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Rapid Parallel Synthesis of Bioactive Folded Cyclotides by Using a Tea-Bag Approach

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We report here the first rapid parallel production of bioactive folded cyclotides by using Fmoc-based solid-phase peptide synthesis in combination with a "tea-bag" approach. Using this approach, we efficiently synthesized 15 analogues of the CXCR4 antagonist cyclotide MCo-CVX-5c. Cyclotides were synthesized in a single-pot, cyclization/folding reaction in the presence of reduced glutathione. Natively folded cyclotides were quickly purified from the cyclization/folding crude mixture by activated thiol Sepharose-based chromatography. The different folded cyclotide analogues were then tested for their ability to inhibit the CXCR4 receptor in a cell-based assay. The results indicated that this approach can be used for the efficient chemical synthesis of libraries of cyclotides with improved biological properties that can be easily interfaced with solution or cell-based assays for rapid screening.

Introduction

Cyclotides are small (28 to 37 residues) globular microproteins that posses a unique head-to-tail cyclized backbone that is stabilized by three disulfide bonds forming a cystine-knot motif.[1,2] The cyclic cystine-knot (CCK) molecular scaffold provides a very rigid molecular platform[3–5] that confers an exceptional stability towards physical, chemical, and biological degradation.[1,2] Cyclotides can be considered to be natural combinatorial peptide libraries that are structurally constrained by the cystine-knot scaffold and head-to-tail cyclization, but in which hypermutation of essentially all residues is permitted—with the exception of the strictly conserved cysteines that hold the knot together.[6–8] In addition, naturally occurring cyclotides have been shown to posses pharmacologically relevant activities.[1,9] Cyclotides have also been engineered to target extra-[10–12] and intracellular[13] molecular targets in animal models. Some of these novel cyclotides have been shown to be orally bioavailable[11] and able to efficiently cross cellular membranes.[14–16] Together, these features make the cyclotide scaffold an excellent molecular framework for the design of new peptide-based therapeutics.[2,17]

Naturally occurring cyclotides are ribosomally produced in plants from precursors that comprise one to three domains.[18–21] However, the mechanism of excision of the cyclotide domains and ligation of the free N and C termini to produce the circular peptides has not yet been completely elucidated, although it has been speculated that asparaginyl endopeptidases are involved in the cyclization process.[22–24] Cyclotides can also be produced recombinantly by using standard microbial expression systems and making use of modified protein-splicing units,[25–28] thus allowing the production of biologically generated libraries of these microproteins for the first time.[26]

The relatively small size of cyclotides also makes it possible to employ chemical tools to generate synthetic combinatorial libraries based on this scaffold for the screening and selection of optimized sequences for a particular biological activity. Chemical libraries have some advantages over biologically produced libraries, for example, chemical libraries are not constrained to natural amino acids and can include both unnatural and D-amino acids, in addition to secondary structures not tolerated by the ribosome. As unnatural and D-amino acids are less susceptible to proteases and peptidases than natural L-amino acids, chemical libraries have the potential to rapidly identify stable and bioactive peptide sequences. In addition, chemical libraries allow the incorporation of post-translational modifications, such as glycosylation and phosphorylation, which are not accessible in bacterial expression systems.

The chemical synthesis of several naturally occurring and engineered cyclotides has been already accomplished by solid-phase peptide synthesis (SPPS) using either Boc-[29,30] or Fmoc-based[10,13,14,31–33] chemistry. All of them use intramolecular native chemical ligation (NCL)[34,35] to cyclize the backbone followed by oxidative folding to produce the natively folded cyclotide. Our group has recently reported that the cyclization and oxidative folding reactions can be efficiently performed in a one-pot reaction when carried out in aqueous phosphate buffer (pH 7.2) in the presence of reduced glutathione (GSH).[10,13,14] Under these conditions, folded cyclotides can be

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produced very efficiently in one step from the corresponding linear precursor with minimal purification;[10,13,14] minimizing the number of steps required to produce cyclotides being key to the production of large libraries. This approach has also been recently used for the rapid and efficient production of other disulfide-rich, backbone-cyclized polypeptides, such as the cyclic defensin RTD-1.[36]

The production of combinatorial peptide-based libraries requires the parallel synthesis of many peptides. The "tea-bag" approach was one the first developed.[37] This approach is well suited for the production of medium-sized libraries ($\approx 3\times10^2$) for antigenic mapping,[38] and to produce amino-acid-scanning[39,40] and positional-scanning libraries.[41] This approach, however, has been mostly used to produce small (10–15 residues) linear peptides, and it has never been explored for the production of libraries of large, complex, folded polypeptides such as cyclotides.

