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Solid-phase synthesis of backbone-cyclized β-helical peptides

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Abstract—This paper describes the synthesis and purification of two 22-residue cyclic peptides, *cyclo*{[(L-Val-D-Val)$_4$-(L-Val-D-Pro-Gly)]$_2$-} **3** and *cyclo*{[(D-Leu-L-Leu)$_4$-(D-Leu-L-Pro-Gly)]$_2$-} **4**, that were designed to fold into double-stranded antiparallel β-helical structures. Due to intramolecular hydrogen bonding and the conformational constraints imposed by the two reverse-turn segments (D-Pro-Gly and L-Pro-Gly, respectively), the linear precursors to **3** and **4** (lin-**3** and lin-**4**) were expected to adopt preorganized conformations that would bring the N and C termini close together and thereby favor ring closure. Precursors lin-**3** and lin-**4** were constructed by stepwise Boc solid-phase peptide synthesis using the commercially available alkanesulfonamide 'safety-catch' linker and cyclized head-to-tail via the method of cleavage-by-cyclization. The crude cyclic peptides were highly hydrophobic and contained minor impurities that could not be removed solely by reversed-phase HPLC (RP-HPLC); however, two-step purification—first by RP-HPLC with *i*-PrOH/water gradients, followed by gel-permeation chromatography (GPC) on Sephadex LH-20 with $CHCl_3$/MeOH—afforded both peptides in pure form (≥95% by ^{1}H NMR) and in acceptable yield (23%). Subsequent ^{1}H NMR experiments supported the expected structures of **3** and **4**. The successful formation of the 66-membered rings of **3** and **4** is consistent with the notion of conformational preorganization in the linear precursors; furthermore, the protocols for synthesis and purification described should prove useful for preparing additional cyclic β-helical peptides, including longer peptides and peptides having polar residues.

1. Introduction

In this article, we present the solid-phase synthesis and backbone-to-backbone cyclization of peptides that are designed to fold into β-helices—i.e., helices formed by peptides composed of alternating D- and L-amino acids (D,L-peptides) and stabilized by β-sheet hydrogen bonding.[1] β-Helical peptides are of interest not only for their ability to form transmembrane ion channels, the most notable example of which is the naturally occurring peptide antibiotic gramicidin A,[2] but also as prospective structural components for new biomolecular architectures. The conformational promiscuity of linear D,L-peptides, however, has limited the usefulness of β-helices; for example, a given D,L-peptide often folds in solution to give a mixture of single-stranded (ss), double-stranded (ds) parallel (↑↑), and ds antiparallel (↑↓) forms.[1–4] In order to overcome these limitations, we sought a means of constraining a D,L-peptide into a single β-helical species. Cyclization is known to provide an important conformational constraint in many natural and designed peptides;[5] here, we use cyclization to prevent interconversion between ss and ds β-helices and to generate a well-defined ds antiparallel species having ca. 5.6 residues per turn (a ↑↓ $\beta^{5.6}$-helix).

2. Experimental design

Conceptually, we designed *cyclo*{[(L-Val-D-Val)$_4$-(L-Val-D-Pro-Gly)]$_2$-} **3** and *cyclo*{[(D-Leu-L-Leu)$_4$-(D-Leu-L-Pro-Gly)]$_2$-} **4** (Fig. 1) by joining two copies of the corresponding linear D,L-peptide with two copies of the reverse-turn[6] sequences D-Pro-Gly and L-Pro-Gly, respectively. In practice, we chose to synthesize the linear precursors to **3** and **4** (lin-**3** and lin-**4**) via stepwise solid-phase peptide synthesis (SPPS) using an alkanesulfonamide safety-catch linker (AS-SCL),[7] originally developed by Kenner[8] and subsequently modified by Ellman,[9,10] and then cyclize the linear peptides with concomitant cleavage

