ORGANIC LETTERS

2001
Vol. 3, No. 11
1681−1684

A Thioester Ligation Approach to Amphipathic Bicyclic Peptide Library

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Received March 25, 2001

ABSTRACT

An efficient approach to synthesize an amphipathic bicyclic peptide library from unprotected peptides is demonstrated through an on-resin intramolecular thioester ligation and an off-resin DMSO-mediated disulfide formation.

Constraining peptides to a discrete set of conformers has been shown to be useful in designing peptidyl drugs with enhanced receptor-binding affinity and selectivity.[1−3] End-to-end lactamization forming cyclic peptides is a conformational constraint commonly found in both nature[4−6] and synthetic design.[7] This constraint has the additional advantages of improving bioavailability and metabolic stability.

For small cyclic peptides consisting of <10 amino acids, an end-to-end constraint alone may suffice to confer conformational rigidity.[8] However, additional constraints are often required for rigidifying larger cyclic peptides through intramolecular cross-linking elements such as non-natural amino acids, disulfide bonds, or side chain-to-side chain lactams.[9] Of these, disulfide bonds, which play a major role in peptide and protein conformational stability, have been found to be compatible with the end-to-end constraint.[10−13] Indeed, naturally occurring macrocyclic peptides consisting of 18−33 amino acids and a cystine-knot of three disulfide bonds have been found in plants and animals.[4−6] Notable examples include kalata,[14] 4 circulins,[15] and θ defensins.[16] Since these highly constrained peptides display a wide range of activity, developing efficient synthetic methods that will obtain sufficient material for structure−activity studies have attracted our attentions.

Previously, we have developed a simplified and efficient scheme for synthesizing cystine-knot macrocyclic peptides based on intramolecular ligation of unprotected peptide thioesters.[15,17,18] Here we describe an extension of this ligation approach of on-resin or off-resin cyclization for preparing a

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10.1021/ol015889i CCC: $20.00
Published on Web 05/09/2001

bicyclic macrocyclic peptide library consisting of 14 amino acids constrained by an end-to-end lactam and an intramolecular disulfide.

An advantage of our approach is its design flexibility, which permits variations of the disulfide constraint to achieve different geometric shapes. To illustrate this design principle and to demonstrate the synthetic efficiency of our approach, we prepared four bicyclic peptides **2a**−**d** based on peptide **1** (Figure 1) by an off-resin cyclization method (Scheme 1,

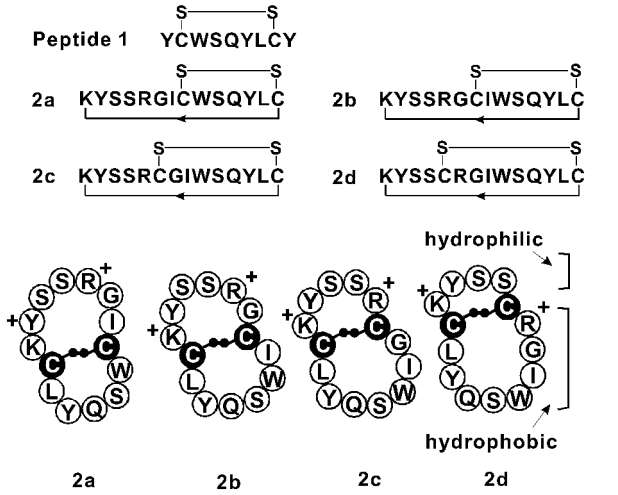
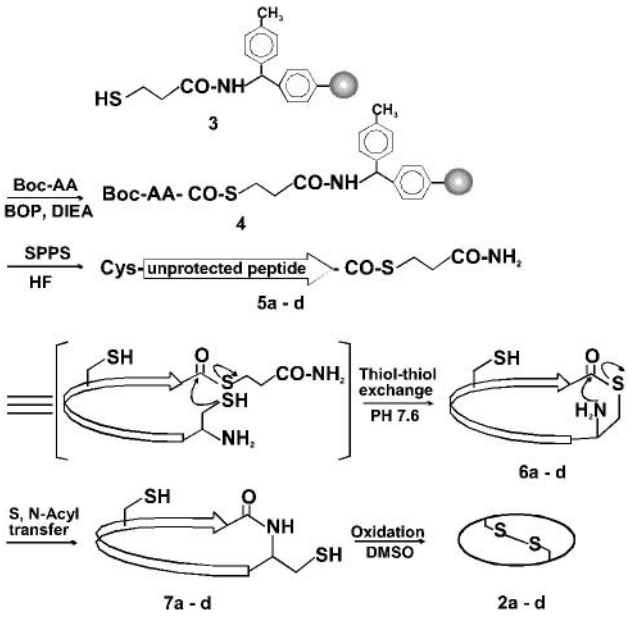


Figure 1. Design of four amphiphatic bicyclic peptide isomers by varying intramolecular disulfide positions.

