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# Highly Potent and Oral Macrocyclic Peptides as a HIV-1 Protease Inhibitor: mRNA Display-Derived Hit-to-Lead Optimization

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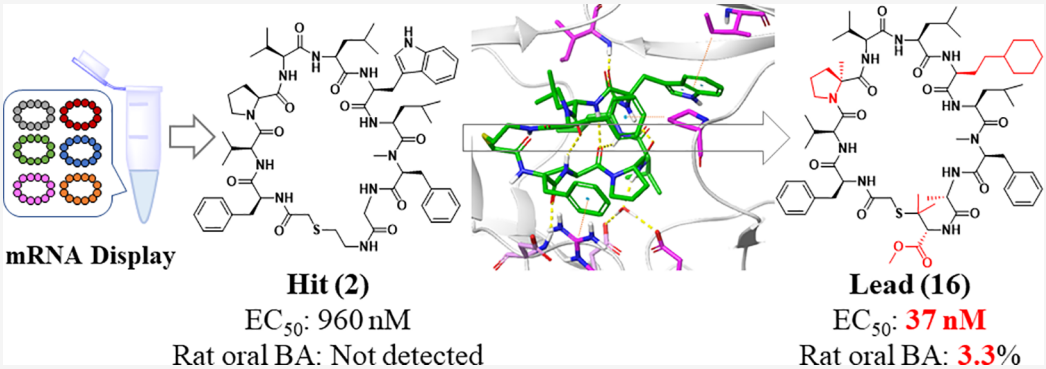


**ABSTRACT:** Human immunodeficiency virus type-1 (HIV-1) protease is essential for viral propagation, and its inhibitors are key anti-HIV-1 drug candidates. In this study, we discovered a novel HIV-1 protease inhibitor (compound **16**) with potent antiviral activity and oral bioavailability using a structure-based drug design approach via X-ray crystal structure analysis and improved metabolic stability, starting from hit macrocyclic peptides identified by mRNA display against HIV-1 protease. We found that the improvement of the proteolytic stability of macrocyclic peptides by introducing a methyl group to the $\alpha$-position of amino acid is crucial to exhibit strong antiviral activity. In addition, macrocyclic peptides, which have moderate metabolic stability and solubility in solutions containing taurocholic acid, exhibited desirable plasma total clearance and oral bioavailability. These approaches may contribute to the successful discovery and development of orally bioavailable peptide drugs.

**KEYWORDS:** *HIV-1 protease inhibitor, Macrocyclic peptide, Antiviral activity, Oral bioavailability, Proteolytic stability, α-Methyl amino acid, mRNA display*

Acquired immunodeficiency syndrome is a chronic, potentially life-threatening condition caused by the human immunodeficiency virus (HIV). HIV infects lymphocytes (mainly expressing CD4 proteins) and macrophages, which are vital to immunity, and aggravates the disease by progressively destroying the immune system.[1,2] HIV-1 protease is one of the key enzymes responsible for the propagation of infectious virus particles[3] and is an important target protein for anti-HIV drugs because it plays a key role in cleaving precursor polyproteins transcribed and translated from the HIV genome.[4,5] After approval of the protease inhibitor saquinavir (Figure 1) in 1995, combination therapy using three antiretroviral agents of different anti-HIV-1 drugs was initiated as the standard of care.[6,7] Darunavir, approved by the Food and Drug Administration in 2006, exhibits potent antiviral activity against drug-resistant HIV-1 clinical isolates.[8−10] However, since darunavir contains a peptide-like core structure that mimics the protease-catalyzed transition state, this has resulted in poor oral bioavailability and metabolic stability. Moreover, it is essential to combine darunavir with ritonavir,[11] a CYP3A4 inhibitor, as a pharmacokinetic (PK) booster in the

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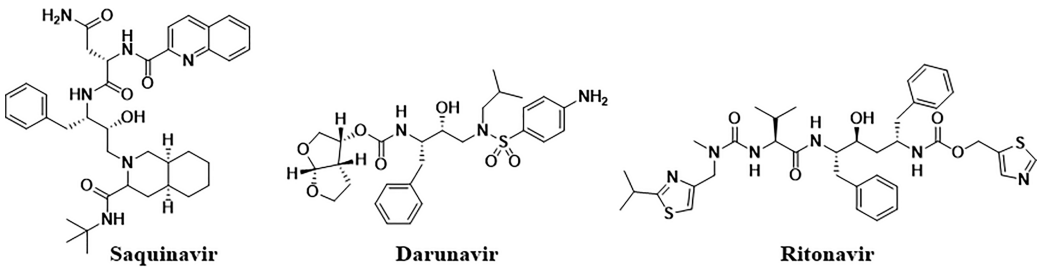