Keywords: Alkanesulfonamide; Safety-catch linker; Cyclic peptide; Solid-phase peptide synthesis; β-Hairpin; β-Turn; β-Helix; HPLC; Gel-permeation chromatography.
Abbreviations: AS-SLC, alkanesulfonamide safety-catch linker; CBC, cleavage-by-cyclization; Boc, *t*-butoxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; PyBop, (benzotriazol-1-yloxy)tris-pyrroli-dinophosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide; HBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide; DMF, *N*,*N*-dimethylformamide; NMP, *N*-methylpyrrolidinone; FSW, flow/shake wash; TFA, trifluoroacetic acid; DIEA, *N*,*N*-diisoprolylethylamine; RP-HPLC, reversed-phase high-performance liquid chromatography; HFIP, hexafluoroisopropanol; HFA·3H_2O, hexafluoroacetone trihydrate; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; IR, infrared spectroscopy; GPC, gel-permeation chromatography.
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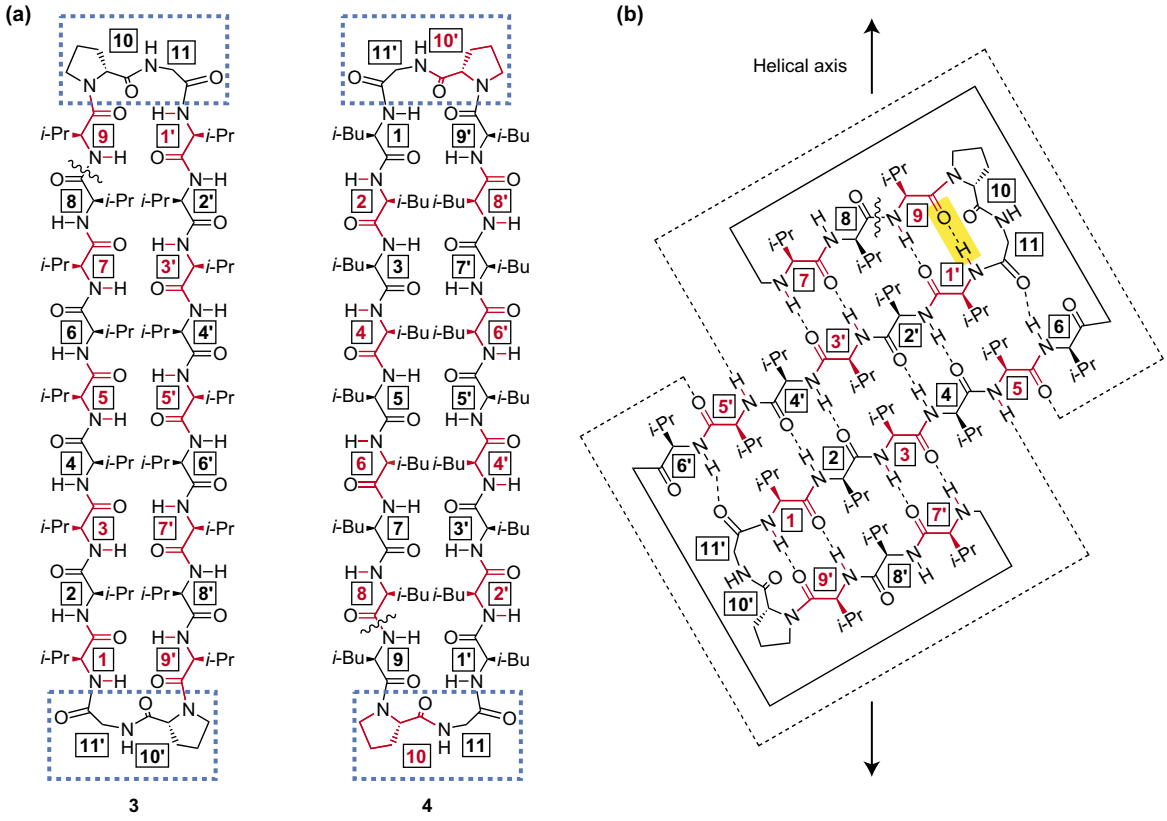