Method A). We designed **2a**−**d** as isomers with similar amino acid compositions that are rigidified by an invariable end-to-end lactam. They differed structurally because of a variable intramolecular disulfide bond that progressively altered the bicyclic conformations.

Scheme 1. Synthetic Routes by Off-Resin Cyclization (Method A) To Synthesize **2a**−**d**



Peptide **1** was reported to be an antagonist of the TNFα-receptor.[19] Since TNFα (157 amino acids) functioning as a homotrimer is a major cytokine that mediates inflammatory and immune functions, the small peptidyl antagonist **1** provides a useful lead for developing analogues. In our hands, peptide **1** was found to be a poor antagonist with low aqueous solubility. Thus, our initial goal was to improve its aqueous solubility.

Structure−activity study[19] showed that the hydrophobic sequence of **1** (WSQYL) was critical for receptor-binding activity and was retained in our design of **2a**−**d**. To increase their aqueous solubility and to facilitate their interactions with the cellular membrane,[8] **2a**−**d** were designed as amphiphatic peptides consisting of a hydrophilic patch with two cationic amino acids and a hydrophobic patch based on the WSQYL sequence.

The off-resin synthetic route[17,13,20−22] to form **2a**−**d** shown in Scheme 1 employed a linear peptide thioester **5a**−**d** containing an N-terminal Cys and a C-terminal thioester. A stepwise solid-phase synthesis gave these precursors in 67−74% yields based on a thiol detachable linker attached to a *p*-methylbenzhydrylamine resin **3**.[18] The attachment of the first amino acid onto the thiol resin was achieved using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and diisopropylethylamine (DIEA). After assembling the peptide chains on resins by Boc/Bzl chemistry with *N*,*N*′-diisopropylcarbodiimide (DIC)/1-hydroxy-benzotriazole (HOBt) as coupling reagent, the unprotected peptide thioesters **5a**−**d** were obtained by cleavage from the thioester resins employing HF/*p*-thiocresol/*m*-cresol (93:2:5, v/v) at 0 °C for 1.5 h. These crude and unprotected peptides were directly used for the off-resin cyclization in the next step.

End-to-end cyclization of the linear peptide thioesters **5a**−**d** to cyclic peptides **7a**−**d** was performed in an aqueous solution buffered with 0.2 M Tris·HCl to pH 7.6, also containing 6 M guanidine-HCl as a denaturant and 3 equiv of water-soluble tris(2-carboxyethyl)phosphine (TCEP) to inhibit the intra- or intermolecular disulfide bond formation.[23] This cyclization is a two-step reaction. The first step forms thiolactone **6a**−**d** through the transthioesterification of an unprotected cysteinyl side chain with the C-terminal thioester. Since there are two cysteinyl side chains, two thiolactones are likely formed. However, the N-terminal (NT)-thiolactone undergoes a spontaneous S,N-acyl transfer to form a stable lactam, whereas the thiolactone formed by the internal Cys is reversible and eventually converts to the NT-thiolactone

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(23) **Cyclization Condition.** Peptide thioesters **5a**−**d** were dissolved in 6 M guanidine·HCl (0.2 M Tris, pH 7.6) buffer with 3 equiv of water-soluble tris(2-carboxyethyl)phosphine (TCEP); the concentration of the reaction solution was 1 mM. The cyclization reactions were complete in 2−3 h.

through a thiol-thiolactone exchange reaction.[24] This proximity-driven cyclization strategy is regiospecific, avoiding the need for a protection/deprotection scheme and enthalpy activation step required in conventional cyclic peptide synthesis.[25−28]

The cyclization yields of **5a**−**d** to **7a**−**d** were nearly quantitative (Table 1) when performed at a relatively diluted concentration of 2 mg/mL. No side products such as dimers or oligomers were observed on the basis of the HPLC profile and MS analysis of other minor peaks (Figure 2). Furthermore, the Cys placements in the sequences of **5a**−**d** had no significant influence on the cyclization yield.

Table 1. Yields and MS Data of Bicyclic Peptide Isomers and Their Disulfide Bond Products

	cyclization (**5a**−**d** to **7a**−**d**)		oxidation (**7a**−**d** to **2a**−**d**)		
entry	yield (%)[a]	MS[b]	yield (%)[c]	MS[d]	reaction time (h)
a	96	1677.25	56	1675.24	48
b	96	1676.16	61	1675.00	28
c	95	1676.33	53	1674.80	32
d	94	1677.06	58	1675.85	30

[a] Crude yields from peptide thioester to cyclic peptide derived from HPLC. [b] Calculated MS: 1676.2. [c] Yields from desired cyclic peptide to disulfide bond products after purification from RP-HPLC. [d] Calculated MS: 1674.2.

