of the libraries (Table 1) was performed in a synthesis block[17] (Coshisoft, Tucson) composed of 42 poly(propylene) syringes equipped with a plastic frit. Each of the syringes was loaded with 150 mg of **2** and acted as an individual reaction vessel. Boc chemistry[18,19] was used to elongate the peptide and included 50% trifluoroacetic acid cleavage and a phosphonium-based[20] condensing agent (BOP/HOBt). The resin was kept in suspension through shaking of the synthesis block with a rotary shaker (3D rotator, CMS). Completions of the couplings were monitored by the Kaiser ninhydrin test[21] on four syringes chosen at random at each step. In the event of a positive (blue) color, the couplings for all vials were repeated.

At each coupling step, either a mixture of protected amino acids was used, consisting of six D- and six L-configuration amino acids, or a single amino acid of the composite was introduced. The amino acids selected were D- and L-versions of Boc-Trp, Boc-Leu, Boc-Glu(OBzl), Boc-Arg(Tos), Boc-Val, and Boc-Pro. These choices were based on a desire both to provide diversity[22] in the classic Hansch characteristics[23] (steric, electronic, and hydrophobic factors) as well as to include

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Table 1. Structure and Endothelin Binding Activities of the 48 Cyclic Peptide Libraries, where Xxx is a Nearly Equimolar Mixture of the 12 Following Amino Acids: D,L-Proline, D,L-Valine, D,L-Leucine, D,L-Tryptophan, D,L-Arginine, and D,L-Glutamic Acid[a]

code	structure	% binding inhibition	code	structure	% binding inhibition
L1	cyclo(Xxx-Xxx-Xxx-L-Pro-D-Asp)	0	**L25**	cyclo(Xxx-L-Pro-Xxx-Xxx-D-Asp)	10.8
L2	cyclo(Xxx-Xxx-Xxx-L-Val-D-Asp)	0	**L26**	cyclo(Xxx-L-Val-Xxx-Xxx-D-Asp)	11.6
L3	cyclo(Xxx-Xxx-Xxx-L-Leu-D-Asp)	0	**L27**	cyclo(Xxx-L-Leu-Xxx-Xxx-D-Asp)	7.0
L4	cyclo(Xxx-Xxx-Xxx-L-Trp-D-Asp)	0	**L28**	cyclo(Xxx-L-Trp-Xxx-Xxx-D-Asp)	3.0
L5	cyclo(Xxx-Xxx-Xxx-L-Arg-D-Asp)	4.0	**L29**	cyclo(Xxx-L-Arg-Xxx-Xxx-D-Asp)	0.3
L6	cyclo(Xxx-Xxx-Xxx-L-Glu-D-Asp)	7.7	**L30**	cyclo(Xxx-L-Glu-Xxx-Xxx-D-Asp)	0
L7	cyclo(Xxx-Xxx-Xxx-D-Pro-D-Asp)	0	**L31**	cyclo(Xxx-D-Pro-Xxx-Xxx-D-Asp)	31.2
L8	cyclo(Xxx-Xxx-Xxx-D-Val-D-Asp)	0	**L32**	cyclo(Xxx-D-Val-Xxx-Xxx-D-Asp)	37.3
L9	cyclo(Xxx-Xxx-Xxx-D-Leu-D-Asp)	0	**L33**	cyclo(Xxx-D-Leu-Xxx-Xxx-D-Asp)	11.5
L10	cyclo(Xxx-Xxx-Xxx-D-Trp-D-Asp)	72.3	**L34**	cyclo(Xxx-D-Trp)-Xxx-Xxx-D-Asp)	4.9
L11	cyclo(Xxx-Xxx-Xxx-D-Arg-D-Asp)	0	**L35**	cyclo(Xxx-D-Arg-Xxx-Xxx-D-Asp)	0
L12	cyclo(Xxx-Xxx-Xxx-D-Glu-D-Asp)	0	**L36**	cyclo(Xxx-S-Glu-Xxx-Xxx-D-Asp)	8.5
L13	cyclo(Xxx-Xxx-L-Pro-Xxx-D-Asp)	0	**L37**	cyclo(L-Pro-Xxx-Xxx-Xxx-D-Asp)	43.6
L14	cyclo(Xxx-Xxx-L-Val-Xxx-D-Asp)	0	**L38**	cyclo(L-Val-Xxx-Xxx-Xxx-D-Asp)	0
L15	cyclo(Xxx-Xxx-L-Leu-Xxx-D-Asp)	76.7	**L39**	cyclo(L-Leu-Xxx-Xxx-Xxx-D-Asp)	0
L16	cyclo(Xxx-Xxx-L-Trp-Xxx-D-Asp)	45.4	**L40**	cyclo(L-Trp-Xxx-Xxx-Xxx-D-Asp)	0
L17	cyclo(Xxx-Xxx-L-Arg-Xxx-D-Asp)	15.1	**L41**	cyclo(L-Arg-Xxx-Xxx-Xxx-D-Asp)	0
L18	cyclo(Xxx-Xxx-L-Glu-Xxx-D-Asp)	1.2	**L42**	cyclo(L-Glu-Xxx-Xxx-Xxx-D-Asp)	0
L19	cyclo(Xxx-Xxx-D-Pro-Xxx-D-Asp)	0	**L43**	cyclo(D-Pro-Xxx-Xxx-Xxx-D-Asp)	8.4
L20	cyclo(Xxx-Xxx-D-Val-Xxx-D-Asp)	0	**L44**	cyclo(D-Val-Xxx-Xxx-Xxx-D-Asp)	0
L21	cyclo(Xxx-Xxx-D-Leu-Xxx-D-Asp)	0	**L45**	cyclo(D-Leu-Xxx-Xxx-Xxx-D-Asp)	0
L22	cyclo(Xxx-Xxx-D-Trp-Xxx-D-Asp)	14.8	**L46**	cyclo(D-Trp-Xxx-Xxx-Xxx-D-Asp)	10.3
L23	cyclo(Xxx-Xxx-D-Arg-Xxx-D-Asp)	0	**L47**	cyclo(D-Arg-Xxx-Xxx-Xxx-D-Asp)	15.2
L24	cyclo(Xxx-Xxx-D-Glu-Xxx-D-Asp)	0	**L48**	cyclo(D-Glu-Xxx-Xxx-Xxx-D-Asp)	0

