ORGANIC LETTERS

2002
Vol. 4, No. 23
4167−4170

A Facile Ligation Approach to Prepare Three-Helix Bundles of HIV Fusion-State Protein Mimetics

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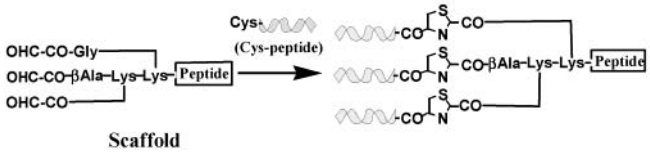
Received September 19, 2002

ABSTRACT



We have designed a facile ligation approach to prepare three-helix bundles mimicking the HIV membrane fusion-state proteins that may be useful as inhibitors and vaccine candidates for blocking HIV infection.

Infection by the human immunodeficiency virus-1 (HIV-1) requires viral and cellular membrane fusion that is mediated by a trimer-of-hairpins in the viral coat protein gp41.[1−3] This trimer is a bundle of six α-helices composed of three helical hairpins, each consisting of heptad repeats of an N-helix paired with an antiparallel C-helix (Figure 1). Compounds that prevent the N- and C-helix pairing are known inhibitors of HIV-1 infection. Synthetic C-peptides corresponding to the C-helix, such as DP178 and C34, potently inhibit membrane fusion by both laboratory-adapted strains and primary isolates HIV-1.[4] A phase-I clinical trial of DP178 resulted in reduced viral loads, suggesting that it has antiviral activity in vivo.[5] N-Peptides such as N36 corresponding to the N-helix are modest inhibitors and are also of interest as candidates for inhibiting viral entry.[6−8]

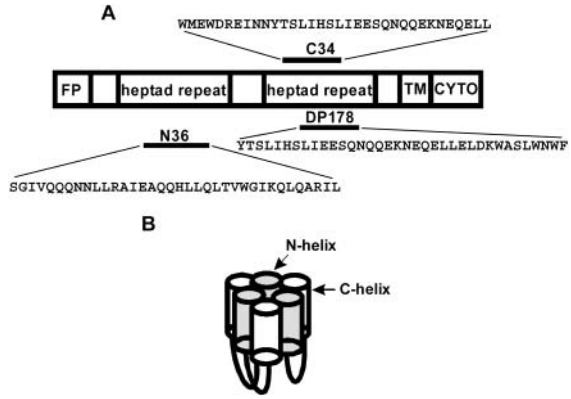


Figure 1. (A) Map of gp41 showing fusion peptide (FP), heptad repeat N-peptide N36, C34, and DP178, transmembrane (TM) and cytoplasmic (CYTO) region. (B) Schematic diagram of gp41 core structure.

For developing synthetic inhibitors or vaccines to block HIV-1 infection, we envisioned that trimeric N- and C-helix peptides might be good mimetics of the fusion-state inter-

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10.1021/ol026932k CCC: $22.00
Published on Web 10/25/2002

mediates of gp41. In this report, we describe a ligation approach to prepare trimeric N- and C-peptides of gp41 as fusion-state protein mimetics. Our approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected N- and C-peptides to an aldehyde scaffold containing three arms. An advantage of this approach is that the monomeric peptides can be readily prepared by synthetic or biosynthetic methods.

The design of the peptide scaffold was centered on the dipeptide Lys[3]-Lys[4] to provide three amino groups for tethering helical N- or C-peptides (Figure 2). To provide

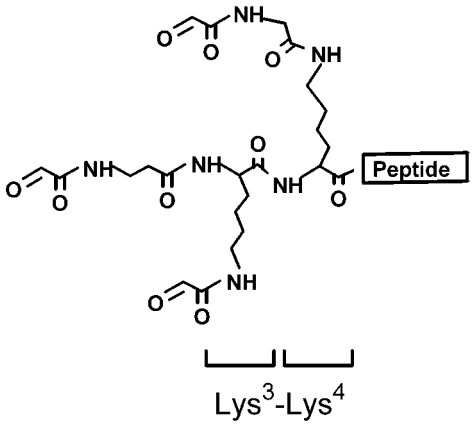

Figure 2. Schematic diagram of aldehyde scaffold.

