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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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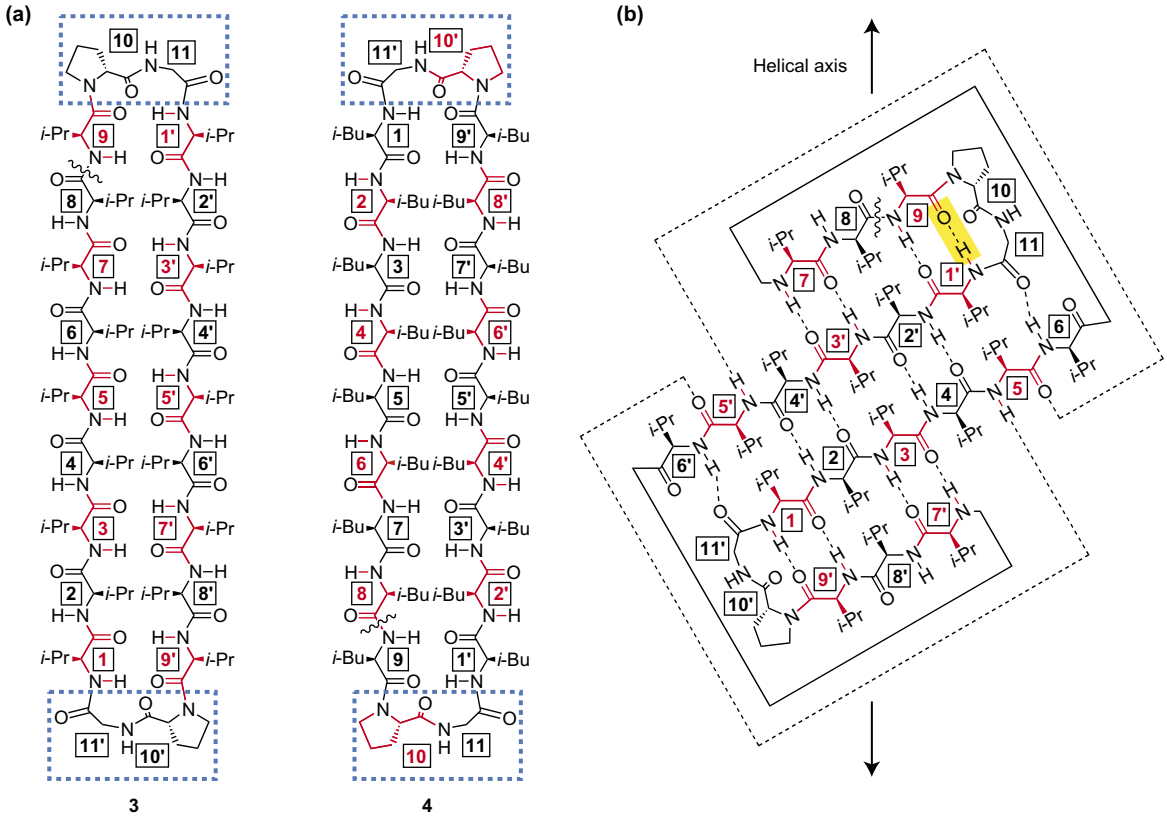


Figure 1. (a) Chemical structures of the peptides prepared in this study: *cyclo*{[(L-Val-D-Val)$_4$-(L-Val-D-Pro-Gly)]$_2$-} **3** and *cyclo*{[(D-Leu-L-Leu)$_4$-(D-Leu-L-Pro-Gly)]$_2$-} **4**. D Residues and Gly are shown in black, while L residues are shown in red and black; residues that comprise 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.

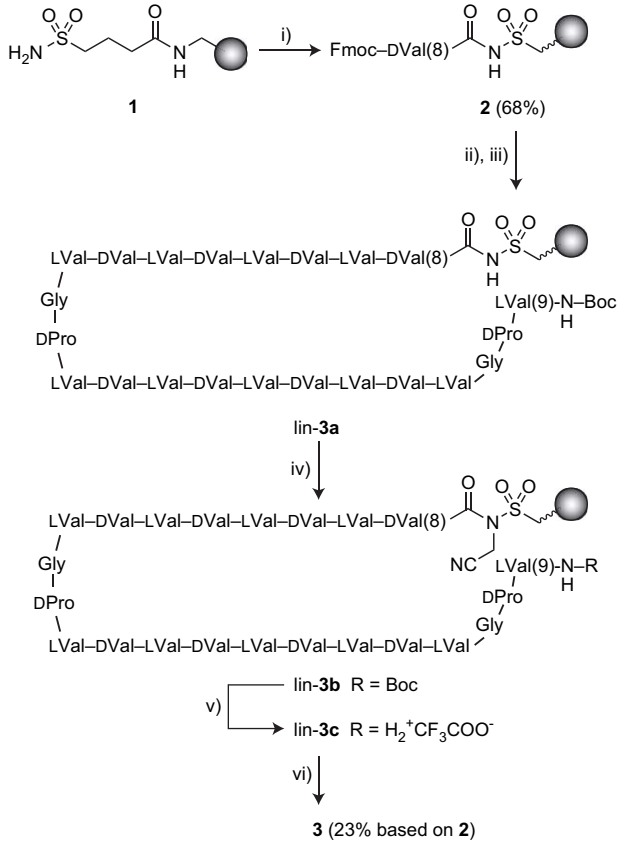
Peptides **3** and **4** also differ in polarity from those prepared earlier via CBC using AS-SCL. The previously reported cyclic peptides all contained at least one polar residue,[11–17] while **3** and **4** are comprised of only nonpolar residues. We, therefore, expected **3** and **4** to be highly 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