[a] The data reflect percent inhibition of ET_1 binding, corrected for nonspecific binding.

the residues found in the potent cyclic peptide antagonist BQ-123 or cyclo(L-Pro-D-Val-L-Leu-D-Trp-D-Asp).[24] When using a mixture, the total excess of amino acids was 4-fold, and the individual ratios were adjusted to values comparable to those reported by Geysen et al.,[25] empirically determined to produce nearly equivalent product ratios.

The cyclic peptides were then prepared in groups of $12 \times 12 \times 12 \times 1$ or 1728-component mixtures. In each of 48 vials, aspartic acid and a second amino acid in one of the remaining four positions were held as constants. This approach is predicted to result in the redundant formation of $20\,736 \times 4$ or 82 944 peptides. Among the 48 vials, any one particular sequence is represented four times, thus providing a possibility of discovering optimized lead candidates in a single series of bioassays, without the need for extensive deconvolution from repeated synthesis. This novel approach was first described by Houghten and colleagues[26,27] and used to rediscover the sequences of leucine and methionine enkephalin among a series of linear pentapeptides.

Following the completion of the synthesis of the linear pentapeptides, the N-terminal Boc protecting group was removed with trifluoroacetic acid and the C-terminal fluorenylmethyl ester α-aspartyl protecting function was removed with 20% piperidine. On-resin cyclization was carried out using BOP/HOBt for 2 h and was repeated three times to obtain a negative Kaiser test. A multiple HF apparatus[28] permitted the simultaneous treatment of 24 libraries using anisole as a scavenger. After removal of HF, the peptide products were extracted with a 30% acetic acid solution. The latter was washed with diethyl ether and lyophilized. Salt free peptides were afforded after reversed phase solid phase extraction using Varian Bond Elut C18. Model studies, monitored by mass spectrometry, suggested that both hydrophobic and hydrophilic cyclic peptides are recoverable intact following the procedure (unpublished observation). The 48 mixtures were lyophilized individually to yield a final weight of peptide product ranging from 21.2 to 34.8 mg.