Figure 1. (a) Chemical structures of the peptides prepared in this study: *cyclo*{[(L-Val-D-Val)$_4$-(L-Val-D-Pro-Gly)]$_2$-} **3** and *cyclo*{[(D-Leu-L-Leu)$_4$-(D-Leu-L-Pro-Gly)]$_2$-} **4**. D Residues and Gly are shown in black, while L residues are shown in red and black; residues that comprise the two symmetry-related halves of the molecules are labeled 1 through 11 and 1′ through 11′. The reverse-turn sequences of **3** and **4** (D-Pro-Gly and L-Pro-Gly, respectively) are boxed in blue. In both **3** and **4**, the amide bond that is formed during CBC (between the carboxyl of residue 8 and the amine of residue 9) is marked with a wavy line. (b) Schematic side view of the anticipated $\uparrow\downarrow\beta^{5,6}$-helical structure of **3**, showing hydrogen-bonding interactions; **4** was expected to have an analogous $\uparrow\downarrow\beta^{5,6}$-helical structure (not shown). We anticipated that, in the linear precursor to **3**, a hydrogen bond between the carboxyl of residue 9 and the NH of residue 1′ (highlighted in yellow) would position the amino group of residue 9 favorably for amide bond formation with the carboxyl of residue 8.

from the resin—a process known as cleavage-by-cyclization (CBC). The AS-SCL has been used previously for the synthesis of cyclic peptides via CBC,[11–17] and resins functionalized with this linker are commercially available, making it a convenient choice for our purposes. We decided to pursue the CBC route rather than the alternative—cleavage of the linear precursor followed by cyclization in solution—in order to save one synthetic step and to avoid the strong acid cleavage that is typically required in Boc SPPS.[18]

At the outset of this work, we noted that the target cyclic peptides **3** and **4** differ in two important ways from those prepared previously via CBC using the AS-SCL. First, prior reports by others had demonstrated CBC of peptides up to 10 residues long,[11–17] while **3** and **4** are both 22 residues long. The ease of macrocyclization reactions tends to correlate inversely with the number of atoms in the ring, and thus the prospect of forming the 66-membered rings of **3** and **4** at first appeared daunting. We reasoned, however, that the two reverse-turn regions of each peptide, together with alternating chirality of the residues and intramolecular hydrogen bonding,[1] would cause the lin-**3** and lin-**4** to fold into preorganized $\uparrow\downarrow\beta^{5,6}$-helical structures that would place the N and C termini close in space and thus encourage ring closure. In a retrosynthetic sense, we made a disconnection between the carboxyl function of residue 8 and the amino group of residue 9 (Fig. 1), so that the forward reaction (ring closure) would take place between residues at the ends of the putative helices and avoid any steric congestion in the middle. Furthermore, we envisaged that hydrogen bonding between the carbonyl of residue 9 and the NH of residue 1′ (Fig. 1b), together with the presence of the nearby reverse-turn residues, would place the amino group of residue 9 in a favorable position for ring closure.

Peptides **3** and **4** also differ in polarity from those prepared earlier via CBC using AS-SCL. The previously reported cyclic peptides all contained at least one polar residue,[11–17] while **3** and **4** are comprised of only nonpolar residues. We, therefore, expected **3** and **4** to be highly hydrophobic. Although the purification of highly hydrophobic peptides is known to be difficult,[19–22] we hoped that, by using high-resolution techniques of separation such as RP-HPLC,[23] we would obtain products pure enough for characterization by high-field NMR.

3. Results

3.1. Solid-phase synthesis of 3 and 4

Here, we describe the synthesis and purification of **3**, but the synthesis and purification of **4** were carried out in an analogous manner. Beginning with 4-sulfamylbutyryl AM resin