In this work, we report the first rapid parallel synthesis of a small library of folded bioactive cyclotides by using a tea-bag approach in combination with optimized cyclization/folding protocols that require minimal purification steps. As a model system, we used the cyclotide MCo-CVX-5c (Figure 1), a potent antagonist of the CXCR4 reporter.[10] Using this approach, we were able to rapidly produce 15 cyclotides based on MCo-CVX-5c that were tested to rapidly evaluate structural–activity relationships.

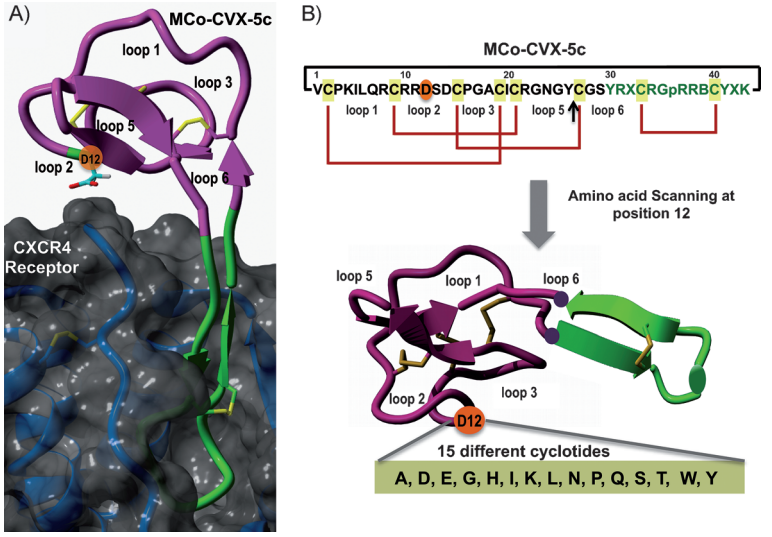

Figure 1. Design of the amino-acid-scanning library for position 12 in loop 2 of cyclotide MCo-CVX-5c (WT). A) Structural model of the molecular complex between MCo-CVX-5c and the CXCR4 receptor, Asp12 is in close proximity to the extracellular surface of CXCR4. B) Sequence and disulfide connectivities (gray) of MCo-CVX-5c and the analogues. The black line indicates the backbone cyclization between the α-NH_2 and the α-carboxylate groups of residues 1 and 43, respectively. The cyclization site is indicated with an arrow. Single-letter codes B, X, and p represent 2-naphthylalanine, citruline, and D-proline, respectively. Molecular graphics were built with Yasara (http://www.yasara.org).

Results and Discussion

Library design

We used the cyclotide MCo-CVX-5c as a model to evaluate the parallel synthesis of cyclotides by the tea-bag approach. This cyclotide was engineered to inhibit the CXCR4 receptor by grafting a topologically modified CVX15-based peptide onto loop six of cyclotide MCoTI-I (Figure 1).[10] This cyclotide has been shown to be a potent CXCR4 antagonist and an efficient HIV-1 cell-entry blocker.[10] In order to improve the activity of this cyclotide, we explored potential mutations located in the neighboring loops to the loop used for the graft that could stabilize the interaction between the cyclotide and the receptor. We first built a model of cyclotide MCo-CVX-5c bound to the CXCR4 receptor (Figure 1 A). This was accomplished, as previously described,[10] by using the solution structure of cyclotide MCoTI-II (PDB ID: 1IB9)[42] and the crystal structure of CVX15 bound to the CXCR4 receptor (PDB ID: 3OE0)[43] as molecular templates. According to this model, residue 12 in loop 2 is in close proximity to the CXCR4 extracellular surface receptor. Accordingly, to explore the effect of this residue on the biological activity of MCo-CVX-5c, we made a small library of cyclotides based on MCo-CVX-5c in which the native Asp residue in this position was mutated to residues containing different chemical functionalities (Figure 1 B). The set of amino acids that were tested comprised negative (Glu) and positive (Lys, His) charged, aliphatic (Ala, Ile, Leu) and aromatic (Tyr, Trp), hydrophilic (Asn, Gln, Ser, Thr), N-alkylated (Pro) and small (Gly) residues.