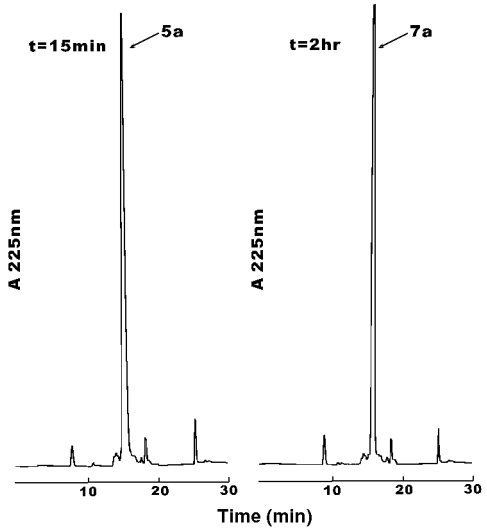

Figure 2. RP-HPLC profiles of the cyclization reaction of **7a** (right panel) from the linear precursor **5a** (the major peak at left panel). The HPLC profiles of **7b**−**d** from **5b**−**d** are similar to those of **5a** and **7a**.

more, the Cys placements in the sequences of **5a**−**d** had no significant influence on the cyclization yield.

Disulfide (SS) oxidation of **7a**−**d** to **2a**−**d** was mediated by dimethyl sulfoxide (DMSO, 10−20 vol %) in an aqueous solution buffered at pH 5−6. This acidic oxidation condition minimizes polymerization. In addition, under our experimental conditions, oxidation-sensitive amino acid residues such as Tyr, Met, and Trp are generally unaffected because of the mildness of this DMSO-mediated process.[29] It also has an advantage over oxidation methods using toxic metals since these are often difficult to remove from the peptide products.[30]

As monitored by analytical RP-HPLC, the controlled oxidation method under acidic conditions was quantitative with one major peak (Figure 3). However, the rates were slow and required 24−48 h to afford the purified bicyclic products **2a**−**d** in 53−61% in isolated yields. The purified bicyclic peptides were confirmed by mass spectrometry (Table 1) and Ellman test.

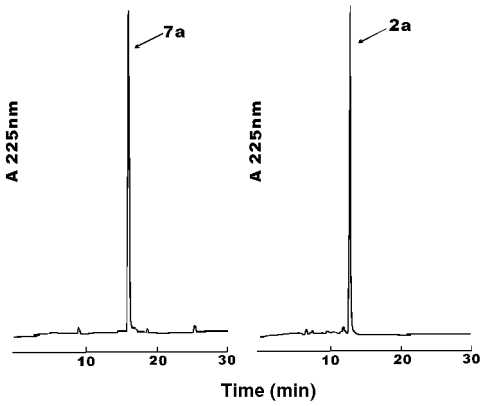

Figure 3. RP-HPLC profiles of the oxidation reaction of **2a** from the cyclic peptide **7a**. The HPLC profiles of **2b**−**d** from **7b**−**d** are similar to those of **2a**.

The oxidation reaction can be accelerated to completion within 3 h by increasing the buffered solution pH to neutral or basic and under high dilution to minimize polymerization. At concentrations >2 mg/mL, precipitation was observed at basic conditions >pH 8, probably due to the rapid and random intermolecular SS bond formation.[31,32] It is interesting to note that there is only a very small difference in the oxidation rates of **2a**−**d** even though the SS bond forms cyclic rings varying from 6 to 10 amino acids.

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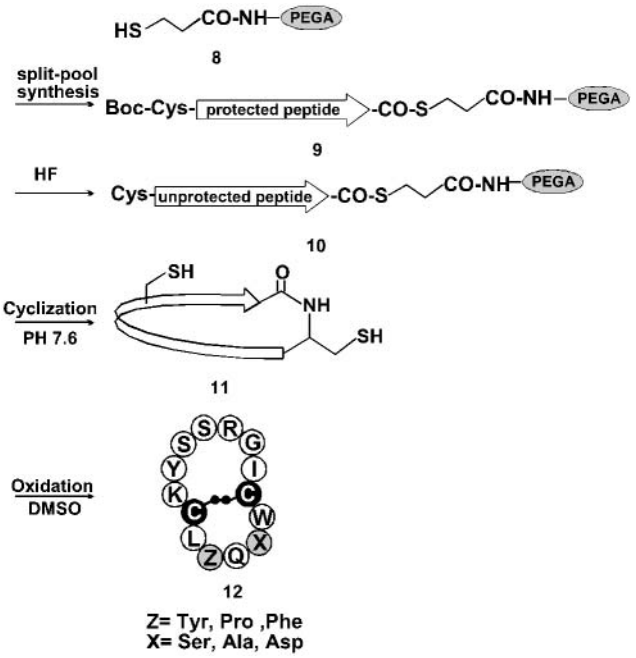
(32) **Oxidation Condition.** Cyclic peptides **7a**−**d** were dissolved in 2 M guanidine·HCl (pH 5−6) with 20% DMSO; the concentration of reaction solution was 0.3 mM. The reactions were complete in 1−2 days.