**Figure 1.** Approved HIV-1 protease inhibitors.

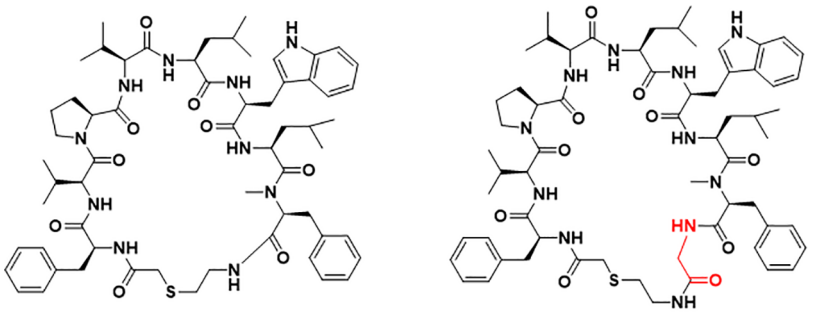


**Figure 2.** Hit compounds identified by mRNA display against HIV-1 protease. [a]Inhibitory activity of HIV-1 protease measured by fluorogenic peptide cleavage assay. [b]Anti-HIV-1 activity assay using the human T cell line (MT-4 cells).

prescription of darunavir; therefore, it is necessary to pay close attention to the drug−drug interaction risk.[12]

Macrocyclic peptides have emerged as a new class of drug discovery modalities and attracted much attention.[13,14] Typically, macrocyclic peptides are considered more likely to acquire strong interactions with a target protein owing to their restricted molecular motion and rigidity compared to linear peptides.[15] Cyclosporine, a natural macrocyclic peptide approved as an oral immunosuppressive drug, penetrates the cell membrane by masking the hydrogen bond donor (HBD) of the amide groups via intramolecular hydrogen bonding.[13] On the other hand, to the best of our knowledge, only a few structure−activity relationship (SAR) studies of macrocyclic peptides for inhibition of intracellular targets or oral bioavailable derivatives have been reported.[16−24] Among them, a macrocyclic hexapeptide with G protein-coupled receptor CXCR7 modulating activity derived from template-fixed $\beta$-hairpin peptidomimetics led to a potent and orally macrocyclic peptide−peptoid hybrid CXCR7 modulator by incorporating an N-linked peptoid and reducing the polarity.[23]

Recently, mRNA display has been used to select peptides from large libraries on the basis of their binding affinity for a target protein and has demonstrated promising results against a variety of protein targets.[25−27] However, large macrocyclic peptides (8−15 mer) tend to be identified by mRNA display and are considered less favorable with respect to drug-likeness, such as cell membrane permeability and oral bioavailability. Recently, MK-0616, a new cholesterol-lowering medicine in a class of drugs called PCSK9 inhibitors, has been studied in clinical trials conducted by Merck.[31] MK-0616 identified by mRNA display is a macrocyclic peptide and has been optimized for the inhibitory potency and stabilization, resulting in tricyclic peptides with picomolar potency against PCSK9. MK-0616 is an orally bioavailable PCSK9 inhibitor with permeation enhancers. So far, it has not been reported that a macrocyclic peptide identified by mRNA display has been optimized to a lead macrocyclic peptide that possesses both intracellular activity and oral bioavailability without the permeation enhancers. Herein, we describe a SAR study of macrocyclic peptides originated from mRNA display for HIV-1 protease inhibitors and optimization to deliver highly potent and orally available macrocyclic peptides.

The HIV-1 protease mRNA display successfully identified hit compounds **1** and **2**, which exhibited the desired enzyme inhibitory activity ($IC_{50}$) (Figure 2). In terms of antiviral activity ($EC_{50}$), compound **2** seemed to be unfavorable regarding membrane permeability owing to its additional HBD derived from an additional glycine unit, although the $IC_{50}$ of compound **2** was better than that of compound **1**. Therefore, we first selected compound **1** as the original compound and then applied the SAR findings to compound **2**.