Library synthesis

All 15 cyclotides (including the control, MCo-CVX-5c, called "WT" from here on; Table 1) were chemically synthesized through Fmoc-based SPPS on a sulfonamide resin[44–46] and a tea-bag approach. We used the peptide bond between residues Tyr26 and Cys27 as the cyclization site. This site has been shown to provide very good yields with different MCoTI-based cyclotide sequences,[13,14] including the WT.[10] The common C-terminal sequence (Tyr26–Ser13) for all cyclotides was synthesized on the 0.1 mmol scale on an automated peptide synthesizer. At this point, the resin was divided into 15 equimolar aliquots each containing around 4–6 μmol ($\approx$25 mg; Figure 2). Each resin was manually coupled in parallel with the corresponding Fmoc-activated amino acid derivative. Once the couplings were complete, each resin was placed in an individual sealed polypropylene tea-bag of around 30×20 mm, properly tagged for identification, and the synthesis was continued on the synthesizer in an ABI433A 0.25 mmol scale reaction vessel, which has a capacity of 42 mL (Figure 2). This reaction vessel was able to accommodate up to nine such tea-bags. The number of tea-bags, however, could easily be increased by using smaller ones. Accordingly, the common N-terminal segment for the 15 different cyclotides was synthesized in two steps, the first with nine and the second with six tea-bags.

Table 1. Nomenclature used for all the MCo-CVX-based cyclotide used in this work. All the cyclotides sequence are based on cyclotide MCo-CVX-5c.

Aaa12[a]	Cyclotide name	Molecular weight [Da] Found	Expected average isotopic
Asp	WT	5056.7 ± 0.8	5056.9
Ala	D12A	5012.8 ± 0.3	5012.9
Glu	D12E	5070.8 ± 0.8	5070.9
Gly	D12G	4998.6 ± 0.6	4998.9
His	D12H	5078.9 ± 0.8	5078.9
Lys	D12K	5069.8 ± 0.6	5070.0
Ile	D12I	5054.8 ± 0.6	5054.9
Leu	D12L	5054.7 ± 0.5	5054.9
Asn	D12N	5055.9 ± 0.5	5055.9
Pro	D12P	5039.5 ± 0.6	5038.9
Gln	D12Q	5069.9 ± 0.1	5069.9
Ser	D12S	5028.7 ± 0.4	5028.9
Thr	D12T	5043.1 ± 1.0	5042.9
Trp	D12W	5127.9 ± 1.0	5128.0
Tyr	D12Y	5104.5 ± 1.0	5105.0

[a] Amino acid at position 12 in loop 2 of cyclotide MCo-CVX-5c (Figure 1).

Once the synthesis of all the analogues was complete, the corresponding peptide sulfonamide-resins were activated in parallel with iodoacetonitrile; this was followed by cleavage with ethyl mercaptoacetate and acidolytic deprotection to give the fully deprotected linear peptide α-thioesters precursors (Figure 2). The crude peptide mixtures were characterized by HPLC and ES-MS, and in all the cases the major product was the corresponding linear precursor thioester (Figures S1 and S2).

Library cyclization/folding and purification

The peptide thioester precursors were directly cyclized and folded in parallel in a one-pot reaction in sodium phosphate buffer (pH 7.2) with 1 mM GSH. Under these conditions WT cyclotide has been shown by NMR spectroscopy to cyclize and adopt a native conformation.[10] The cyclization/folding reactions were followed by HPLC and shown to be complete in 24–96 h. Most of the cyclotides were able to cyclize and fold with yields similar to that of WT (≈80%), only cyclotides D12I, D12L, D12Y, and D12W showed lower yields, with several late-