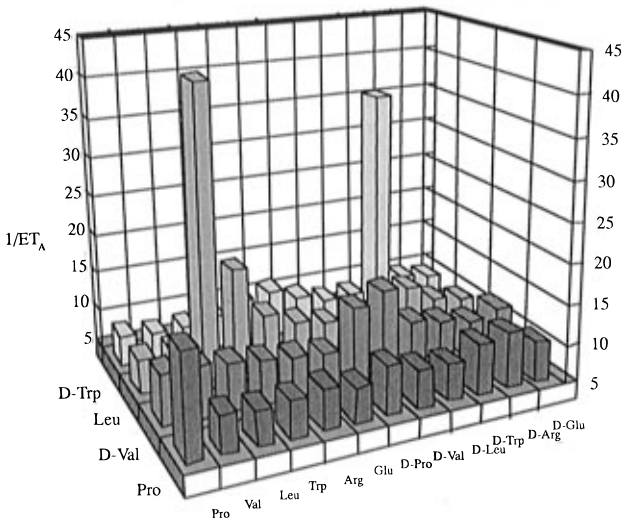

Figure 1. Rediscovering Banyu cyclic pentapeptide endothelin antagonist cyclo(Pro-D-Val-Leu-D-Trp-D-Asp). Relative potencies of 48 vials containing 1728 cyclic peptides are given as reciprocals of ET_A binding concentrations, after correction for nonspecific binding.

Biological assays for endothelin receptor binding were performed *in vitro* using a cell culture assay. In order to assess biological activity of peptide libraries, we performed competitive radioligand binding assay with [^{125}I]ET-1 in CHO cell monolayers expressing recombinant human endothelin-A (ET_A) receptors. Each library was added to the bioassay at a total peptide concentration of 800 μM. This resulted in a nominal concentration of individual cyclic peptide species of approximately 0.5 μM in the bioassay, assuming a uniform combinatorial synthesis. The results of the screening from a single-point assay allowed the determination of the most active amino acid for each position. Maximal enhancement of activity (Figure 1) was observed for L-Pro at the first position, D-Val at the second, L-Leu at the third, and D-Trp at the fourth. This combination in fact corresponds exactly to the residues found in the Banyu

antagonist[24] BQ-123. Nevertheless, a more modest activity was also observed for D-Pro at the second position *n* and for L-Trp at the third. We were aware that the activity of a library containing two slightly active molecules could have a global potency higher than the activity measured for a mixture containing the most active peptide. Furthermore, the occurrence in a library of several molecules with different activity intensity is very probable since it is commonly observed that only a few amino acids in a peptide sequence are truly critical for the biological response. In this respect, the peptide with the apparently preferred combination of amino acids in a positional scan assay may not be the optimum combination for maximum potency.

Accordingly, four new peptides (**P1**−**P4**) were synthesized based upon the four apparently best combinations. The four analogs were synthesized using solid phase methods using the same approach as with the peptide mixtures. Purification by reversed phase high-performance liquid chromatography (RP-HPLC) afforded four compounds whose structures were further defined by electrospray mass spectrometry (ES-MS) (see Experimental Section) and by comparison to the authentic compound in the case of BQ-123.

Next, dose−activity response studies were carried out on each of these four single compounds. The dose−activity response curves (Figure 2) from a seven-point assay indicated the highest endothelin receptor binding activity for **P3** which corresponds to the original Banyu compound. **P4**, which substitutes an L-tryptophan for the L-leucine in BQ-123, possessed only a modest endothelin receptor binding activity, while **P1** and **P2**, both of which contain two prolines, were totally inactive. The lack of activity for **P1** and **P2** either could reflect false positives in the binding assay or might suggest possible synergism among these complex mixtures.

