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Design and Synthesis of Dimeric HIV-1 Integrase Inhibitory Peptides

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Abstract—Dimers of known HIV-1 integrase inhibitory hexapeptide H-His-Cys-Lys-Phe-Trp-Trp-NH_2 containing different lengths of cross linkers in the place of cysteine residue, were designed, and synthesized. The inhibitory potency of these dimeric peptides is consistently higher than the lead hexapeptide. The dimeric peptide with djenkolic acid linker exhibited IC_{50} values of 5.3 and 6.5 μM, for 3′-end processing and strand transfer, respectively.

HIV-1 integrase is essential for the HIV replication cycle,[1] furthermore it is a key enzyme for the ability of the HIV virus to infect non-dividing cells.[2] The mechanism of integrase enzymatic activity involves two steps. First, the enzyme removes two nucleosides from each of the viral DNA ends (3′-processing) then, after migration of the DNA-protein complex to the nucleus, integrase catalyzes the insertion of the viral DNA through its 3′-end into the cellular chromosome (strand transfer, referred to as integration or 3′-end joining). In addition to reverse transcriptase and protease, integrase has also been the focus of attention for HIV antiviral chemotherapy. Integrase is an attractive target because it has no counterpart in mammalian cells; therefore, selective integrase inhibitors should not produce any side effects. Many integrase inhibitors have been reported to date.[3] However, progress with the design of effective inhibitors of HIV-integrase is slower than in the case of reverse transcriptase or protease inhibitors and no clinically useful inhibitors have yet been reported.

During our structure–activity studies of analogues of the hexapeptide integrase inhibitor, His-Cys-Lys-Phe-Trp-Trp-NH_2 (**1**), identified earlier by peptide library methodology by Plasterk and coworkers,[4] we found that a dimeric peptide (**2c**) was a more potent inhibitor of integrase than the monomeric hexapeptide **1**.[5] This suggests that a dimeric peptide may occupy the two neighboring catalytic sites in the integrase oligomer. In order to study the relationship between the linker length or linker functionality, and the inhibitory activity of dimeric peptide, we designed several linkers and developed an efficient strategy for dimeric peptide synthesis. Here, we present the syntheses and results of HIV-1 integrase inhibitory activity assays of these newly generated dimeric peptides **2a–e** (Fig. 1) with five different dimerization linkers: L-lanthionine **4a**, L-cystine **4b**, L-cystathionine **4c**, L-homolanthionine **4d**, and L-djenkolic acid **4e**. Linkers **4a** and **4c** have been found previously in natural product peptides, and are used frequently as non reducible analogues of **4b**[6–8] mostly as a part of cyclic structures. Linkers **4d**[9] and **4e**[10] have found only infrequent usage, mostly as components in cyclic peptides.

The lead peptide **1** was synthesized by an automated SPPS using Rink amide resin and Fmoc chemistry; the dimeric cystine containing peptide **2b** was isolated as the oxidative byproduct after preparative HPLC of **1**.

The method of synthesis of dimeric peptides **2a**, **2c**, **2d**, and **2e** is presented on Scheme 1 (synthesis of the L-homolanthionine containing peptide **2d** is shown as an example). The di-Fmoc derivatives **5a**, **5c**, **5d**, and **5e** were prepared by reaction of the corresponding amino