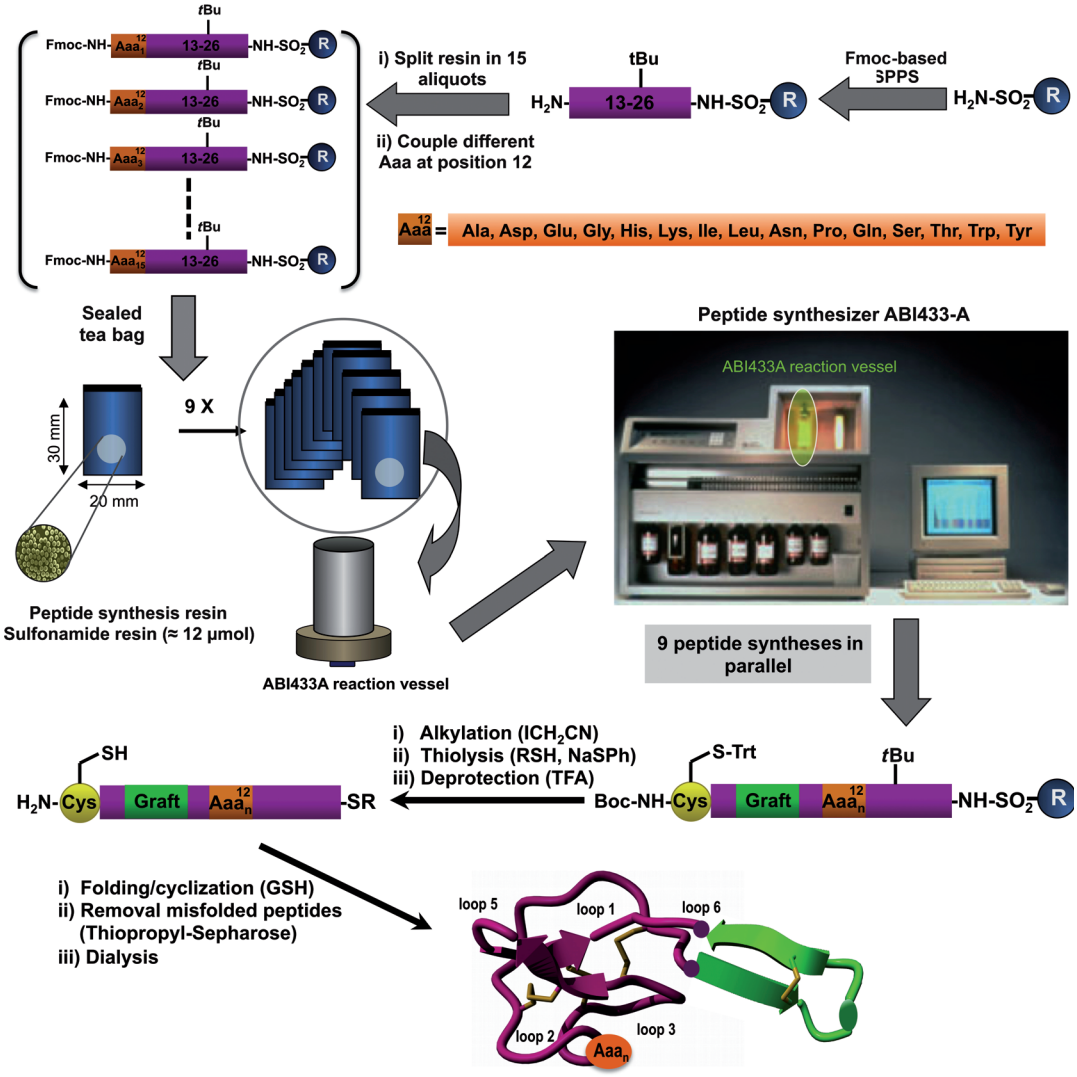

Figure 2. Synthetic scheme for the parallel production of the amino-acid-scanning library at position 12 of MCo-CVX-5c by using a tea-bag approach.