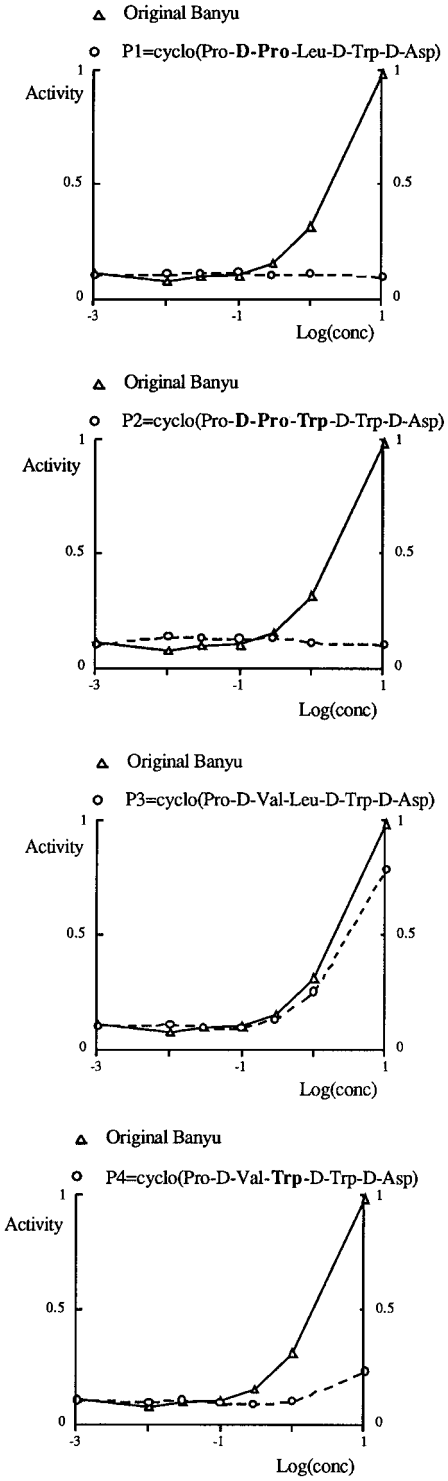

Figure 2. Dose−activity response curves where inhibitory activity of authentic cyclo(Pro-D-Val-Leu-D-Trp-D-Asp) (BQ-123) is compared to four synthetic analogs with purification protocols comparable to library components.

Conclusions

The major objective of this study was to validate our synthetic approach for the preparation of relatively pure cyclic peptide libraries. The major synthesis features, including side chain attachment of the first amino acid to the resin, and on-resin cyclization appear as reasonable and effective choices for the preparation of head-to-tail cyclic peptide libraries.

Our study presented two secondary goals: on the one hand, validation of the positional scanning approach for medium size cyclic peptide libraries and, on the other hand, an attempt to find a cyclic peptide with higher endothelin receptor binding activity than BQ-123. While no improved BQ-123 analogs were unveiled in the process, the rediscovery of the Banyu endothelin antagonist compound among the 82 944 cyclic peptides of the "self-deconvoluting" library confirms that this strategy might prove useful for the rapid identification of suitable targets for new receptor classes where no prior constrained leads are available.

Experimental Section

Instruments and Materials. ^{1}H-NMR spectra (300 MHz, $CDCl_3$) were recorded on a Varian Inova-300 spectrometer. TLC were run on Merck silica gel 60 F254 plates. Melting points were determined on a Unimelt Thomas Hoover capillary melting point apparatus and are uncorrected. Specific optical rotations were determined on a Jasco Model 700 instrument at the sodium D line. Amino acid analyses were performed on a Dionex D-300 amino acid analyzer and a Dionex CP-3 programmer at the Medical Center of the University of Louisville after the peptide mixtures were hydrolyzed in 6 N HCl at 110 °C for 20−24 h in sealed, evacuated hydrolysis tubes. Molecular weight determinations were made by ES-MS at the University of Michigan. HPLC was performed on a Hitachi 655A-22 instrument with a Vydac C18 column using a linear gradient of (A) water containing 0.05% TFA and (B) acetonitrile containing 0.05% TFA, at a flow rate of 1 mL/min with UV detection at 254 nm unless otherwise specified. UV spectra were recorded on a Hewlett Packard 8452A diode array spectrometer.

N^{α}-(*tert*-Butyloxycarbonyl)-D-Aspartic Acid β-Fluorenylmethyl Ester (1).[29] DCC (12.4 g, 60.1 mmol) was added portionwise to a stirred solution of N^{α}-(*tert*-butyloxycarbonyl)-D-aspartic acid[30] (14 g, 60 mmol) in 100 mL of ethyl acetate at 0 °C. The reaction mixture was kept at 0 °C for 1 h and at room temperature for 1 h. The solution was filtered and the solvent evaporated to dryness to obtain N^{α}-(*tert*-butyloxycarbonyl)-D-aspartic anhydride. The latter was dissolved in 100 mL of dry THF with 12.4 g (63.2 mmol) of 9-fluorenylmethanol. Next 11.5 mL (66 mmol) of DIEA was added, and the solution was stirred overnight. The solvent was evaporated and the remaining solid partitioned between AcOEt and a 5% citric acid solution. The aqueous solution was acidified to pH 4 with 4 N HCl. The organic phase was washed with a 5% citric acid solution and then water, dried ($MgSO_4$), and concentrated until the appearance of the first crystals. The product **1** was crystallized from ethyl acetate/hexane, filtered, washed with hexane, and dried *in vacuo*: yield 13.4 g (54%); TLC R_f ($CHCl_3$−EtOH−AcOH = 90:8:2) 0.63; mp 156°C; $[\alpha]^{20}_D$ +15.2° (*c* 1.00, THF); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.50 (s, 9H, Me_3C), 2.95 (ddd, 2H, CH_2COOH), 4.24 (t, 1H, $COOCH_2CH$), 4.50 (multiplet, 2H, $COOCH_2CH$), 4.68 (t, 1H, NHC*H*), 5.55 (d, 1H, NH), 7.30−7.79 (cluster, 8H, aryl). Calcd for $C_{23}H_{25}NO_6$, MW = 411.47; C, H, N.