pseudosymmetry and to minimize steric hindrance, we added a β-Ala spacer to the α-amine of Lys[3] and a Gly to the ϵ-amine of Lys[4] so that all three amino arms were equally spaced from the Lys[4] C-α carbon. Each amino arm was capped at the amino terminus with a Ser residue to serve as an aldehyde precursor for chemoselective ligation. For the dual purpose of designing viral-entry inhibitors and vaccine, a 14-residue peptide as a T-helper epitope from tetanus toxoid (amino acid 830−844)[9] was appended onto the C-terminus of the scaffold to enhance the immunogenicity of the trimeric protein mimetics. A tripeptide spacer Ser-Ser-Ala was inserted between the scaffold and T-helper sequences to increase the aqueous solubility of the hydrophobic T-helper epitope.[10]

Thiazolidine ligation requires an N-terminal cysteine-peptide to couple to an aldehyde scaffold. Although we and others have developed various chemoselective ligation methods based on mutually reactive weak base-electrophile reactions for coupling unprotected peptides to scaffolds,[11−16] we have found thiazolidine ligation to be suitable. It can be performed in a broad pH range (pH 1−8) in both aqueous

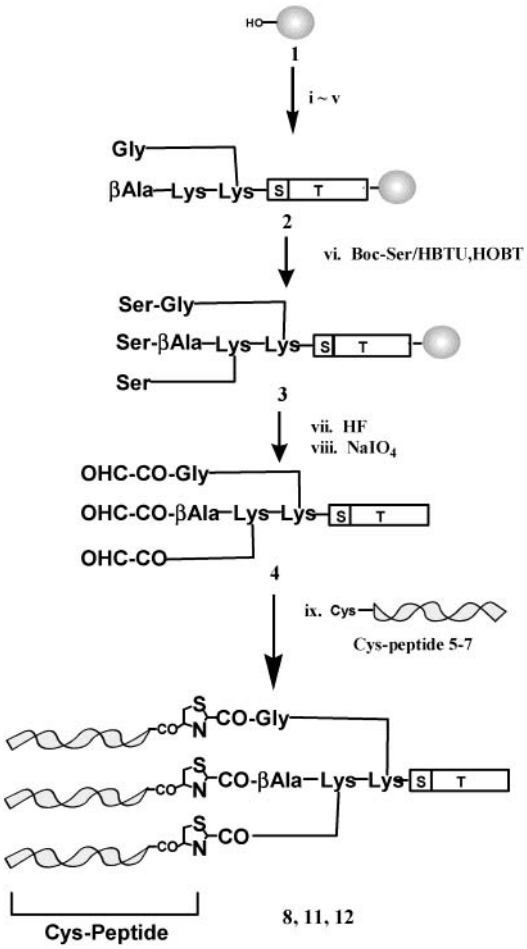

Figure 3. Synthetic scheme of three-helix bundle. (i) Stepwise assembly of peptide chain S and T (T, T-helper sequence; S, spacer sequence). (ii) Fmoc-Lys(Boc)-OH. (iii) Boc-Gly-OH. (iv) Fmoc-Lys(Boc)-OH. (v) Boc-βAla-OH. **5**, **8**: Cys-peptide = Cys-N36. **6**, **11**: Cys-peptide = Cys-C34. **7**, **12**: Cys-peptide = Cys-DP178.

or nonaqueous conditions, which has an advantage to minimize insolubility of the hydrophobic and amphipathic building blocks in our reactions. The thiazolidine ligation method is highly regiospecific. Side chains of lysine, arginine, and other amino acids are excluded from this reaction,[15] but the N-terminal cysteine reacts with aldehyde to form a stable five-member thiazolidine.