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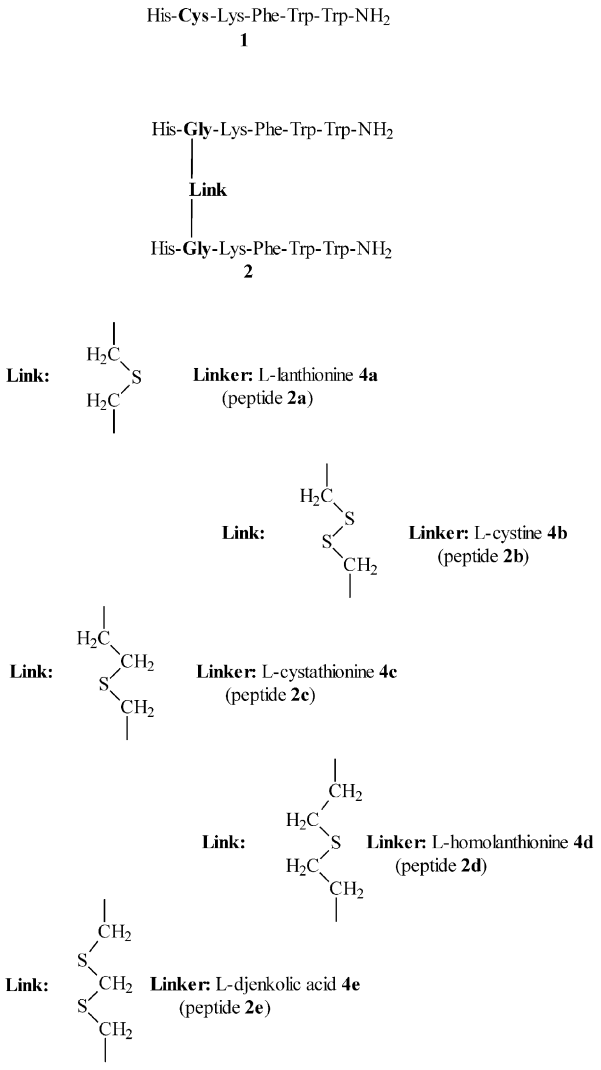
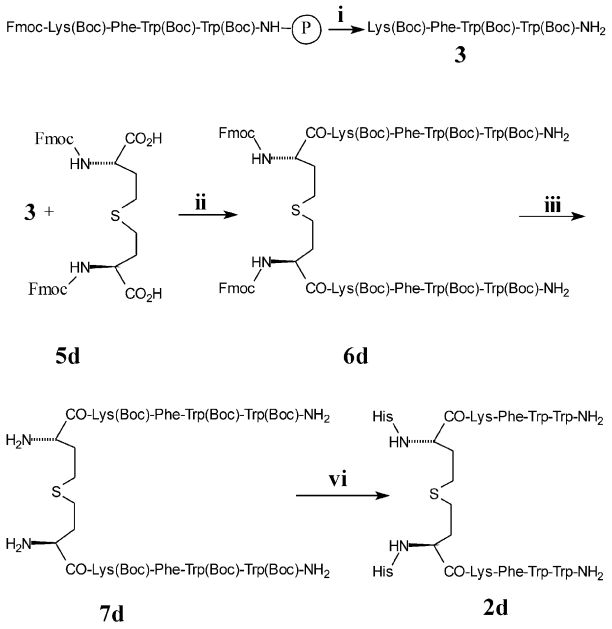


Figure 1. Schematic representation of prepared peptides.



Scheme 1. The synthesis of dimeric peptide **2d**: (i) 1% TFA, 1% EDT in DCM (15×2 min); (ii) FEP, DIEA in DCM (3 h, −10 °C→rt); (iii) 50% piperidine in DMF (1 h, rt), preparative HPLC; (vi) (1) Boc-His(Boc)-OH, HATU, HOAt, DIPEA (3 h rt); (2) 2.5% TIS, 2.5% H_2O in TFA (2 h, rt).