Boc-Asp(*O*-benzyl poly(styrene) resin)-OFm-Acylated Resin (2). Hydroxymethyl resin (9.0 g, 0.8 mequiv/g) (Peninsula; 1% cross-linked) was swollen in 75 mL of distilled pyridine. Next 4.7 mL (30 mmol) of diisopropylcarbodiimide was added to a solution of 12.0 g (29.1 mmol) of **1** and 4.0 g (29.6 mmol) of HOBt in 100 mL of dry THF. The mixture was stirred for 20 min before addition to the resin. After 6 h of reaction, the resin was washed with 3 × 40 mL of methanol followed by 3 × 40 mL of DCM. The resin was capped by stirring for 90 min in 100 mL of DCM containing 5.5 mL (58.2 mmol) of acetic anhydride and 4.5 mL (55.7 mmol) of pyridine. The resin was then filtered, washed as described previously, and dried *in vacuo*: yield 11.2 g; substitution yield 0.52 mmol/g.

General Procedure for the Synthesis of Libraries L1−L48 Cyclo(Xxx-Xxx-Xxx-Xxx-D-Asp), where Xxx = D,L-Pro, D,L-Val, D,L-Leu, D,L-Trp, D,L-Arg, and D,L-Glu. The libraries were synthesized in two separate batches (**L1−L24** followed by **L25−L48**) on 150 mg (0.078 mmol of aspartic acid) of resin **2** in each reactor.

Peptide chain elongation: The resin was washed with DCM (3 × 2 min × 1 mL/syringe) and shaken with 50% TFA in DCM (5 min × 1 mL/syringe). The TFA treatment was repeated for an additional 25 min; the resin was washed with DCM (5 min × 1 mL/syringe), neutralized with DIEA−DCM (1:9) (2 min × 1 mL/syringe), and washed again with DCM. For each coupling, the protected amino acids were individually weighed, combined, and divided into individual syringes according to the amino acid ratios given in the Supporting Information. For each syringe, the proportionate values would be Boc-L-Trp (7.97 mg, 0.026 mmol), Boc-L-Leu (5.68 mg, 0.023 mmol), Boc-L-Glu(OBzl) (9.97 mg, 0.030 mmol), Boc-L-Arg(Tos) (16.4 mg, 0.038 mmol), Boc-L-Val (4.34 mg, 0.020 mmol), Boc-L-Pro (4.30 mg, 0.020 mmol), Boc-D-Trp (7.97 mg, 0.026 mmol), Boc-D-Leu (5.68 mg, 0.023 mmol), Boc-D-Glu(OBzl) (9.97 mg, 0.030 mmol), Boc-D-Arg(Tos) (16.4 mg, 0.038 mmol), Boc-D-Val (4.34 mg, 0..020 mmol), and Boc-D-Pro (4.30 mg, 0.020 mmol). Coupling reagent amounts used were BOP (138.1 mg, 0.312 mmol) and HOBt (42.2 mg, 0.312 mmol), dissolved with the amino acids in 1 mL of DMF/syringe. Each milliliter of amino acid solution also contained 54.5 μL of DIEA (0.312 mmol). The mixture was stirred for 1 min prior to transfer to individual syringes. The coupling of single amino acid was achieved with a 4-fold excess of amino acid, BOP (138.06 mg, 0.312 mmol), HOBt (42.15 mg, 0.312 mmol), and DIEA 54.5 μL (0.312 mmol) in 1 mL of DMF. The block was shaken for 1 h, and the completion of the couplings was monitored by testing of four randomly chosen syringes using the Kaiser ninhydrin test.