Macrocyclic peptides consist of several amino acids and structural conversion sites. The X-ray crystal structure analysis of a protein−ligand complex is useful in the structure-based drug design to improve enzyme inhibitory activity. First, we used X-ray crystal structure analysis to improve the $IC_{50}$ of compound **1**. The cocrystal of compound **1** with a homodimer HIV-1 protease was successfully obtained by extensive screening of the conditions for cocrystallization. X-ray crystallographic analysis suggested that compound **1** forms a water-mediated interaction with aspartate residues (Asp25 and Asp25′) in the active center of the HIV-1 protease. In addition, hydrogen bonding between the backbone amide carbonyl of 1-Phe and the backbone amide (−NH) of Asp29′, the cation−$\pi$


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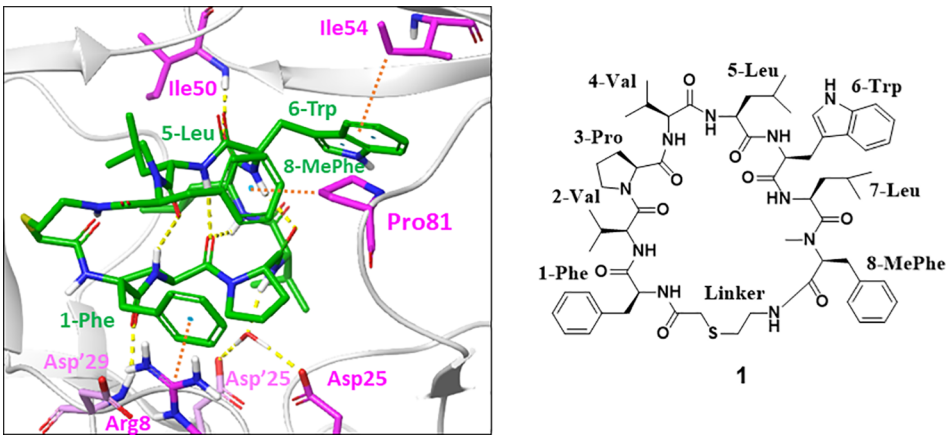
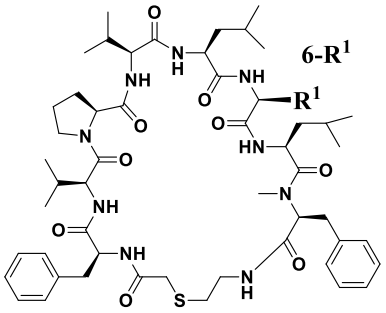
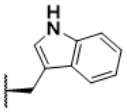
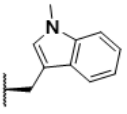
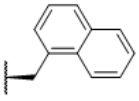



**Figure 3.** Interactions between HIV-1 protease (gray ribbon) and compound **1** (green sticks). The residues of HIV-1 protease interacting with compound **1** are highlighted as thin pink sticks and dark pink sticks. Yellow and orange dashed lines indicate hydrogen bonds and CH−$\pi$ or cation−$\pi$ interactions, respectively.

**Table 1. Optimization of the 6th Position of Compound 1**




| Compound | 6-R$^1$ | IC$_{50}$ (nM)[a] | EC$_{50}$ (nM)[b] |
|---|---|---|---|
| 1 |  | 8.05 | 610 |
| 3 |  | 7.86 | 1500 |
| 4 |  | 0.69 | 320 |

[a]Inhibitory activity of HIV-1 protease measured by the fluorogenic peptide cleavage assay. [b]The anti-HIV-1 activity assay using the human T cell line (MT-4 cells).

interaction between side chains of 1-Phe and Arg8, the hydrogen bonding between the backbone amide carbonyl of 5-Leu and the backbone amide (−NH) of Ile50, and CH−$\pi$ interactions between side chains of 6-Trp and Ile54 and 8-MePhe and Pro81 were also confirmed. Besides, compound **1** formed four intramolecular hydrogen bonds (Figure 3).