Scheme 2. An On-Resin Cyclization Scheme (Method B) to a Small Llibrary of Nine Bicyclic Peptides Based on **2a**



An on-resin cyclization method (Method B, Scheme 2) was used to simplify the synthetic process for preparing a small library of nine bicyclic peptides based on **2a.** Peptide chains were assembled on a nondetachable thiol linker attached to an aminomethyl PEGA resin **8** by Boc/Bzl chemistry using the split-pool method to prepare nine analogues differing in two positions. After deprotection by HF/*p*-thiocresol/*m*-cresol (93:2:5, v/v) at 0 °C for 1 h, the cyclic peptides **11** were released from resins in an aqueous solution buffered with 0.2 M Tris·HCl at pH 7.6 containing 6 M guanidine·HCl.[33] Disulfide bond formation was directly carried out at pH 5−6 with 20% DMSO by diluting the above buffer to 2 M guanidine. The identity of the library **12** obtained after desalting with RP-HPLC was confirmed by MALDI-MS (Table 2). Analytical HPLC revealed all the expected peptides in eight peaks (Figure 4), because under this elution condition one of the peaks contains two compounds (entries 12-3 and 12-4, Table 2). No polymers were observed on MS.

Table 2. MS Data of a Library of Nine Bicyclic Peptides Based on **2a**

entry	X	Z	MS calcd	MS obsd	HPLC t_R (min)
12-1	Ser	Tyr	1674.2	1675.98	19.307
12-2	Ser	Pro	1608.1	1609.89	23.372
12-3	Ser	Phe	1658.2	1659.97	13.769
12-4	Ala	Tyr	1658.2	1659.97	14.712
12-5	Ala	Pro	1592.1	1594.13	15.291
12-6	Ala	Phe	1642.2	1643.93	9.047
12-7	Asp	Tyr	1702.2	1704.00	15.291
12-8	Asp	Pro	1636.1	1637.61	19.912
12-9	Asp	Phe	1686.2	1687.38	10.657

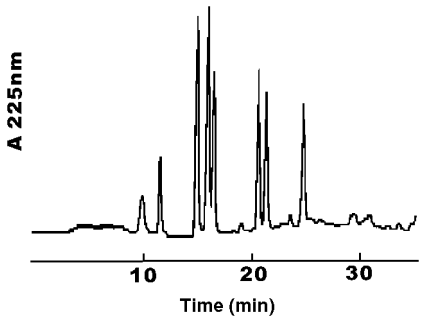


Figure 4. RP-HPLC profile of the library **12** of nine bicyclic peptides

The bicyclic peptides **2a**−**d** as well as the bicyclic library (12-1 to 12-9**)** exhibited properties expected in our design for amphipathic peptides. For example, they showed excellent aqueous solubility. In addition, they displayed the membranolytic affinity of an amphipathic peptide. In our design, **2a** possesses a net of two cationic amino acids. Since cationic amphipathic peptides often display membranolytic activity against microbes, an antimicrobial assay would validate our amphipathic design. Indeed, **2a** displayed moderate microbicidal activity with minimal inhibition concentrations (MICs) of 50.5, 23.4, and 50.5 μM against three test organisms, *Escherichia coli* (a Gram-negative bacterium), *Staphylococucus aureus* (a Gram-positive bacterium), and *Candida albicans* (a yeast), respectively. In contrast, peptide **1**, the lead compound, was inactive (MICs > 500 μM). When assayed as a mixture, the bicyclic peptide library derived from **2a** showed the expected diminished activity because of the loss of a cationic charge (Asp was used in the library). It displayed MICs of 39.2 and 111.2 μM against *S. aureus* and *C. albicans*, respectively, but was inactive against *E. coli*. Interestingly, the reduced monocyclic forms of both **2a** and its library were substantially more active as antimicrobials with MICs ranging from 9 to 18 μM against the three test organisms. It should be pointed out that this observation is consistent with the character of the nonspecific membranolytic activity and specific receptor-binding requirements of bioactive peptides. In sum, we have demonstrated the efficiency of an off-resin synthesis of bicyclic peptide isomers, as well as an on-resin synthesis of a small library by intramolecular ligation. Such an approach holds promise for generating large bicyclic peptide libraries applicable for drug discovery.

Acknowledgment. We thank for Dr. Jinglong Yang for the antimicrobial assays. This work was supported in part by U.S. Public Health Service Grants CA36544 and AI46164.

OL015889I

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