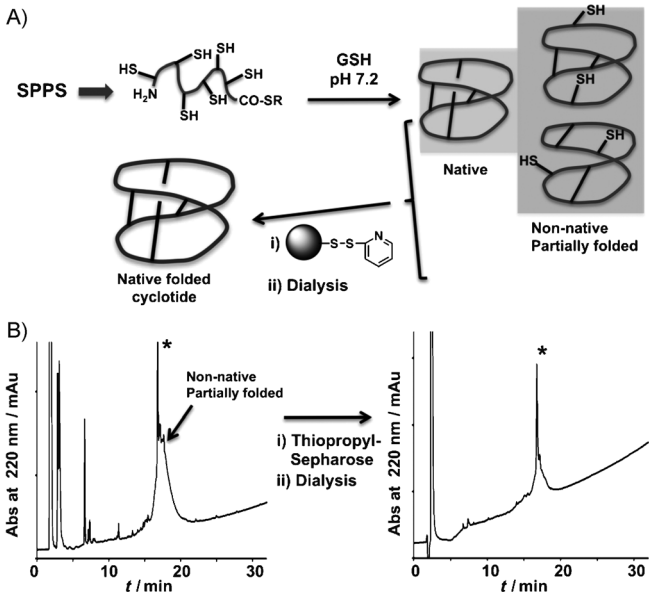

Figure 3. Purification of folded cyclotides from the one-pot cyclization–folding reaction by using activated thiopropyl–Sepharose beads. A) Principle for the removal of unfolded and/or partially folded cyclotides from the cyclization–folding reaction by using activated thiopropyl–Sepharose beads. Unfolded and/or partially folded peptides have free thiol groups that are captured by and immobilized onto the Sepharose beads. Fully folded cyclotides do not contain any free thiols and therefore are not captured. The capture reaction also produces 2-mercaptopyridone, which can easily be removed in a separate dialysis step. B) Analytical HPLC analysis of the purification of cyclotide D12L by using activated thiopropyl–Sepharose beads followed by dialysis. The cyclization–folding reaction in this cyclotide analogue produced some late-eluting by-products that were attributed to partially folded cyclotides and GSH adducts. All of these by-products contain free-thiol groups and were efficiently captured by the activated Sepharose beads to yield folded cyclotide with a high level of purity, as determined by HPLC and ES-MS (Figures S3 and S4). HPLC analysis was performed with a linear gradient of 0–70% solvent B over 30 min. An asterisk marks the product corresponding to the folded cyclotide as determined by ES-MS analysis.

eluting by-products that were attributed to partially folded and/or GSH adducts, as determined by ES-MS analysis (Figures 3 and S4). The formation of alternative structures with shuffled S–S bonds was not observed for any of the cyclotides studied in this work. The conditions used in this work for the oxidative folding, which involve thermodynamic control under slightly reductive conditions, favor the accumulation of products containing the more stable cyclotide scaffold.[10] Removal of partially folded and/or GSH–peptide adducts was readily accomplished by using activated thiopropyl–Sepharose chromatography. Activated thiopropyl–Sepharose beads contain a reactive disulfide that reacts covalently with molecules containing free thiol groups, thereby removing any partially folded product from the crude reaction (Figure 3A). Accordingly, fully folded cyclotides are eluted from the column along with 2-mercaptopyridone, which can be easily removed by dialysis. As shown in Figure 4, this approach was successfully used to produce 14 of the 15 cyclotides in high purity (≈80–90%, as determined by HPLC and ES-MS). For most cell-based assays this level of purity is usually satisfactory.[10] In addition, we tested the activity of some of the cyclotides purified by HPLC and compared them with the activities obtained with the same cyclotides purified by just using the activated thiopropyl–Sepharose, and the values were similar.[10]

Only cyclotide, D12W, was obtained with a lower degree of purity (≥70%, as determined by HPLC and ES-MS). This was attributed to the higher reactivity of Trp-containing peptides of the linear peptide precursor. It is also important to note that this purification approach can be easily used in parallel to purify a single cyclotide compound, but also, and more importantly, to purify mixtures of cyclotides, as it relies only on the ability of the polypeptide/s to adopt a cyclotide fold with no free thiol groups. Consequently, this approach to remove unfolded and partially folded compounds is potentially compatible with the production of positional scanning libraries from the cyclotide scaffold.

Biological activity against the CXCR4 receptor

To evaluate the effect of the different mutations on the biological activity of the corresponding cyclotide analogues, we tested the ability of the CVX15-grafted cyclotides to inhibit SDF1α-mediated CXCR4 activation by using a CXCR4–β-lactamase U2OS cell-based fluorescence assay (Figure 5). All the cyclotides tested were able to inhibit SDF1α-mediated CXCR4 activation in a dose-dependent manner, with IC_{50} values ranging from 3.5±0.1 (D12H) to 45±8 nM (D12Y). The empty scaffold cyclotide MCoTI-I has already been shown to have no inhibitory activity in this assay,[10] and so was not included in this study. The small molecule AMD3100[47] was also included as positive control. As previously described, the activity of the WT cyclotide was similar to that of AMD3100 in this type of assay, thus indicating the relatively high potency of this cyclotide. As expected, the D12E mutation did not lead to a change in activity. Most of the other mutations tested in this work did not significantly change the activity of the resulting cyclotides. Intriguingly, replacing the original Asp amino acid by a positively charged residue had no or very little effect on the biological activity of the resulting cyclotides. For example, cyclotide D12K (IC_{50}=9±2 nM) was only about two times less potent than the WT cyclotide, and cyclotide D12H (IC_{50}=3.5±0.5 nM) had practically the same activity as the WT cyclotide. Cyclotide D20P (IC_{50}=20±3 nM) also showed a slight decrease in activity, being about four times less active than WT); this could be attributed to the conformational restrictions imposed by the Pro residue. Among the analogues tested in this study, only cyclotides D12Y (IC_{50}=45±8 nM) and D12W (IC_{50}=30±4 nM) led to a significant decrease in biological activity, being both almost 10 times less active than the WT cyclotide. Together, these results indicate that position 12 is quite tolerant to mutations, and only the introduction of aromatic residues (Trp and Tyr) seems to have a significant detrimental effect on the biological activity of the resulting cyclotides.