As the X-ray crystal structure suggested that the indole NH of 6-Trp in compound **1** was not involved in the interaction with HIV-1 protease, we expected that the removal of the indole NH of 6-Trp would not affect the IC$_{50}$. As expected, *N*-methylation of the indole moiety of 6-Trp to 1-methylindole was tolerable (compound **3**, Table 1). Furthermore, the replacement with 2-naphthalene exhibited a 12-fold potent IC$_{50}$ (compound **4**). The reason for the enhanced activity may be that the 2-naphthalene ring filled the lipophilic pocket around the sixth position. Although compound **4** showed remarkable inhibitory activity, there was room for improvement regarding the antiviral activity, as a significant gap between IC$_{50}$ and EC$_{50}$ can be seen. Typically, peptides are susceptible to hydrolysis. Since compound **4** may be decomposed by intracellular proteolytic enzymes, as indicated by its moderate stability (46%) in human liver S9 without NADPH (to exclude oxidative metabolic pathways) (Figure 4), we attempted to enhance the EC$_{50}$ of the human T cell line (MT-4 cells) by improving proteolytic enzyme stability. It is known that alkylation at the $\alpha$-position of a constituent amino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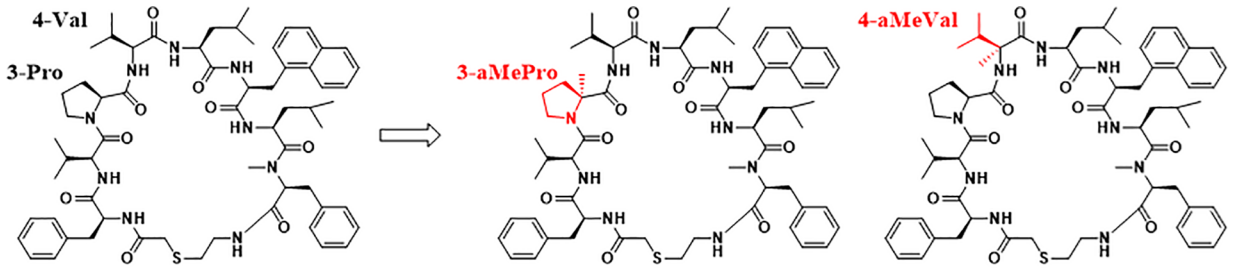


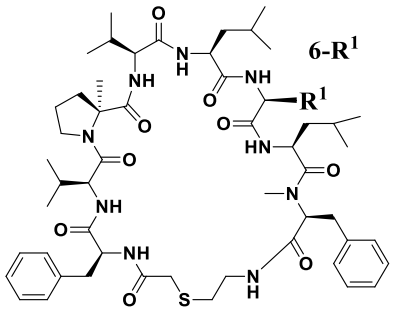

**4**
$IC_{50}$ (nM)[a] : 0.68
$EC_{50}$ (nM)[b] : 320
Metabolic Stability
Human liver S9 (%)[c] : 46%

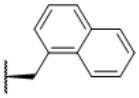
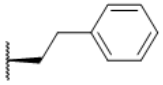
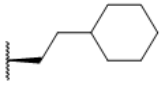
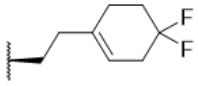
**5**
$IC_{50}$ (nM)[a] : 0.78
$EC_{50}$ (nM)[b] : 30
Metabolic Stability
Human liver S9 (%)[c] : 86%

**6**
$IC_{50}$ (nM)[a] : 0.44
$EC_{50}$ (nM)[b] : 120
Metabolic Stability
Human liver S9 (%)[c] : 69%

**Figure 4.** αMe introduction at the 3rd and 4th positions of compound **4**. [a]Inhibitory activity of HIV-1 protease measured by the fluorogenic peptide cleavage assay. [b]Anti-HIV-1 activity assay using the human T cell line (MT-4 cells). [c]Metabolic stability using human liver S9.

**Table 2. Optimization of the 6th Position of Compound 5**




| Compound | 6-$R^1$ | $IC_{50}$ (nM)[a] | $EC_{50}$ (nM)[b] |
|---|---|---|---|
| 5 |  | 0.78 | 30 |
| 7 |  | 0.45 | 28 |
| 8 |  | 0.29 | 12 |
| 9 |  | 0.21 | 10 |

[a]Inhibitory activity of HIV-1 protease measured by the fluorogenic peptide cleavage assay. [b]The anti-HIV-1 activity assay using the human T cell line (MT-4 cells).

acid in a peptide improves protease tolerance owing to steric hindrance.[28] Therefore, methylation at the α-position was carried out for each amino acid involved in compound **4**. To our delight, methylation at the α-position improved stability against the S9 fraction and also demonstrated antiviral activities when Pro at the third position and Val at the fourth position were converted into α-methyl L-proline (αMePro, compound **5**) and α-methyl L-valine (αMeVal, compound **6**), respectively (Figure 4). In contrast, α-methyl amino acids at the other positions significantly decreased both $IC_{50}$ and $EC_{50}$ (data not shown). In order to further improve antiviral activity, we optimized the side chain at the sixth position of compound **5**. As shown in Table 2, the nonaromatic substituents enhanced the $EC_{50}$ along with their improved $IC_{50}$ (compounds **8** and **9**), and the $IC_{50}$ and $EC_{50}$ of compound **7** substituted by the phenethyl moiety was comparable with those of compound **5**.