Cyclic Peptide Library Cyclizations Using BOP Reagent. The resin in each syringe was washed with DCM, shaken for 20 min with 1 mL of 20% piperidine in DMF, filtered, and washed again with DCM. The Boc group was cleaved with TFA and the resin neutralized as described above. Next the condensing agents were preactivated as follows: BOP (3.31 g, 7.48 mmol), HOBt (1.012 g, 7.48 mmol), and DIEA (1.3 mL, 7.48 mmol) in 24 mL of DMF. The mixture was stirred for 1 min and distributed to the 24 syringes, and the synthesis block was shaken for 2 h. The cyclization step was repeated two more times to obtain a negative Kaiser test in four randomly chosen syringes. The resin was washed with DCM and dried *in vacuo* overnight.

Cyclic Peptide Library Cleavage from Resin Using Anhydrous Hydrogen Fluoride. The cleavages were achieved in a 24-chamber multiple HF apparatus. **L1−L24** and **L25−L48** were cleaved separately. For each syringe, the resin was placed in a cleavage vessel with 200 μL of anisole and 5 mL of anhydrous HF. The mixture was stirred at 0 °C for 1 h before removal of HF. For each reaction vessel, the resin was transferred to a fritted glass funnel, and the peptide products were extracted with 50 mL of 30% acetic acid each. The acetic acid solution was washed twice with 25 mL of diethyl ether and lyophilized to obtain salty flakes. The peptide libraries were obtained after solid phase extraction on reversed phase Varian Bond Elut C18; the remaining salts were washed out with water, and the peptides were extracted with 50 mL of acetonitrile−water (1:1) and lyophilized to afford white or off-white powders. Yields ranged from 21 to 35 mg for the individual syringe reactions (theory = 47.6 mg based on an average MW of 610 for BQ-123 or 49.6 mg if calculated on the number-average MW of 636). The amino acid compositions of **L10**, **L17**, **L30**, and **L45** were randomly checked by amino acid analysis.

Cyclo(Pro-D-Pro-Leu-D-Trp-D-Asp) (P1), Cyclo(Pro-D-Pro-Trp-D-Trp-D-Asp) (P2), Cyclo(Pro-D-Val-Leu-D-Trp-D-Asp) (P3), and Cyclo(Pro-D-Val-Trp-D-Trp-D-Asp) (P4). The synthesis, cleavage, and purification of these four compounds were performed on 150 mg of resin for each peptide in a fashion identical with that reported for the libraries. HPLC analysis of the crude cyclic peptide products following solid phase extraction varied 45−60%, and no further purification of these products was attempted prior to bioassay. The compounds were characterized using high-resolution electrospray mass spectroscopy and HPLC. Compound **P3**, corresponding to authentic BQ-123, was cochromatographed against authentic BQ-123 (kindly provided by Dr. K. Darlak).

For purposes of more complete characterization, each of the four crude samples was further purified as follows. Reversed phase HPLC purifications of samples of **P1−P4** were performed using a linear gradient of (A) water containing 0.05% TFA and (B) acetonitrile containing 0.05% TFA (30−90% B, 30 min) at a flow rate of 1 mL/min on an analytical Vydac C18 reversed phase column (4 mm × 250 mm) with UV monitoring at 254 nm. Samples were submitted for mass spectral analysis and amino acid analysis and provided the following results.

P1: Asp 1.2 (1), Pro 2.3 (2), Leu 0.7 (1), Trp 0.8 (1); ES-MS (positive mode) M + Na^+ = 631.7; ES-MS (negative mode) 607.3 (calcd for $C_{31}H_{40}N_6O_7$ 608.28).

P2: Asp 1.0 (1), Pro 2.2 (2), Trp ND (2); ES-MS (positive mode) M + Na^+ = 706.6; ES-MS (negative mode) 680.5 (calcd for $C_{36}H_{39}N_7O_7$ 681.28).

P3: Asp 1.0 (1), Pro 1.1 (1), Leu 0.8 (1), Trp ND (1), Val 1.0 (1); ES-MS (positive mode) M + Na = 632.3; ES-MS (negative mode) 609.5 (calcd for $C_{31}H_{42}N_6O_7$ 610.30).

P4: Asp 1.0 (1), Pro 1.1 (1), Trp ND (2), Val 0.9 (1); ES-MS (positive mode) M + Na = 706.6; ES-MS (negative mode) 682.4 (calcd for $C_{36}H_{41}N_7O_7$ 683.30).