1,[9,10] we anchored the C-terminal residue, D-Val(8), as the preformed Fmoc-amino acid fluoride (Scheme 1). We chose to use the acid fluoride rather than in situ activation with PyBop for the sake of convenience: the former allowed us to carry out the coupling at room temperature in a SPPS vessel,[24] while the latter requires the coupling to be carried out at −20 °C in a round-bottom flask.[9,10] Furthermore, the presence of the Fmoc protecting group let us determine the yield of the anchoring reaction using the quantitative variant of the Fmoc UV absorbance assay.[25] In our hands, double coupling with Fmoc-D-Val-F[26] furnished the Fmoc-amino-acylated resin **2** in 68% yield; we attribute this modest yield to the steric hindrance posed by the β-branched side chain of D-Val. Consistent with this interpretation, de Visser et al. reported a yield of 64% for the anchoring of β-branched Fmoc-L-Thr(^{t}Bu)-F to **1**,[12] while during the synthesis of peptide **4**, we found that double coupling of the non-β-branched Fmoc-L-Leu-F proceeded in 91% overall yield.



Scheme 1. (i) 2×Fmoc-D-Val-F, DIEA, CH_2Cl_2, room temperature; (ii) 4×20% piperidine in DMF; (iii) Boc SPPS; (iv) ICH_2CN, DIEA, NMP; (v) 2×TFA (neat); (vi) DIEA, THF. (In **2** and lin-**3a–c**, the residues that react during ring closure, D-Val(8) and L-Val(9), are numbered as in Figure 1, while, for clarity, the numbering of all other residues is omitted.)

Following the removal of the Fmoc group with 20% piperidine in DMF,[27] we continued the synthesis at the appropriate scale using manual Boc SPPS and following the in situ neutralization protocol of Kent,[28] with the exception that the more efficient coupling reagent HATU was used in place of HBTU,[29] NMP was used in place of DMF, and a combination of flow and shake washes (FSW) was used in place of a single flow wash (see Section 6 for details).

After we had coupled the final residue, L-Val(9), we activated the C terminus of the resin-bound linear peptide lin-**3a** via cyanomethylation with ICH_2CN and DIEA in NMP[9,10] to give activated species lin-**3b**. We then removed the *N*-terminal Boc group with neat trifluoroacetic acid (TFA)[28] and effected CBC by suspending the TFA-peptidyl resin lin-**3c** in THF and adding 3 equiv DIEA.[11] The CBC reaction was accompanied by the formation of a white precipitate, which we collected by filtering the resin and washing copiously with $CHCl_3$. Evaporation of the $CHCl_3$ yielded crude **3**.

3.2. Purification and characterization of 3 and 4

Crude peptide **3** proved insoluble in solvents such as MeOH, EtOH, *i*-PrOH, and CH_3CN that are typically used for RP-HPLC; we attribute this insolubility to the highly hydrophobic nature of the peptide. However, dissolution of the crude peptide in HFIP at a concentration of ca. 80 mg mL^{-1}, followed by twofold dilution with HFA·3H_2O, furnished a solution that was suitable for use in RP-HPLC. Initial attempts at RP-HPLC using a C4 column with CH_3CN/water failed to elute any peaks, but switching to *i*-PrOH/water and also using a C4 column gave predominantly a single, broad peak (Fig. 2), which we collected as two fractions (fractions *a* and *b*) and analyzed using MS and ^{1}H NMR spectroscopy.

Both RP-HPLC fractions showed the anticipated molecular ions by MALDI-MS. 1D ^{1}H NMR spectroscopy revealed that fraction *a* consisted of a ca. 10:1 ratio of major and minor species (Fig. 3a), which we tentatively assigned as the desired peptide **3** and an epimerized product, respectively; this assignment is supported by the absence of additional peaks in the mass spectrum, which suggests that the minor species has the same molecular weight as the desired peptide **3**. In addition, NMR spectroscopy indicated that fraction *b* contained the desired peptide **3**, the putative epimerized material, and at least one additional contaminant (Fig. 3b). We note that the chemical shifts of the corresponding amide protons in Figure 3a and b differ somewhat due to differing amounts of water and impurities in the two samples. When