Conclusions

In summary, we have shown for the first time that bioactive folded MCoTI-based cyclotides can be efficiently produced in

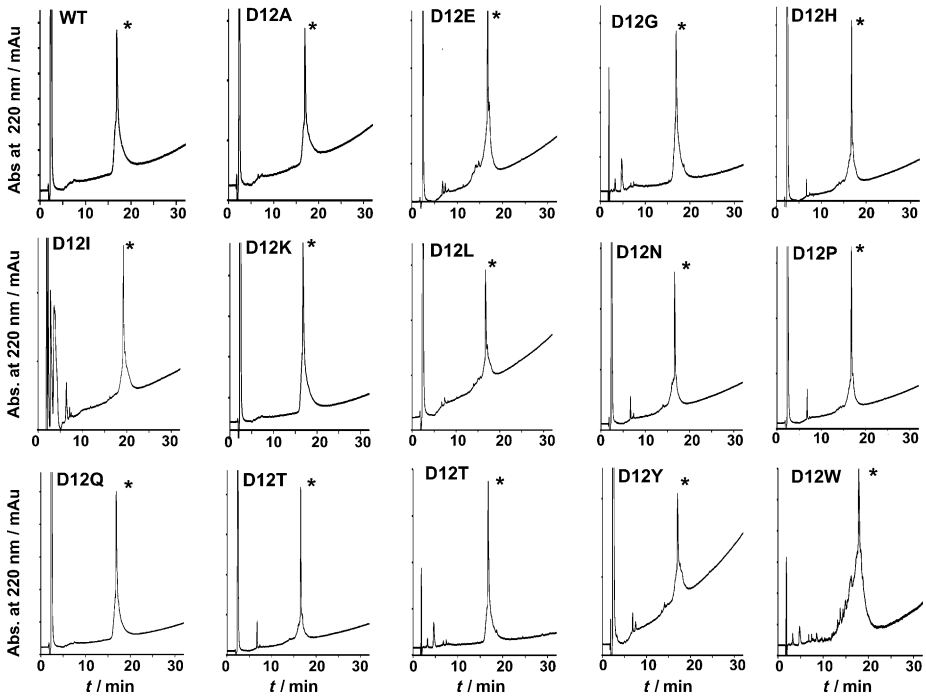

Figure 4. Analytical HPLC analysis of all the cyclotides used in this work after they had been purified by activated thiopropyl–Sepharose beads and dialysis. HPLC analysis was performed with a linear gradient of 0–70% solvent B over 30 min. An asterisk marks the product corresponding to the folded cyclotide as determined by ES-MS analysis.

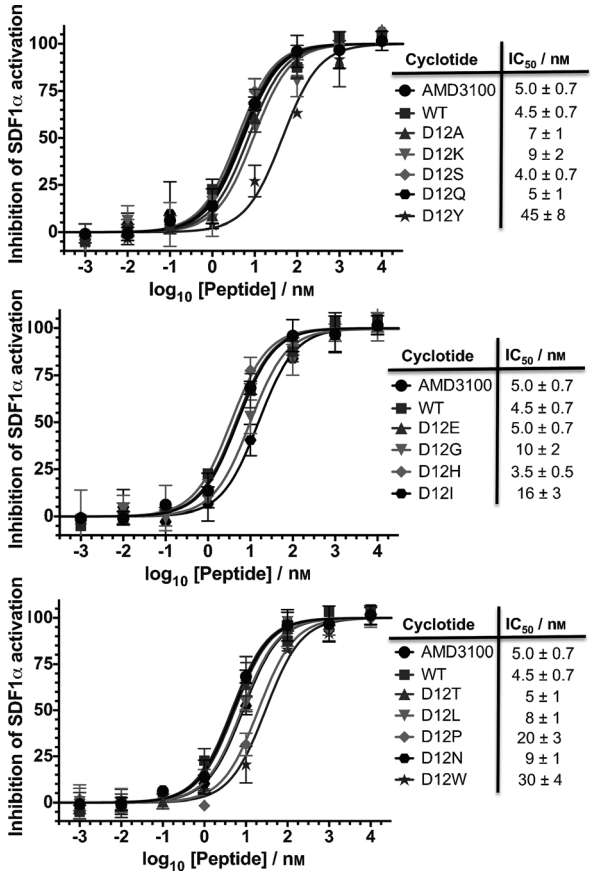

Figure 5. Biological characterization of all the MCo-CVX-based cyclotides produced in this work. Competitive inhibition of SDF1α-mediated CXCR4 activation by the different cyclotide analogues. The small-molecule CXCR4 antagonist AMD3100 was used as control. The assay was performed in CXCR4-bla U2OS cells as described in the Experimental Section.