P1: TLC R_f (nBuOH−AcOH−H_2O = 8:1:1) 0.51; HPLC t_R 17.46 min (linear gradient 5−90% B, 30 min), 8.21 min (linear gradient 30−90% B, 30 min). **P2**: TLC R_f (nBuOH−AcOH−H_2O = 8:1:1) 0.49; HPLC t_R 18.20 min (linear gradient 5−90% B, 30 min), 9.47 min (linear gradient 30−90% B, 30 min). **P3**: TLC R_f (nBuOH−AcOH−H_2O = 8:1:1) 0.87; HPLC t_R 19.36 min (linear gradient 5−90% B, 30 min), 10.36 min (linear gradient 30−90% B, 30 min). **P4**: TLC R_f (nBuOH−AcOH−H_2O = 8:1:1) 0.88; HPLC t_R 19.95 min (linear gradient 5−90% B, 30 min), 11.27 min (linear gradient 30−90% B, 30 min).

Radioligand Binding Assay. CHO cells stably expressing human endothelin-A receptor cDNA were cultured in monolayers on 24-well plates as previously described.[31,32] Confluent monolayers of cells were preincubated with cyclic peptide analogs at 37 °C for 10 min in a binding buffer (DMEM/0.3% bovine serum albumin) containing 2% DMSO. Then [^{125}I-Tyr13]ET-1 was added to each well at 20 pM, and the cells were incubated at 37 °C for an additional 1 h. Cells were washed four times with ice-cold binding buffer and then lysed with 0.1 N NaOH. Cell-bound radioactivity was determined using a γ-counter. Nonspecific binding was defined in the presence of 1 μM unlabeled ET-1 and was approximately 1% of the levels of the specific binding in the absence of competitor peptides. Assays were carried out in triplicate. Synthetic cyclic peptide libraries were solubilized in a small volume of DMSO and then diluted with binding buffer to achieve a final concentration of total peptides of approximately 0.8 mM in assay wells.

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Supporting Information Available: Tables S1 and S2 containing the ratio of amino acids used in mixtures and amino acid analysis results for six randomly selected samples of cyclic peptide mixtures (2 pages). Ordering information is given on any current masthead page.

References

(1) Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gordon, E. M. Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries. *J. Med. Chem.* **1994**, *37*, 1233−1251.

(2) Gordon, E. M.; Barrett, R.; Dower, W. J.; Fodor, S. P. A.; Gallop, M. A. Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions. *J. Med. Chem.* **1994**, *37*, 1385−1401.

(3) Terrett, N. K.; Gardner, M.; Gordon, D. W.; Kobylecki, R. J.; Steele, J. Combinatorial synthesis - The design of compound libraries and their application to drug discovery. *Tetrahedron* **1995**, *51*, 8135−8173.

(4) Thompson, L. A.; Ellman, J. A. Synthesis and applications of small molecule libraries. *Chem. Rev.* **1996**, *96*, 555−600.

(5) DeWitt, S.; Kiely, J.; Stankovic, C.; Schroeder, M.; Cody, D.; Pavia, M. "Diversomers": an approach to nonpeptide, nonoligomeric chemical diversity. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6909−6913.

(6) Bunin, B.; Plunkett, M.; Ellmann, J. The combinatorial synthesis and chemical and biological evaluation of 1,4-benzodiazepine library. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4708−4712.

(7) Cho, C.; Moran, E.; Stephans, J.; Fodor, S.; Adams, C.; Sundaram, A.; Jacobs, J.; Schultz, P. An unnatural biopolymer. *Science* **1993**, *261*, 1303−1305.

(8) Ladner, R. C. Constrained peptides as binding entities. *Tibtech* **1995**, *13*, 426−430.

(9) Olivera, B.; Rivier, J.; Clark, C.; Ramilo, C.; Corpuz, G.; Abogadie, F.; Mena, E.; Woodward, S.; Hillyard, D.; Cruz, L. Diversity of conus neuropeptides. *Science* **1990**, *249*, 257−263.

(10) O'Neil, K. T.; Hoess, R. H.; Jackson, S. A.; Ramachandran, N.; Mousa, S. A.; DeGrado, W. F. Identification of GP-IIB/IIIA Binding Peptides Using a Conformationally Constrained Phage Peptide Library. *Proteins* **1992**, *14*, 509−515.

(11) Rovero, P.; Quartara, L.; Fabbri, G. Synthesis of cyclic peptides on solid support. *Tetrahedron Lett.* **1991**, *32*, 2639−2642.

(12) Kates, S. A.; Solé, N. A.; Johnson, C.; Hudson, D.; Barany, G.; Albericio, F. A novel, convenient, three phase synthesis of cyclic peptides. *Tetrahedron Lett.* **1993**, *34*, 1549−1552.