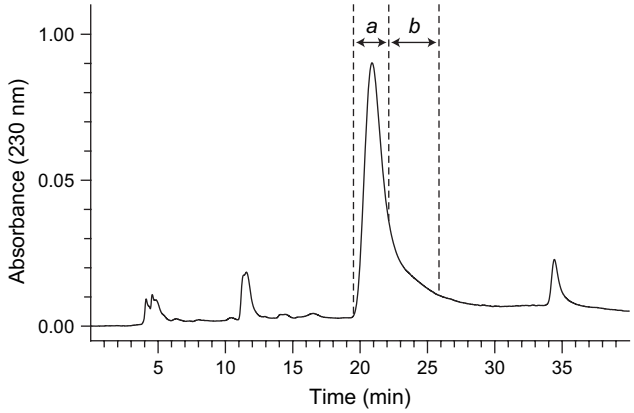


Figure 2. RP-HPLC profile of as-synthesized crude peptide **3**. The major peak was collected as two fractions, *a* and *b*. Conditions: 22×250 mm C4 column; 20 mL min^{-1}; gradient=50% B→50% B (1 min), 50→100% B (30 min), 100→100% B (5 min); solvent A=0.1% TFA and 1% *i*-PrOH in H_2O, solvent B=0.07% TFA and 90% *i*-PrOH in H_2O. The data were baseline-corrected by subtracting a blank chromatogram run with the same gradient.

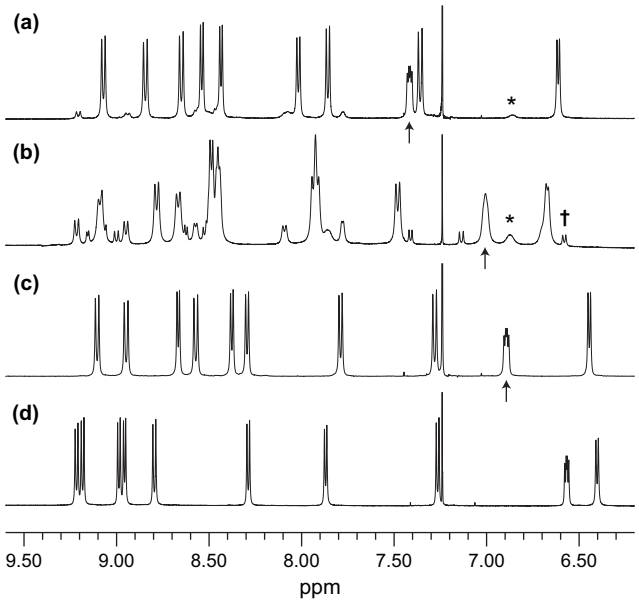


Figure 3. NH region of the 1D ^{1}H NMR spectra of peptides **3** and **4** (500 or 600 MHz, ca. 10 mM, 295 K, $CDCl_3$; residual $CHCl_3$ appears as a singlet at 7.24 ppm). (a) RP-HPLC fraction *a* (Fig. 2a), prior to repurification by GPC, which contains signals corresponding to a ca. 10:1 ratio of desired peptide **3** and putative epimerized material (one peak due to this material is marked with an asterisk). (b) RP-HPLC fraction *b* (Fig. 2b), prior to repurification by GPC, which shows signals from peptide **3**, the putative epimerized material (one peak due to this material is marked with an asterisk), and at least one additional contaminant (one peak due to this contaminant is marked with a dagger sign). (c) RP-HPLC fraction *a* (Fig. 2a) after repurification by GPC, which contains only signals arising from the desired peptide **3** (the spectrum of RP-HPLC/GPC-purified fraction *b* was identical). (d) RP-HPLC/GPC-purified peptide **4**, which contains only signals arising from the desired peptide. In (a–c), the chemical shifts of the amide protons of **3** vary due to different amounts of water and impurities in each sample. For example, the NH peak of Gly (easily identified by its multiplicity and marked in each spectrum with an arrow) appears at 7.42, 7.01, and 6.89 ppm in (a), (b), and (c), respectively.

we sought to purify both fractions further using RP-HPLC, we found that repeated chromatography on a C4, C8, or C18 column failed to completely remove the putative epimerized material.

We next attempted to repurify fraction *a* of the RP-HPLC-purified material using gravity-driven GPC on Sephadex LH-20.[30] These efforts were complicated by the lack of a suitable pure solvent that would satisfy the following criteria: (1) the solvent must be able to dissolve the partially purified peptide, and (2) the solvent must be sufficiently polar to prevent irreversible adsorption of the peptide to the LH-20 stationary phase.[30] One obvious choice was $CHCl_3$, in which the peptide is soluble to ca. 20 mM; $CHCl_3$, however, is a nonpolar solvent. Moreover, $CHCl_3$ is denser than LH-20 and therefore inconvenient for GPC using gravity-driven flow. After some experimentation, we determined that the solvent mixture $CHCl_3$/MeOH (65:35) satisfies both of the criteria stated above; in addition, this mixture is less dense than LH-20. We found that a single pass of fraction *a* through a 2.5×55 cm column of LH-20 in $CHCl_3$/MeOH gave material that was ≥95% pure as estimated by 1D ^{1}H NMR (Fig. 3c). Furthermore, even fraction *b* of the RP-HPLC-purified material, which contained ca. 30% contaminant (Fig. 3b), could be purified in this manner. Using this two-step method of purification, we obtained both **3** and **4** in 23% yield based on Fmoc-aminoacyl resin (e.g., **2**).