Next, the SAR findings from the SAR exploration as described above were applied to the structural optimization of compound **2**, which exhibited a more potent $IC_{50}$ compared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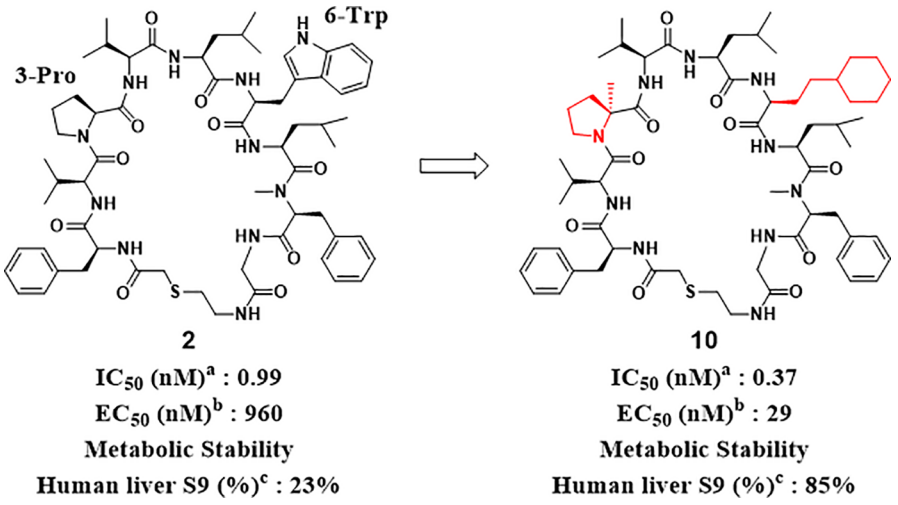
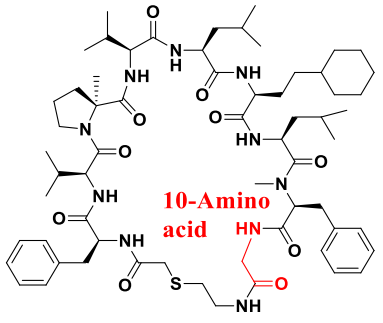



**Figure 5.** αMe introduction at the 3rd position and L-homocyclohexylalanine introduction at 6th position. [a]Inhibitory activity of HIV-1 protease measured by the fluorogenic peptide cleavage assay. [b]Anti-HIV-1 activity assay using the human T cell line (MT-4 cells). [c]Metabolic stability using human liver S9.

with that of compound **1**. As expected, compound **10**, which consisted of αMePro at the third position and L-homocyclohexylalanine at the sixth position, exhibited remarkable potency with a high stability to the human liver S9 fraction (Figure 5). In addition, we optimized the amino acid at the 10th position of compound **10** and found that Ala instead of Gly gave more potent compound **11** while Leu and Phe resulted in the diminished potencies (compounds **12** and **13**) (Table 3). Since thioethers are typically susceptible to oxidative metabolism, the introduction of steric hindrance adjacent to the sulfur atom was conducted to improve liver metabolic stability (Table 4). A vicinal dimethyl group at the α-position of the sulfur atom improved liver microsomal metabolic stability (compounds **14** and **15**). Interestingly, compound **16** bearing a methoxycarbonyl group, which is well-known as a metabolically labile functional group, still maintained the stability of compound **15** with a slight drop of potency.

**Table 3. Optimization at the 10th Position of Compound 10**




10 ~ 13

| compound | 10-amino acid | $IC_{50}$ (nM)[a] | $EC_{50}$ (nM)[b] |
|---|---|---|---|
| **10** | Gly | 0.37 | 29 |
| **11** | Ala | 0.28 | 24 |
| **12** | Leu | 2.30 | 62 |
| **13** | Phe | 2.32 | 56 |

[a]Inhibitory activity of HIV-1 protease measured by the fluorogenic peptide cleavage assay. [b]The anti-HIV-1 activity assay using the human T cell line (MT-4 cells).