parallel by using a tea-bag approach in combination with highly efficient cyclization-folding protocols. The approach described in this work also includes an efficient purification procedure to rapidly remove unfolded or partially folded cyclotides from the cyclization-folding crude. This procedure can be easily used in parallel to purify individual compounds, but also, and more importantly, to purify cyclotide mixtures. These qualities make it potentially compatible with the synthesis of positional scanning libraries in order to efficiently screen large libraries.

These protocols were successfully used to produce a small library of bioactive cyclotides based on the cyclotide MCo-CVX-5c (WT), which is a potent CXCR4 antagonist recently developed in our group.[10] Biological evaluation of this library allowed rapid assessment of the effects of single mutations on the activity of the corresponding cyclotide analogues. The results of the mutation of position 12 of loop 2 indicated that none of the mutations improved on the activity of the WT cyclotide, but that this position was quite tolerant of most of the mutations tested, except for those containing aromatic side chains. These results open up the possibility of studying other positions and/or loops within this bioactive cyclotide through the use of amino-acid- or positional-scanning libraries. In summary, our results demonstrate for the first time the efficient parallel synthesis of bioactive folded cyclotides thereby providing a powerful chemical tool for evaluating structure–activity relationships in large complex polypeptides in a rapid and efficient fashion.

Experimental Section

Analytical HPLC was performed on a HP1100 series instrument with 220 and 280 nm detection by using a Vydac C18 column (5 mm, 4.6×150 mm) at a flow rate of 1 mL min^{-1}. All runs used linear gradients of 0.1% aqueous trifluoroacetic acid (TFA, solvent A) versus 0.1% TFA, 90% acetonitrile in H_2O (solvent B). UV/Vis spectroscopy was carried out on an Agilent 8453 diode-array spectrophotometer, and fluorescence analysis was performed on a Jobin Yvon Flurolog-3 spectroflurometer. Electrospray mass spectrometry (ES-MS) analysis was routinely applied to all cyclized peptides. ES-MS experiments were performed on a Applied Biosystems API 3000 triple quadrupole mass spectrometer.

Preparation of Fmoc-Tyr(*t*Bu)-F: Fmoc-Tyr(*t*Bu)-F was prepared by using diethylaminosulfur trifluoride (DAST), as previously described[14] and quickly used afterwards. Briefly, DAST (160 μL, 1.2 mmol) was added dropwise at 25 °C under a nitrogen current to a stirred solution of Fmoc-Tyr(*t*Bu)-OH (459.6 mg, 1 mmol) in dry CH_2Cl_2 (10 mL) containing dry pyridine (81 μL, 1 mmol). After 20 min, the mixture was washed with ice-cold water (3×20 mL). The organic layer was separated and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure to give the corresponding Fmoc-amino acyl fluoride as white solid that was immediately used.

Loading of 4-sulfamylbutyryl AM resin with Fmoc-Tyr(*t*Bu)-F: The first residue was loaded by using Fmoc-Tyr(*t*Bu)-F as previously described.[10] Briefly, 4-sulfamylbutyryl AM resin (420 mg, 0.33 mmol; Novabiochem) was swollen for 20 min with dry CH_2Cl_2 and then drained. A solution of Fmoc-Tyr(*t*Bu)-F (≈461 mg, 1 mmol) in dry CH_2Cl_2 (2 mL) and di-isopropylethylamine (DIEA; 180 μL, 1 mmol) was added to the drained resin, and the mixture was allowed to react at 25 °C for 1 h. The resin was washed with dry CH_2Cl_2 (5×5 mL), dried and kept at −20 °C until use.