Unprotected peptides N36, DP178, and C34 with a Cys at their N-terminals were prepared by Fmoc chemistry using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIEA) as coupling reagents. After assembling the peptide chains on resin, the unprotected peptides were obtained by cleavage from the resin employing trifluoroacetic acid (TFA)/phenol/water/1,2-ethanedithiol (EDT)/triisopropyl-silane (TIS) 86.5:5:5:2.5:1 for 4 h. The crude peptides were purified by

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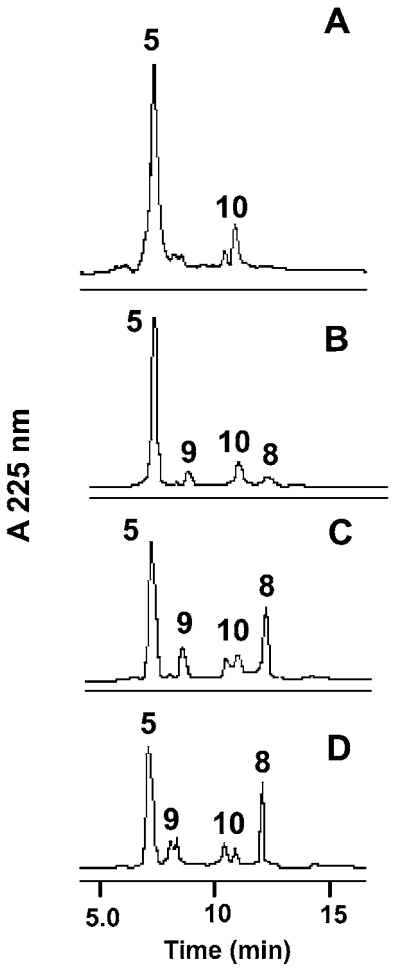

Figure 4. HPLC profiles of ligation of scaffold **4** and Cys-N36 **5**. **9**, monomer; **10**, dimer; and **8**, trimer. (A) 4 h in HPLC eluent, pH 2, (B) 24 h in HPLC eluent, pH 2, (C) 4 h in HOAC, and (D) 18 h in HOAC.

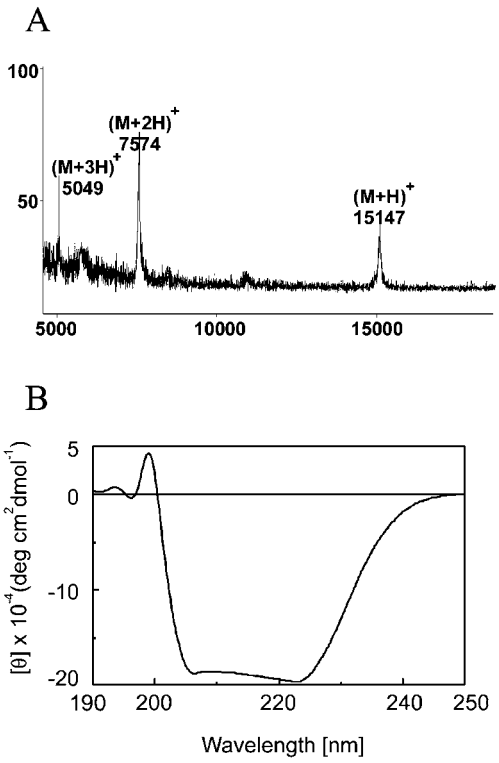

Figure 5. (A) MS analysis and (B) CD spectrum (in triflouroethanol) of three-helix bundle protein mimetic **8**.

HPLC, and the purified unprotected peptides were then used as starting materials for the thiazolidine ligation.