Figure 3c and d, respectively, shows the 1D ^{1}H NMR spectra of peptides **3** and **4** after RP-HPLC/GPC purification and thorough drying. Both spectra consist of a single set of sharp, well-dispersed resonances, with no minor peaks that would indicate multiple conformers or oligomers interconverting on the NMR time scale. Three additional observations concerning the NMR spectra support the expected ↑↓ $\beta^{5.6}$-helical structures of **3** and **4**. First, both the ^{1}H and proton-decoupled ^{13}C spectra are consistent with the anticipated twofold symmetrical structures of the 22-mers: the NH region of each ^{1}H spectrum (Fig. 3c and d, δ 6.44–9.23) contains the expected 10 NH peaks, while the ^{13}C spectra (see Section 6) each contain 11 peaks in the carbonyl region (C′, δ 169–175) and 11 peaks in the C$^\alpha$ region (δ 47–62). Second, both ^{1}H spectra (Fig. 3c and d) show 8 NH resonances downfield from 7.2 ppm, chemical shifts consistent with the 16 hydrogen bonds[3,31,32] anticipated for the ↑↓ $\beta^{5.6}$-helical structures of **3** and **4**. Finally, the majority of the $^3J_{NH-H\alpha}$ values in the ^{1}H spectra are greater than 8 Hz, which is typical for residues having β-sheet-like ϕ dihedral angles.[33] The detailed structural analysis of **3** and **4** using 2D ^{1}H NMR spectroscopy and structure calculations is reported elsewhere.[34]

4. Discussion

Over the past two decades, a number of studies have established CBC as an effective strategy for the solid-phase synthesis of cyclic peptides. Several linkers have been used for this purpose, including those having amine-labile anchors such as the Kaiser oxime[35] and thioester[36] linkers, as well as 'safety-catch' linkers that employ aryl hydrazide,[37,38] catechol,[39,40] or alkanesulfonamide[11–17] functions. Safety-catch linkers are particularly useful for the synthesis of cyclic peptides because they exploit amine-stable anchors that prevent premature cyclization during elongation of the peptide chain; at the conclusion of the synthesis, however, these same anchors can be activated for nucleophilic attack in order to effect CBC. Resins functionalized with the AS-SCL are commercially available, and Yang and Morriello have reported the use of such resin for the synthesis of cyclic peptides by CBC.[11] Subsequent reports by Noort,[12] Guo,[13–16] and Ganesan[17] have begun to define the scope of this approach.

The 22-residue peptides **3** and **4**, both of which contain 66-membered rings, are noteworthy in that they are among the longest cyclic peptides yet prepared via CBC using the AS-SCL. In both cases, the CBC reaction proceeded smoothly without resorting to low-loading resins ('pseudo-high-dilution'[12] conditions). These results support our expectation that the linear precursors to **3** and **4** would adopt preorganized ↑↓ $\beta^{5.6}$-helical conformations in which the N and C termini are close together. We note that Guo et al. have advanced similar arguments to rationalize the success of CBC for backbone-to-backbone cyclization of linear precursors having unprotected ornithine side chains.[13–16]

As mentioned above, the as-synthesized crude peptides **3** and **4** were contaminated with minor products that could

not be removed by repeated RP-HPLC. The purification of highly hydrophobic peptides is notoriously difficult;[19–22] nevertheless, we were able to purify **3** and **4** to near homogeneity using a facile two-step procedure of RP-HPLC followed by GPC on Sephadex LH-20. In our case, the GPC step could be carried out with gravity-driven flow and without the use of an in-line UV detector (see Section 6 for details), thus minimizing capital outlay. We note that Rijkers et al. have reported a similar two-step RP-HPLC/GPC strategy for the purification of hydrophobic *S*-palmitoyl peptides.[41] The success of this twofold approach is likely due to the orthogonal nature of the separation techniques: RP-HPLC operates by adsorption of molecules onto a stationary phase on the basis of molecular hydrophobicity,[23] while GPC works by partitioning of molecules into a porous stationary phase on the basis of molecular shape and size.[42] In the case of **3** and **4**, one of the hard-to-remove contaminants is probably an epimer resulting from loss of configuration at residue 8 during the CBC step, and we note that GPC on Sephadex media is known to be capable of resolving peptides that differ only in the chirality of a single residue.[43] An alternative strategy would be to avoid epimerization altogether by using the aryl hydrazide linker, which is reported to suppress epimerization during CBC.[37,38] For our future work, however, we wish to synthesize cyclic β-helical peptides having a range of side-chain functionalities, and CBC using the aryl hydrazide linker requires an oxidation step, a process that may not be compatible with sensitive side-chain groups.