Chemical synthesis of the cyclotide-based library: All peptides were synthesized as described in Figure 2 by solid-phase synthesis on an automatic peptide synthesizer ABI433A (Applied Biosystems) by using the Fast-Fmoc chemistry with a 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/DIEA activation protocol on a 0.1 mmol scale on a Fmoc-Tyr(*t*Bu)-sulfamylbutyryl AM resin. Side-chain protection compatible with Fmoc chemistry was employed, as previously described,[10] for the synthesis of peptide α-thioesters by the Fmoc-protocol, except for the N-terminal Cys residue, which was introduced as Boc-Cys(Trt)-OH. Before the coupling of residue 12, the peptide–resin was manually resuspended in CH_2Cl_2/DMF (1:1) and split into 15 equal aliquots. Each peptide–resin aliquot was placed in a separate 1 mL polypropylene column (Qiagen). The resins were manually deprotected with 20% 4-methylpiperidine in DMF (3×5 min) and then individually coupled with each of the different 15 Fmoc-Aaa-OH (Figures 1 and 2). Couplings were performed with HBTU and DIEA for 45 min. The resins were then washed, dried, placed in individual tagged tea-bags (30×20 mm) and sealed. The synthesis was continued in the peptide synthesizer 0.25 mmol reaction vessel, which can hold up to nine different tea-bags of the dimensions reported above. This required two synthetic steps, the first with nine and the second with six tea-bags in the reaction vessel. Following chain assembly, alkylation, thiolytic cleavage and side-chain deprotection were performed for individual peptides in 1 mL polypropylene columns, as previously described.[14] Briefly, protected peptide–resin (≈10 mg) was first alkylated twice with ICH_2CN (17.4 μL, 0.24 mmol; previously filtered through basic alumina) and DIEA (8.2 μL, 0.046 mmol) in *N*-methylpyrrolidone (NMP; 0.22 mL) for 24 h. The resin was then washed with NMP (3×5 mL) and CH_2Cl_2 (3×5 mL). The alkylated peptide resin was cleaved from the resin with $HSCH_2CH_2CO_2Et$ (20 μL, 0.18 mmol) in the presence of a catalytic amount of sodium thiophenolate (NaSPh, 0.3 mg, 2.2 μmol) in DMF/CH_2Cl_2 (1:2, *v/v*, 0.12 mL) for 24 h. The resin was then dried at reduced pressure. The side-chain-protecting groups were removed by treating the dried resin with TFA/H_2O/tri-isopropylsilane (TIS; 95:3:2, *v/v/v*, 0.5 mL) for 3–4 h at room temperature. The resin was filtered, and the linear peptide thioester was precipitated in cold Et_2O. The crude material was dissolved in a minimal amount of H_2O/MeCN (4:1) containing 0.1% TFA and characterized by HPLC and ES-MS as the desired grafted MCoTI-I linear precursor α-thioester (Figure S1 and S2). Cyclization and folding were accomplished by flash dilution of the linear α-thioester TFA crude to a final concentration of ≈20–50 μM into freshly degassed reduced glutathione (GSH, 2 mM), sodium phosphate buffer (0.1 M, pH 7.2) for 96 h.

Purification of folded cyclotides by using activated thiopropyl–Sepharose beads: Following oxidative folding, the pH of the cyclization-folding crude was raised to 8.0 by slowly adding an aqueous solution of Na_3PO_4 (0.2 M). After 4 h, the resulting crudes were treated with pre-swollen activated thiopropyl–Sepharose beads (20 μL of swollen beads per mL of cyclization-folding crude, Pharmacia) for 1 h. The beads were then drained and washed with 10% HOAc in water (2×200 μL). The combined liquid fractions were pooled and dialyzed in a slide-A-lyzer mini dialysis (Thermoscientific) with a cut-off of 3.5 kDa against HOAc (0.1 M, 2×14 mL). The dialyzed samples were then analyzed by HPLC and ES-MS for purity (Figures 4 and S4), lyophilized and stored at −20 °C. Purified cyclotides were quantified by UV/Vis spectroscopy at 280 nm by using a molar absorptivity coefficient of 10470 M^{-1} cm^{-1} for all the cyclotides except for D12Y (11810 M^{-1} cm^{-1}) and D12W (16020 M^{-1} cm^{-1}).

Cell-based CXCR4 competitive-binding assays: Briefly, Tango CXCR4-bla U2OS cells (Life Technologies) were seeded at 11000 cells per well in 384-well tissue culture plate for 24 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% FBS. Cells were pre-treated with various concentrations of inhibitors for 30 min prior to the addition of SDF-1α (30 nM), then incubated for 5 h at 37 °C. β-Lactamase substrate LiveBLAzer-FRET B/G Substrate (Invitrogen) was incubated with treated cells for 2 h at room temperature, and the fluorescence signal was measured by Synergy H1 plate reader (Bio Tek) at 409/460 nm (substrate cleaved) and 409/530 nm (substrate uncleaved).

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Keywords: CXCR4 · cyclotides · protein design · protein engineering · protein–protein interactions

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