Finally, compounds with potent $EC_{50}$ and good metabolic stability in rat hepatic microsomes were evaluated in rat in vivo PK studies (Table 5). Notably, compounds **15** and **16** achieved desirable plasma total clearance (5.7 and 8.2 mL/min/kg, respectively) and oral bioavailability (0.2% and 3.3%, respectively) in rats. Interestingly, there was a great difference in the oral bioavailability between compounds **15** and **16** although they have similar physicochemical properties and in vitro data. Using a preliminary Simcyp animal (version 21) simulation,[33] we confirmed that a difference in in vivo distribution ($Vd_{ss}$ and $t_{1/2}$) between compounds **15** and **16** could affect the in vivo duration after oral administration to rats and improve oral bioavailability. In addition, low plasma concentration after oral administration to rats and the LLOQ (10 nM) of LC-MS/MS might overestimate the differences of the oral bioavailability. Although the oral bioavailability of compound **16** (3.3%) was less than that of the previous macrocyclic hexapeptide (compound **25**,[23] 18%), the plasma total clearance of compound **16** (8.2 mL/min/kg) was superior to that of compound **25** (136 mL/min/kg); thus, the oral plasma exposure corrected by the dose of compound **16** was superior to that of the previous macrocyclic hexapeptide. The solubility of our macrocyclic peptides was improved by the presence of taurocholic acid, suggesting that macrocyclic peptides can be dissolved by micellization in the presence of bile acids, as reported previously.[32] A previous macrocyclic hexapeptide (compound **25**[23]) was administered in a self-emulsifying drug delivery system (SEDDS) formulation to enhance oral bioavailability, although compound **16** was administered in suspension. Therefore, the application of the SEDDS to compound **16** is expected to improve its oral bioavailability by enhancing the solubility. On the other hand, we consider that the disadvantage of the use of the self-microemulsifying drug delivery system (SMEDDS) is to address the issue of the chemical stability during the storage of the SMEDDS formulation because a marketed SMEDDS formulation such as Neoral (cyclosporine) is a capsule filled liquid and the chemical stability in the liquid should be required.[34] In addition, the exposed polar surface area (EPSA) of compounds **15** and **16** was less than 100, although the EPSA of compound **2** was greater than 100. EPSA can be used as a


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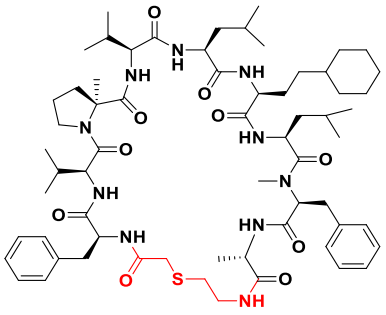
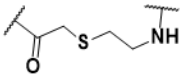
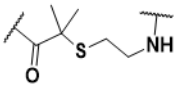
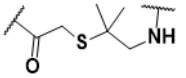
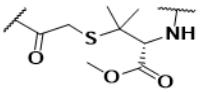
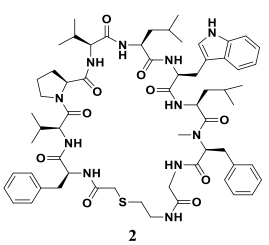
**Table 4. Optimization of the Thioether Linker of Compound 11**




| Compound | Linker | $IC_{50}$ (nM)[a] | $EC_{50}$ (nM)[b] | Metabolic stability rat/human hepatic microsomes (%)[c] |
|---|---|---|---|---|
| 11 |  | 0.28 | 24 | 53/8 |
| 14 |  | 15.30 | 150 | 46/28 |
| 15 |  | 0.62 | 11 | 60/20 |
| 16 |  | 3.52 | 37 | 63/23 |

[a]Measured by the fluorogenic peptide cleavage assay. [b]Anti-HIV-1 activity assay using MT-4 cells. [c]Metabolic stability using human and rat hepatic microsomes.