5. Conclusion

The foregoing results show that CBC using the commercially available AS-SCL is an effective route for the solid-phase synthesis of backbone-cyclized ↑↓ $\beta^{5,6}$-helical peptides. The successful preparation of the 66-membered-ring-containing peptides **3** and **4** suggests that the linear precursors also possess significant ↑↓ $\beta^{5,6}$-helical structure, which presumably facilitates ring closure by placing the N and C termini close in space. In the case of **3** and **4**, two-step purification via RP-HPLC followed by GPC yielded these highly hydrophobic peptides in pure form. We are currently working to establish the generality of these procedures by preparing longer cyclic β-helical peptides and peptides having polar side chains.[11–17] We anticipate that the latter peptides will find application as ligands for macromolecular targets and as building blocks for new protein architectures.

6. Experimental

6.1. Materials

Before use, DIEA was distilled first from ninhydrin and then from CaH_2,[44] and ICH_2CN was filtered through a plug of basic alumina;[9,10] otherwise, all materials were used as received from the source indicated: 4-sulfamylbutyryl AM resin, EMD Biosciences, San Diego, CA, USA; anhydrous CH_2Cl_2 and THF, Sigma–Aldrich, Milwaukee, WI, USA; anhydrous NMP (Biotech Grade, over 4 Å molecular sieves), Pharmco, Brookfield, CT, USA; HATU, Applied Biosystems, Foster City, CA, USA; HFIP, TCI America, Portland, OR, USA; $HFA \cdot 3H_2O$, Sigma–Aldrich; and Sephadex LH-20, Amersham Biosciences, Piscataway, NJ, USA. $CHCl_3$ used in GPC was of HPLC-grade and stabilized with ca. 50 ppm pentene.

6.2. General procedures for manual solid-phase peptide synthesis. Procedure for flow/shake washes (FSW)

A single round of FSW consisted of a brief (ca. 10–15 s) vacuum-assisted flow wash (ca. 5 mL s^{-1}), taking care not to let the resin go dry, followed by a 5 s shake with ca. 10 mL solvent.

6.3. Representative procedures for manual solid-phase peptide synthesis. Synthesis of *cyclo*{[(L-Val-D-Val)$_4$-(L-Val-D-Pro-Gly)]$_2$-} (3)

6.3.1. Anchoring the first amino acid. 4-Sulfamylbutyryl AM resin[9,10] (1.0 mmol, 0.91 g of 1.1 mequiv g^{-1}, 1 equiv) was placed in a SPPS vessel[18,28] having a coarse porosity sintered glass frit and Teflon stopcock. Anhydrous CH_2Cl_2 (10 mL) was added and the resin was allowed to swell by shaking for 1 h. The CH_2Cl_2 was drained to the top of the resin, taking care not to let the resin go dry, and the resin was rinsed using two rounds of FSW. A solution of Fmoc-D-Val-F[26] (1.02 g, 3 mmol, 3 equiv) in anhydrous CH_2Cl_2 (6 mL) was added, followed by DIEA (0.35 mL, 2 mmol, 2 equiv),[24] and the reaction mixture was shaken for 1 h and then drained. After 2×FSW with CH_2Cl_2, the coupling was repeated as before. The resin was again rinsed via 2×FSW with CH_2Cl_2, followed by Et_2O, and then dried overnight under vacuum. Two ca. 5 mg samples of the aminoacyl resin were removed and subjected to the quantitative Fmoc UV absorbance assay,[25] by which the loading of the resin was determined to be 0.60 mmol g^{-1} (coupling yield=68%). A portion of the resin (600 mg) was removed and transferred to another SPPS vessel, and the synthesis was carried forward at the appropriate scale (0.36 mmol).

6.3.2. Elongation of the peptide chain. The Fmoc group of the aminoacyl resin obtained by using the procedure given in Section 6.3.1 was removed by shaking with 20% piperidine in DMF for 4×3 min,[27] and the resin was rinsed via 2×FSW with NMP. The synthesis was then carried forward using Boc manual SPPS according to the following modified version of the in situ neutralization protocol:[28,29]

Boc-amino acid (1.44 mmol, 4 equiv) and HATU (0.521 g, 1.37 mmol, 3.8 equiv) were dissolved in anhydrous NMP (3 mL) and DIEA was added (0.38 mL, 2.16 mmol, 6 equiv). The mixture was shaken until a homogeneous solution was obtained and then allowed to sit for an additional 1 min in order to preactivate the amino acid. The resulting solution of activated amino acid was added to the peptidyl resin; the resin was shaken for 30 min and then rinsed via 2×FSW with NMP. The resin was tested for the presence of unreacted amines using the qualitative ninhydrin test[18] (for primary amines) or the qualitative chloranil test[18,45] (for secondary amines), and couplings that yielded a positive test were repeated. If the resin continued to give a positive test after recoupling, it was capped by treatment with Ac_2O (0.34 mL, 3.6 mmol, 10 equiv) and DIEA (0.31 mL,

1.8 mmol, 5 equiv) in NMP (5 mL) for 2 h. When all the amino groups had reacted, the *N*-terminal Boc group was deprotected by shaking for 2×1 min with neat TFA and the resin was rinsed via 3×FSW with NMP. The next Boc-amino acid was then coupled as before.