(13) Kates, S. A.; Solé, N. A.; Albericio, F.; Barany, G. Solid phase synthesis of cyclic peptides. In *Peptides: Design, Synthesis and Biological Activity*; Basava, C., Anantharamaiah, G. M., Eds.; Birkhäuser: Boston, 1994; pp 39−58.

(14) Darlak, K.; Romanovskis, P.; Spatola, A. Cyclic peptide libraries. In *Peptides: Chemistry, Structure, and Biology*; Hodges, R., Smith, J., Eds.; ESCOM: Leiden, 1994; pp 981−983.

(15) Spatola, A. F.; Darlak, K.; Romanovskis, P. An approach to cyclic peptide libraries: reducing epimerization in medium size during solid phase synthesis. *Tetrahedron Lett.* **1996**, *37*, 591−594.

(16) Green, J.; Bradley, K. Studies on the acylation of hydroxy-functionalized resins using Fmoc amino acids activated using diisopropylcarbodiimide/HOBt or as acid fluorides. *Tetrahedron* **1993**, *49*, 4141−4146.

(17) Krchnak, V.; Vagner, J. Color monitored solid-phase multiple peptide synthesis under low-pressure continuous-flow conditions. *Pept. Res.* **1990**, *3*, 182.

(18) Fields, G.; Tian, Z.; Barany, G. In *Synthetic Peptides: A User's Guide;* Grant, G., Ed.; W. H. Freeman and Co.: New York, 1992; Chapter 3.

(19) Gairi, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. Use of BOP reagent for the suppression of diketopiperazine formation in Boc/Bzl solid-phase peptide synthesis. *Tetrahedron Lett.* **1990**, *31*, 7363−7366.

(20) Castro, B.; Dormoy, J.; Evin, G.; Selve, C. Reactif de couplage peptidique IV (1)-L'hexafluorophosphate de benzotriazolyl N-oxytrisdimethylamino phosphonium (B.O.P.). (Peptide coupling reagents. IV. N-[Oxytris(dimethylamino)phosphonium]benzotriazole hexafluorophosphate.) *Tetrahedron Lett.* **1975**, 1219−1222.

(21) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595−598.

(22) Hellberg, S.; Sjöström, M.; Skagerberg, B.; Wold, S. Peptide quantitative structure-activity relationship: a multivariate approach. *J. Med. Chem.* **1987**, *30*, 1126−1135.

(23) Hansch, C. In *Drug Design*; Ariens, E., Ed.; Academic Press: New York and London, 1971; Chapter 2.

(24) Ihara, M.; Noguchi, K.; Saeki, T.; Fukuroda, T.; Tsuchida, S.; Kimura, S.; Fukami, T.; Ishikawa, K.; Nishikibe, M.; Yano, M. Biological profiles of highly potent novel endothelin antagonists selective for ET_A receptor. *Life Sci.* **1992**, *50*, 247−255.

(25) Geysen, H. M. U.S. Patent 5 194 392, 1993.

(26) Houghten, R.; Pinilla, C.; Blondelle, S.; Appel, J.; Dooley, C.; Cuervo, J. Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature (London)* **1991**, *354*, 84−86.

(27) Dooley, C. T.; Houghten, R. A. The use of positional scanning synthetic peptide combinatorial libraries for the rapid determination of opioid receptor ligands. *Life Sci.* **1993**, *52*, 1509−1517 and references therein.

(28) Houghten, R.; Bray, M.; Degraw, S.; Kirby, C. Simplified procedure for carrying out simultaneous multiple hydrogen fluoride cleavages of protected peptide resins. *Int. J. Pept. Protein Res.* **1986**, *27*, 673−768.

(29) Al-Obeidi, F.; Sanderson, D.; Hruby, V. Synthesis of β- and γ-fluorenylmethyl esters of respectively N^{α}-Boc-L-aspartic acid and N^{α}-Boc-L-glutamic acid. *Int. J. Pept. Protein Res.* **1990**, *35*, 215−218.

(30) Stewart, J.; Young, J. *Solid Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, 1984; pp 61−62.

(31) Xu, D.; Emoto, N.; Giaid, A.; Slaughter, C.; Kaw, S.; deWit, D.; Yanagisawa, M. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* **1994**, *78*, 473−485.

(32) Sakurai, T.; Yanagisawa, M.; Takuwa, Y.; Miyazaki, H.; Kimura, S.; Goto, K.; Masaki, T. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **1990**, *348*, 732−735.

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