**Table 5. Pharmacokinetic Properties of Compounds in Rats[a]**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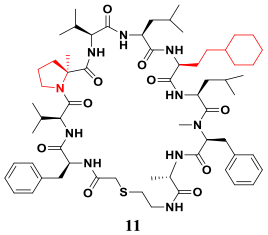
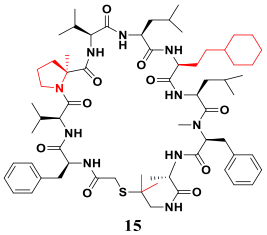
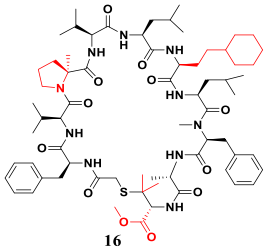

11


15


16

| compound | metabolic stability rat/human (%)[b] | $CL_{tot}$ (mL/min/kg)[c] | $Vd_{ss}$ (L/kg) | $t_{1/2}$ (h) | $F$ (%)[c] | serum fu (%) | solubility ($\mu$M) pH 1.2/pH 6.8/pH 6.8 with TCA | EPSA[d] |
|---|---|---|---|---|---|---|---|---|
| **2** | 29/3 | 6.8 | 0.1 | 0.7 | ND | 1.1 | ND/ND/26 | 113 |
| **11** | 53/18 | 11.4 | 0.8 | 1.6 | ND | 0.1 | ND/ND/NT | 88 |
| **15** | 60/20 | 5.7 | 0.7 | 2.0 | 0.2 | 0.1 | ND/ND/NT | 93 |
| **16** | 63/23 | 8.2 | 1.9 | 3.9 | 3.3 | 0.1 | ND/ND/10 | 92 |

[a]ND: not detected; NT: not tested. [b]Metabolic stability using human and rat hepatic microsomes. [c]All compounds were administered to rats by intravenous administration at 1 $\mu$mol/kg solution and oral administration at 2 $\mu$mol/kg suspension. [d]Exposed polar surface area (EPSA) analysis by supercritical fluid chromatography.

filter with a cutoff value of 100 to predict acceptable permeability for the bRo5 chemical series.[29,30] We consider that compound **2** does not have significant permeability. It is necessary to optimize the lipophilicity of the compound to achieve high passive membrane permeability, as reported previously.[30] In the future, lead-to-candidate optimization to improve the solubility and permeability of macrocyclic peptides or the application of SEDDS will be required. Although further improvement in oral bioavailability is still required, we conducted SAR exploration and optimization of



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the strong binders from mRNA display and successfully obtained highly potent and orally available HIV-1 protease inhibitors as a new series.

In summary, we discovered the macrocyclic peptide series as a novel class of HIV-1 protease inhibitors through mRNA display technology. The structure-based drug design approach using the X-ray cocrystal structure allowed one to enhance the potency by removing one HBD. In the effort to enhance the antiviral activity, we found that proteolytic stability can be essential to fill the gap between cell-free inhibitory activity and cell-based antiviral activity. Methyl installation to the $\alpha$-position of Pro delivered highly potent antiviral active compounds accordingly. In addition, the improvement of metabolic stability by modification of the linker part gave compounds **15** and **16** with desirable potencies, solubility in solutions containing taurocholic acid, and relatively lower EPSA values, and these compounds successfully demonstrated desirable plasma total clearance and oral bioavailability in the rat PK test. We strongly believe that this study would provide valuable insights to other peptide drug discovery projects aimed at intracellular targets and oral bioavailability.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.2c00310.

Synthetic procedure for peptides, amino acids, and resins, characterization of peptides, protocols of biological assays, pharmacokinetic studies, and supporting figures of the X-ray structures (PDF)

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### Notes

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## ■ ABBREVIATIONS

HIV-1, human immunodeficiency virus type-1; SBDD, structure-based drug design; CYP3A4, cytochrome P450 3A4; PK, pharmacokinetic; HBD, hydrogen bond donor; SAR, structure−activity relationship; CXCR7, C-X-C chemokine receptor type-7; $IC_{50}$, enzyme inhibitory activity; $EC_{50}$, antiviral activity; Asp, L-aspartic acid; Phe, L-phenylalanine; Arg, L-arginine; Leu, L-leucine; Trp, L-tryptophan; Ile, L-isoleucine; MePhe, *N*-methyl L-phenylalanine; Pro, L-proline; Val, L-valine; Gly, glycine; Ala, L-alanine; SEDDS, self-emulsifying drug delivery system; EPSA, exposed polar surface area

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