6.3.3. Activation and cyclization. When the last Boc-amino acid had been coupled, the *N*-terminal Boc group was left on, and the C terminus was activated by shaking the resin for 24 h with ICH_2CN (0.66 mL, 9.08 mmol, 25 equiv) and DIEA (0.63 mL, 3.63 mmol, 10 equiv) in NMP (5 mL).[9,10] The resin was rinsed via 3×FSW with NMP, the *N*-terminal Boc group was removed as before, and the resin was washed via 3×FSW with NMP and 3×FSW with anhydrous THF. The resin was then transferred to a round-bottom flask; anhydrous THF (7 mL) was added, followed by DIEA (127 μL, 1.09 mmol, 3 equiv), and the resulting reaction mixture was stirred magnetically under Ar. Although a white precipitate began to form within 1 h, the mixture was stirred for 3 days in order to ensure complete reaction.

The crude peptide was collected by filtering the resin through a medium porosity sintered glass funnel and rinsing copiously with $CHCl_3$ (ca. 100 mL). Removal of the $CHCl_3$ under reduced pressured afforded crude **3** (431 mg).

6.4. Representative procedure for RP-HPLC. Chromatography of crude peptide 3

Crude peptide **3** (431 mg) was dissolved in HFIP (ca. 5 mL) and diluted with $HFA\cdot 3H_2O$ (ca. 10 mL). The resulting solution was clarified by filtration using a 0.45-μm in-line syringe filter, and then subjected to RP-HPLC in 3 mL injections on a 22×250 mm C4 column run at 20 mL min^{-1} using a gradient of 60% B to 77% B over 17 min (solvent A=0.1% TFA and 1% *i*-PrOH in H_2O; solvent B=0.07% TFA and 90% *i*-PrOH in H_2O). The absorbance of the eluant was monitored at 230 nm, and the major peak was collected as two fractions as indicated in Figure 2. Yield: fraction *a*, 145 mg; fraction *b*, 73 mg.

6.5. Representative procedure for GPC. Chromatography of HPLC-purified peptide 3

Fraction *a* of RP-HPLC-purified peptide **3** (140 mg) was dissolved in 10 mL $CHCl_3$/MeOH (65:35 v/v), and 5 mL of this solution was loaded onto a 2.5×55 cm column of LH-20 in $CHCl_3$/MeOH (65:35 v/v; degassed separately before mixing) using a Pasteur pipette, taking care not to let the top of the resin go dry.[42] The column was then run overnight using gravity-driven flow at ca. 1 mL min^{-1}. Fractions of 5 mL were collected, and peptide-containing fractions were identified by transferring an aliquot to a quartz cuvette and reading the absorbance at 245 nm. Pure **6** eluted first, followed by the putative epimerized material, and the two species were typically separated by 2–3 fractions that contained no peptide. The remaining 5 mL of fraction *a* was chromatographed in an identical manner. Fraction *b* of the HPLC-purified material was dissolved in 5 mL $CHCl_3$/MeOH 65:35 v/v and chromatographed in a single aliquot as described above. In this manner, a total of 170 mg of pure **3** was obtained (0.083 mmol, 23% based on **2**).

6.6. Synthesis of peptide 4

For the synthesis of **4**, resin **1** (0.45 g, 0.5 mmol, 1 equiv) was loaded with Fmoc-L-Leu-F[26] (0.53 g, 1.5 mmol, 3 equiv) in 91% yield as described above for **3**. The remainder of the synthesis and purification of **4** was then carried out analogously to the procedures used for **3**, to give 245 mg pure **4** (0.104 mmol, 23% yield).

6.7. Spectral characterization of peptides 3 and 4

Mass spectral analysis was performed by the Laboratory for Biological Mass Spectrometry of Texas A&M University (College Station, TX, USA). Samples of RP-HPLC/GPC purified **3** and **4** were dried under vacuum for at least 12 h prior to NMR analysis. ^1H NMR and proton-decoupled ^{13}C NMR spectra were referenced to $CHCl_3$ (7.24 ppm) and $CDCl_3$ (77.0 ppm), respectively. The ^1H NMR resonances were assigned using 2D NMR spectroscopy as described elsewhere.[34] We were able to partially assign the ^{13}C NMR resonances by comparison of the 1D proton-decoupled ^{13}C spectra with residue-dependent chemical shift values from the literature,[46] and by cross-correlating the shifts observed for **3** and **4**; we did not attempt more to fully assign the ^{13}C resonances. Due to the twofold symmetry of **3** and **4**, we observed NMR signals corresponding to only half the total number of residues. IR spectra were recorded on a Nicolet 750 FTIR spectrometer at a resolution of 8 cm^{-1}, and samples were held in a liquid cell having BaF_2 windows and a path length of 50 μm. IR spectra were corrected for background by taking the ratio of the sample spectra to a blank spectrum.