acids with FmocOSu (2.3 equiv) and Et_3N (5 equiv) in acetonitrile/water (1:1 vol) mixture (rt, 3 h). The products were purified by MPLC[11] and the purity of each product was >95% (determined by RP HPLC, C_8 column). The dimeric amino acids **4c** and **4e** are commercially available.[12] The amino acids **4a** and **4d** were prepared from the methyl esters of Boc_2-L-cystine and Boc_2-L-homocystine by the sulfur extrusion reaction with tris(diethylamino)phosphine in dry benzene[13] (conditions for cystine derivative: 1 h, rt; for homocystine derivative: reflux 5 min, then 24 h at 30 °C), purification by MPLC and deprotection (LiOH in THF/water, rt, 1 h, then 50% TFA in DCM −10 °C to rt, 2 h). ^{1}H- and ^{1}H,^{1}H-COSY NMR spectra of **5a** (mp 111–114 °C, $[\alpha]_D^{20} = +10°$, *c* 0.15 in MeOH), **5c** (mp 129–130 °C, $[\alpha]_D^{20} = -6°$, *c* 0.15 in MeOH), **5d** (mp 198–204 °C dec., $[\alpha]_D^{20} = -26°$, *c* 0.45 in DMF), and **5e** (mp 123–125 °C, $[\alpha]_D^{20} = -57°$, *c* 0.45 in DMF) confirmed their structures (^{1}H NMR, 400 MHz, in $(CD_3)_2SO$; **5a**: 7.88 (4H, d, 7.4 Hz), 7.72 (4H, d, 7.4 Hz), 7.41 (4H, dd, 2×7.4 Hz), 7.32 (4H, dd, 2×7.4 Hz), 4.31–4.11 (8H, m), 3.01–2.95 (2H, m), 2.85–2.77 (2H, m); **5c**: 7.88 (4H, d, 7.5 Hz), 7.71 (4H, d, 7.5 Hz), 7.40 (4H, dd, 2×7.5 Hz), 7.32 (4H, dd, 2×7.5 Hz), 4.34–4.18 (8H, m), 2.94 (1H, dd, 13.2 Hz, 4.2 Hz), 2.77 (1H, dd, 13.2 Hz, 9.7 Hz), 2.65–2.53 (2H, m), 1.98–1.84 (2H, m); **5d:** 7.88 (4H, d, 7.4 Hz), 7.71 (4H, d, 7.4 Hz), 7.41 (4H, dd, 2×7.4 Hz), 7.32 (4H, dd, 2×7.4 Hz), 4.29–4.17 (6H, m), 4.12–4.07 (2H, m) 2.62–2.47 (4H, m), 1.98–1.83 (4H, m); **5e**: 7.88 (4H, d, 7.5 Hz), 7.72 (4H, d, 7.5 Hz), 7.41 (4H, dd, 2×7.5 Hz), 7.32 (4H, dd, 2×7.5 Hz), 4.34–4.18 (8H, m), 3.83 (2H, s), 3.11 (2H, dd, 13.7 Hz, 4.8 Hz), 2.83 (2H, dd, 13.7 Hz, 9.9 Hz)). The observed monoisotopic masses for **5a**, **5c**, **5d**, and **5e** (FAB-MS) were in good agreement with their theoretical masses (in parentheses): **5a** 653.5, 675.5 (653.2 M + H^+, 675.2 M + Na^+) Da; **5c** 667.2 (667.2 M + H^+) Da; **2d** 681.2 (681.5 M + H^+) Da; **2e** 699.4 (699.2 M + H^+) Da.

Typical Procedure for Dimeric Peptides Synthesis

The tetrapeptide Lys(Boc)-Phe-Trp(Boc)-Trp(Boc)-NH_2 (**3**) was synthesized by manual SPPS on Sieber amide resin (Aldrich, 0.35 mmol/g) using Fmoc chemistry, cleaved from the resin by treatment with, 1% TFA, 1% EDT in DCM (15×2 min), and purified[14] by MPLC.[15] The di-Fmoc protected linker **5** was coupled with **3** (2.5 equiv) in DCM solution, with 2-fluoro-1-ethylpyridinium tetrafluoroborate (FEP)[16] as a coupling reagent (FEP gave better results than HATU/HOAt) and DIPEA. The coupling product **6** was purified by RP HPLC, after deprotection of N-terminal NH_2 groups (50% piperidine in DCM). After purification and lyophilization, the peptide **7** was coupled with Boc-His (Boc)-OH in DMF (HATU/HOAt/DIPEA). After completion of the reaction water was added to precipitate the fully protected peptide. This peptide was washed three times with distilled water, dried under