The aldehyde scaffold was generated efficiently by oxidation of the N-terminal Ser with sodium periodate under very mild aqueous conditions.[17] Its precursor was prepared by a combination of Fmoc and Boc chemistries,[18] by first assembling the T-helper and spacer sequences by Boc chemistry on a resin support using HBTU/1-hydroxybenzotriazol (HOBt) as coupling reagent and the remaining sequencing using the Lys^3-Lys^4 Fmoc chemistry with Fmoc-Lys(Boc) as a protected intermediate to afford peptide-resin. Treatment of this peptide-resin with 55% TFA removed the side-chain-protecting Boc group, and Boc-Gly was coupled to the ϵ-amine of Lys^4. Removal of Fmoc from the α-amino group and followed by sequential coupling with second Fmoc-Lys-(Boc) and a Boc-β-Ala gave peptide resin **2**. Next, coupling of Boc-Ser(*t*-Bu) afforded the scaffold precursor **3** with three Ser at the N-terminals and a T-helper at the C-terminal. HF treatment removed all protecting groups and cleaved the peptide from the resin support, and the crude product was purified by reverse-phase HPLC. The scaffold precursor **3** was hydrophobic because of the T-helper peptide and $NaIO_4$ oxidation was performed in acetonitrile containing 0.2 M acetate buffer (pH 5.5) 50:50 (v/v) and was completed in 10 min. The reaction mixture was then injected into a semipreparative HPLC to remove formaldehyde formed in the reaction, and the purified aldehyde-scaffold **4** was obtained in >90% yield. To avoid degradation of the aldehyde moiety, the desired HPLC fractions containing the aldehyde were used directly for ligation with Cys-N36 peptide **5** (Figure 3).

Thiazolidine ligation was performed by mixing 3.3 molar excess of Cys-N36 peptide **5** with aldehyde-scaffold **4** in the HPLC eluent containing 0.1% TFA at pH 2, which was deaerated and kept under nitrogen. Under this highly dilute condition, the thiazolidine ligation was slow and incomplete after 24 h, with all three products, monomer **9** (MH^+ calcd 6730, found 6730), dimer **10** (MH^+ calcd 10938, found 10938), and trimer **8** found in the reaction mixture (Figure 4A and B).

A new stereocenter at the C2 carbon of the thiazolidne ring was generated, and the diastereomers were observed as broad peaks in HPLC profiles. The ligation reaction was accelerated by lyophilizing the mixture to dryness and then dissolving the residue in a minimal amount of acetic acid (100 μL/1 mg) to afford trimer **8** (MH^+ calcd 15147, found 15147, Figure 5A) in 43% yield in 4 h (Figure 4C) based

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on the aldehyde scaffold as a limiting agent. Prolonging the ligation did not improve on the yield, with the HPLC profile showing dead-end products of monomers and dimers because of the degraded side products of the tri-aldehyde scaffold (Figure 4D).

Typically, 3−5 molar excess of Cys-peptide was dissolved in about 600 μL of HPLC eluent containing 1 mg of aldehyde scaffold **4**. The reaction mixture was kept under nitrogen overnight (18−24 h) and then lyophilized to dryness. To the lyophilized solid was added 100 μL of glacial acetic acid, and the solution was kept under nitrogen. After 4 h, 1 mL of water was added, and the diluted ligation solution was applied onto a semipreparative HPLC to obtain ligation product. In the same condition, thiazolidine ligation of Cys-C34 **6** and Cys-DP178 **7** with the aldehyde scaffold **4** afforded the corresponding trimeric products **11** and **12** (**11** MH^+ calcd 15359, found 15359; **12** MH^+ calcd 16130, found 16130) in 60−65% yield. Circular dichroism measurements of the trimeric N36 **8** showed that it displayed strong helical structures in trifluoroethanol consistent with stable trimeric coil-coil helices (Figure 5B).

Preliminary results showed that the three-helix bundle protein mimetic of DP178 inhibited HIV-1 infection with $IC_{50} < 0.5$ nM and was 10-fold more potent than the monomeric DP178.[19]

In conclusion, the thiazolidine ligation of unprotected peptide segments on a peptide scaffold provides a useful approach to prepare three-helix bundle fusion-state protein mimetics for HIV inhibitors and anti-HIV vaccine candidates. These protein mimics have three N- or C-peptides attached on a symmetric scaffold to force a folded structure mimicking a three-helix bundle fusion-state protein. The peptide scaffold also provides flexibility to incorporate T-helper epitopes for synthetic vaccines or other peptide cargoes for testing other biological functions.

Acknowledgment. This work was in part supported by U.S. Public Health Service, CA36544 and AI46174. We thank Dr. Jin-Long Yang for biological assay.

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(19) The m-tropic strain viruses were treated with peptide and then incubated with HeLa-CD4/LTR-lac Z indicator cell for 72 h. Anti-HIV activity of peptides was expresses as IC_{50}.