6.7.1. *cyclo*{[(L-Val-D-Val)$_4$-(L-Val-D-Pro-Gly)]$_2$-} (3). ^1H NMR (500 MHz, 289 K, 10 mM in $CDCl_3$): *L-Val(1)*, NH (7.28, d, J=9.6 Hz), $C^{\alpha}H$ (4.74), $C^{\beta}H$ (2.12), $C^{\gamma}H_3$ (0.95, 0.91); *D-Val(2)*, NH (8.68, d, J=6.3 Hz), $C^{\alpha}H$ (4.64), $C^{\beta}H$ (2.32), $C^{\gamma}H_3$ (1.03, 0.99); *L-Val(3)*, NH (8.58, d, J=9.8 Hz), $C^{\alpha}H$ (4.89), $C^{\beta}H$ (2.00), $C^{\gamma}H_3$ (0.92, 0.84); *D-Val(4)*, NH (8.38, d, J=6.8 Hz), $C^{\alpha}H$ (4.76), $C^{\beta}H$ (2.10), $C^{\gamma}H_3$ (0.95); *L-Val(5)*, NH (8.96, d, J=10.1 Hz), $C^{\alpha}H$ (5.11), $C^{\beta}H$ (2.06), $C^{\gamma}H_3$ (0.90, 0.81); *D-Val(6)*, NH (8.30, d, J=7.5 Hz), $C^{\alpha}H$ (4.62), $C^{\beta}H$ (2.10), $C^{\gamma}H_3$ (0.94, 0.90); *L-Val(7)*, NH (9.14, d, J=9.1 Hz), $C^{\alpha}H$ (4.03), $C^{\beta}H$ (1.91), $C^{\gamma}H_3$ (0.89); *D-Val(8)*, NH (6.45, d, J=6.4 Hz), $C^{\alpha}H$ (4.92), $C^{\beta}H$ (2.20), $C^{\gamma}H_3$ (1.06, 0.93); *L-Val(9)*, NH (7.78, d, J=8.4 Hz), $C^{\alpha}H$ (4.39), $C^{\beta}H$ (2.13), $C^{\gamma}H_3$ (1.05, 0.95); *D-Pro(10)*, $C^{\alpha}H$ (4.40), $C^{\beta}H$ (2.27, 2.05), $C^{\gamma}H$ (2.02), $C^{\delta}H$ (4.27, 3.63); *Gly(11)*, NH (6.91), $C^{\alpha}H$ (4.31, 3.60). ^{13}C NMR (75 MHz, 295 K, 10 mM in $CDCl_3$): δ 173.2 (C′), 172.5 (C′), 172.1 (C′), 171.8 (C′), 171.7 (C′), 171.4 (C′), 171.3 (C′), 171.2 (C′), 170.8 (C′), 170.7 (C′), 169.5 (C′), 61.7 (D-Pro10 C^{α}), 59.0 (Val C^{α}), 59.0 (Val C^{α}), 58.9 (Val C^{α}), 58.8 (Val C^{α}), 58.6 (Val C^{α}), 58.0 (Val C^{α}), 57.2 (Val C^{α}), 56.6 (Val C^{α}), 56.2 (Val C^{α}), 48.0, 42.5, 32.4, 32.4, 32.2, 31.7, 31.7, 31.6, 31.3, 30.6, 30.5, 29.6, 25.9 (D-Pro10 C^{γ}), 20.1 (Val C^{γ}), 19.9 (Val C^{γ}), 19.8 (Val C^{γ}), 19.7 (Val C^{γ}), 19.6 (Val C^{γ}), 19.5 (Val C^{γ}), 19.4 (Val C^{γ}), 19.3 (Val C^{γ}), 19.3 (Val C^{γ}), 19.3 (Val C^{γ}), 19.2 (Val C^{γ}), 19.0 (Val C^{γ}), 18.9 (Val C^{γ}), 18.7 (Val C^{γ}), 18.4 (Val C^{γ}), 17.8 (Val C^{γ}), 17.7 (Val C^{γ}), 17.2 (Val C^{γ}) ppm. IR (liquid, BaF_2, 295 K, 2 mM in $CDCl_3$): ν 3413 (amide A, nonhydrogen-bonded), 3278 (amide A, hydrogen-bonded),

3066 (amide B), 1682 (amide I parallel component), 1635 (amide I perpendicular component), 1543 (amide II) cm^{-1}. HRMS (MALDI) *m/z* calcd for $C_{104}H_{183}N_{22}O_{22}$ $[M+H]^+$: 2092.3877, found: 2092.3845; calcd for $C_{104}H_{182}N_{22}NaO_{22}$ $[M+Na]^+$: 2114.3697, found: 2114.3662; calcd for $C_{104}H_{182}N_{22}KO_{22}$ $[M+K]^+$: 2130.3436, found: 2130.3501.