Table 1. Results of in vitro HIV-1 integrase inhibitory assay for peptides 1, **2a–e**

	Peptides [IC_{50}] (μM)[a]					
	1	**2a**	**2b**	**2c**	**2d**	**2e**
3′-End processing	102±20	67±7	81±10	77±21	59±7	5.3±0.7
Strand transfer	90±15	19±6	58±15	87±23	63±7	6.5±1.9

[a]Values are means of at least three experiments, and are given with standard deviations.

reduced pressure, and deprotected by mixing with a solution of 2.5% TIS and 2.5% water in TFA. After 2 h, the solvents were removed under reduced pressure, the residue was washed six times with ether and dissolved in water/acetonitrile mixture (0.05% TFA), and lyophilized, The product was dissolved again in 0.05% TFA in water, kept at rt 6 h (to complete Trp residue deprotection), and then purified by preparative RP HPLC (Vydac C_4 or C_{18} column). Purity of all peptides was between 90 and 95%, (RP HPLC on C_8 and C_4 columns), MALDI-TOF-MS spectra verified molecular masses of all peptides. The observed monoisotopic masses were in good agreement with theoretical masses (in parentheses): **1** 905.3 (905.4 $M+H^+$) Da; **2a** 1775.3 (1775.8 $M+H^+$) Da; **2b** 1808.0 (1807.8 $M+H^+$) Da; **2c** 1789.7 (1789.9 $M+H^+$) Da; **2d** 1803.9 (1803.9 $M+H^+$) Da; **2e** 1821.8 (1821.8 $M+H^+$) Da.

HIV-1 Integrase Inhibition Assay

The HIV-1 integrase assays were performed as described previously,[17] with the following modifications. The peptides were pre-incubated with 500 nM HIV-1 integrase for 20 min at room temperature in a buffer containing 50 mM MOPS, pH 7.2, 7.5 mM NaCl, 7.5 mM $MnCl_2$, and 5% glycerol. Reactions were started by adding 20 nM of the 5′-end ^{32}P-labeled 21-mer double-stranded DNA template in a final volume of 10 μL, and reactions were carried out for 1 h at 37 °C. Reactions were quenched by adding 10 μL of denaturing loading dye (formamide 99%, SDS 1%, bromophenol blue 0.2 mg/mL, xylene cyanol FF 0.2 mg/mL). Samples were loaded onto 20% (19:1) denaturing polyacrylamide gels. Gels were dried, exposed overnight and analyzed using Molecular Dynamics PhosphorImager (Sunnyvale, CA, USA). The densitometric analysis was performed using ImageQuant v1.2 from Molecular Dynamics software package. Each lane was quantified to determine the amount of 3′-processing and strand transfer products (Table 1).

In conclusion, HIV-1 integrase inhibitory assays on peptides **1** and **2a–2e** demonstrate that the dimeric peptides possess higher inhibitory potency than monomeric peptide **1** for the integrase enzyme 3′-processing and also for the strand transfer process. The linker moieties of the dimeric peptides in this study include the thioethers, the dithio linkage and the dithiomethylene linkages. The peptide backbone to backbone lengths consist of four, five, or six bonds. Significantly, the dithiomethylene linker containing dimer (peptide **2e**) showed an approximately 20-fold higher potency compared to the monomer peptide **1**. This inhibitory potency was sustained both in the 3′-processing and the strand transfer process of the integrase enzyme. The mechanistic implications of inhibitory action suggest that the dimeric inhibitory peptide may act as a bivalent inhibitor, simultaneously occupying two neighboring catalytic sites in tandem, with an entropic advantage, within the integrase oligomeric complex. Indeed, recent X-ray structure of a small molecule inhibitor bound to the three integrase core domains showed that the active site is very close to the active site of the neighboring integrase subunit.[18]

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