6.7.2. *cyclo*{[(D-Leu-L-Leu)$_4$-(D-Leu-L-Pro-Gly)]$_2$-} 4. ^1H NMR (600 MHz, 283 K, 12 mM in $CDCl_3$): *D-Leu(1)*, NH (7.28, d, J=8.9 Hz), $C^{\alpha}H$ (4.75), $C^{\beta}H$ (1.64, 1.54), $C^{\delta}H_3$ (0.86, 0.80); *L-Leu(2)*, NH (8.92, d, J=7.1 Hz), $C^{\alpha}H$ (4.62), $C^{\beta}H$ (1.77, 1.37), $C^{\gamma}H$ (1.48), $C^{\delta}H_3$ (0.93, 0.87); *D-Leu(3)*, NH (8.80, d, J=9.3 Hz), $C^{\alpha}H$ (4.81), $C^{\beta}H$ (1.54, 1.42); *L-Leu(4)*, NH (8.35, d, J=8.4 Hz), $C^{\alpha}H$ (4.79), $C^{\beta}H$ (1.59, 1.14), $C^{\gamma}H$ (1.37), $C^{\delta}H_3$ (0.81, 0.82); *D-Leu(5)*, NH (9.22, d, J=9.4 Hz), $C^{\alpha}H$ (4.85), $C^{\beta}H$ (1.62, 1.35), $C^{\delta}H_3$ (0.86, 0.79); *L-Leu(6)*, NH (8.95, d, J=8.3 Hz), $C^{\alpha}H$ (4.67), $C^{\beta}H$ (1.71, 1.32), $C^{\gamma}H$ (1.43), $C^{\delta}H_3$ (0.87); *D-Leu(7)*, NH (9.23, d, J=9.0 Hz), $C^{\alpha}H$ (4.32), $C^{\beta}H$ (1.61, 1.37), $C^{\gamma}H$ (1.58), $C^{\delta}H_3$ (0.87); *L-Leu(8)*, NH (6.44, d, J=6.9 Hz), $C^{\alpha}H$ (4.99), $C^{\beta}H$ (1.59, 1.48), $C^{\delta}H_3$ (0.94); *D-Leu(9)*, NH (7.85, d, J=7.7 Hz), $C^{\alpha}H$ (4.60), $C^{\beta}H$ (1.71), $C^{\gamma}H$ (1.63), $C^{\delta}H_3$ (0.98, 0.94); *L-Pro(10)*, $C^{\alpha}H$ (4.40), $C^{\beta}H$ (2.28, 2.03), $C^{\gamma}H$ (2.06), $C^{\delta}H$ (4.74, 3.60); *Gly(11)*, NH (6.70, dd, J=4.8, 7.9 Hz), $C^{\alpha}H$ (4.22, 3.44). ^{13}C NMR (75 MHz, 295 K, 12 mM in $CDCl_3$): δ 174.7 (C′), 173.2 (C′), 172.5 (C′), 172.2 (C′), 171.5 (C′), 171.4 (C′), 171.4 (C′), 171.2 (C′), 170.7 (C′), 170.6 (C′), 169.8 (C′), 61.8 (l-Pro10 C^{α}), 51.9 (Leu C^{α}), 51.9 (Leu C^{α}), 51.3 (Leu C^{α}), 51.1 (Leu C^{α}), 51.0 (Leu C^{α}), 51.0 (Leu C^{α}), 50.9 (Leu C^{α}), 50.8 (Leu C^{α}), 50.1 (Leu C^{α}), 47.7, 43.8, 43.3, 43.1, 42.6, 42.4, 42.3, 42.0, 41.6, 41.0, 40.2, 29.4, 25.2, 25.1, 25.0, 25.0, 24.9, 24.8, 24.8, 24.7, 24.6, 24.5, 23.7 (Leu C^{δ}), 23.6 (Leu C^{δ}), 23.3 (Leu C^{δ}), 23.2 (Leu C^{δ}), 23.2 (Leu C^{δ}), 23.0 (Leu C^{δ}), 22.9 (Leu C^{δ}), 22.8 (Leu C^{δ}), 22.7 (Leu C^{δ}), 22.7 (Leu C^{δ}), 22.7 (Leu C^{δ}), 22.4 (Leu C^{δ}), 22.4 (Leu C^{δ}), 22.3 (Leu C^{δ}), 22.2 (Leu C^{δ}), 21.8 (Leu C^{δ}), 21.7 (Leu C^{δ}), 21.7 (Leu C^{δ}) ppm. IR (liquid, BaF_2, 295 K, 2 mM in $CDCl_3$): ν 3417 (amide A, nonhydrogen-bonded), 3267 (amide A, hydrogen-bonded), 3070 (amide B), 1682 (amide I parallel component), 1639 (amide I perpendicular component), 1551 (amide II) cm^{-1}. HRMS (MALDI) *m/z* calcd for $C_{122}H_{219}N_{22}O_{22}$ $[M+H]^+$: 2344.6694, found: 2344.6670; calcd for $C_{122}H_{218}N_{22}NaO_{22}$ $[M+Na]^+$: 2366.6514, found: 2366.6489; calcd for $C_{122}H_{218}N_{22}KO_{22}$ $[M+K]^+$: 2382.6253, found: 2382.6099.

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