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Short communication

Symmetry-based inhibitors of HIV-1 protease. Design, synthesis and preliminary structure-activity studies of acylated 2,3-diamino-1-hydroxypropanes and 2,4 diamino-1-hydroxybutanes

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Abstract – Two series of peptidomimetics containing a novel C_2 pseudosymmetrical hydroxyalkyldiamino core structure were prepared from amino acid starting materials and tested for inhibitory activity against the HIV-1 protease (HIV-1 Pr) and the virus in cell culture. In the 2,3-diamino-1-hydroxypropane series, compound **6a**, containing P1/P1^1 benzyl and P2/P2^1 Fmoc substituents, displayed modest HIV-1 Pr inhibition (IC_{50} = 430 nM). The corresponding 2,4-diamino-1-hydroxybutane derivative (**6b**) was the best inhibitor of the series (IC_{50} = 160 nM). Interestingly, **6a** and **6b** showed satisfactory inhibition of HIV replication in cell culture (ED_{50} = 340 and 110 nM, respectively), a result which suggests good cell membrane penetration by this class of compounds. © 1999 Éditions scientifiques et médicales Elsevier SAS

aspartyl protease / HIV-1 inhibitors / peptidomimetic

1. Introduction

Human immunodeficiency virus (HIV) has been identified as the probable causative agent of AIDS. The different therapeutic strategies for intervention of this disease have been reviewed recently [1]. A critical role in the viral replication is played by the HIV protease (Pr), an enzyme responsible for the processing of the polyproteins to structural proteins and enzymes essential for the viral maturation and infectivity [2]. This essential role makes it a promising target for chemotherapy of AIDS [3–7]. Since the initial characterisation of this enzyme, rapid progress has been achieved toward the development of potent and selective inhibitors [8–10]. The C_2-symmetry of HIV-1 Pr, which functions as a dimer with each subunit contributing an amino acid triad Asp-Thr-Gly to the active site, stimulated the design of symmetry-based inhibitors. On the basis of the hydroxyethylene core as a tetrahedral transition-state replacement for the peptide substrate, various potent and selective C_2-symmetric inhibitors have been designed [3, 8, 11, 12]. Recently, following the Kempf strategy [10] and the aminodiol inhibitors discovery [13], we disclosed a new class of HIV-Pr inhibitor which incorporates a C_2 symmetric 1,1-diamino-2-hydroxyethane core (gSer) as its key structural feature [14]. A member of this class, represented by the lead compound LC *(figure 1)*, displays significant HIV-1 Pr inhibition ($IC_{50} \approx 450$ nM).

Subsequently, dynamics simulations [15] indicated a "pseudosymmetric" mode of binding, suggesting that LC contains a core functionality capable of hydrogen bonding (OH, and NH of gSer) to the catalytic aspartate (Asp 25^1/Asp 25) residues of the enzyme and P1/P2 hydrophobic groups for placement in the S1/S2 subsites. Furthermore, these studies revealed that a lengthening of the gSer core structure in LC, by one or two methylene groups, would result in more potent inhibitors that would

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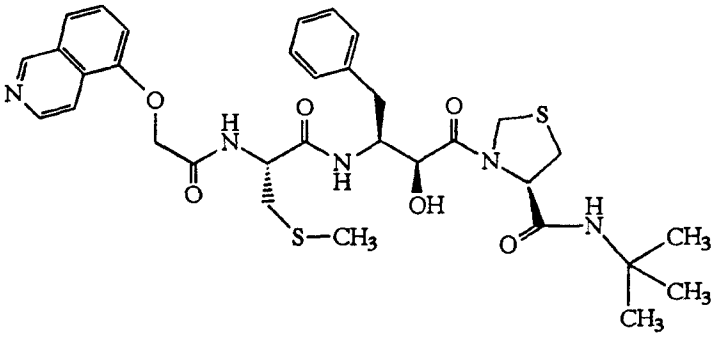
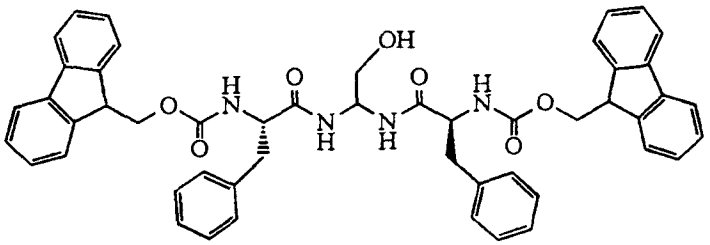
Abbreviations: Standard abbreviations for amino acid derivatives and peptides are according to IUPAC-IUB Biochemical Nomenclature [25]. Other abbreviations are: Ben, benzofuran-2-carbonyl; Boc, tert-butyloxycarbonyl; DMF, dimethylformamide; DMSO, dimethylsulfoxide; EtOAc, ethyl acetate; Et_2O, diethyl ether; Fmoc, fluorenylmethyloxycarbonyl; Ind, indol-2-carbonyl; MeOH, methyl alcohol; NMM, N-methyl-morpholine; OSu, N-hydroxysuccinimidyl ester; Pyp, pyperidin-2-carbonyl; THF, tetrahydrofuran; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; WSC, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide.

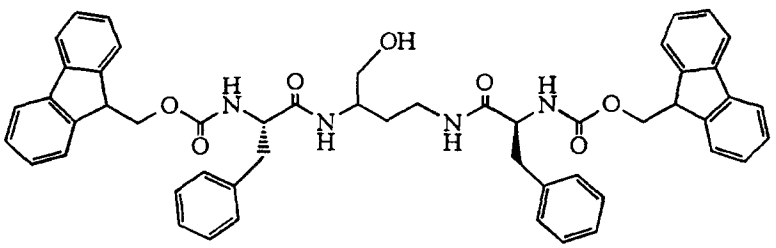


KNI-272



Fmoc-Phe-gSer-Phe-Fmoc

LC



6b

Figure 1. Chemical structures of KNI-272, symmetrical lead compound (LC) and its 2,4-diamino-1-hydroxybutane core structure containing analogue **6b**.

occupy, at least in part, the subsites of the enzyme and which are capable of forming further H bonds to the protein backbone. According to this prediction, pseudo-symmetric analogues of LC (*figure 2* and *table I*) containing 2,3-diamino-1-hydroxypropane or 2,4-diamino-1-hydroxybutane core units with different terminal groups were designed, prepared, and their interaction with recombinant HIV-1 Pr and anti HIV-1 activity were evaluated. For simplicity, the P1/P1^{I} and P2/P2^{I} nomenclature is used throughout the paper, although there is only

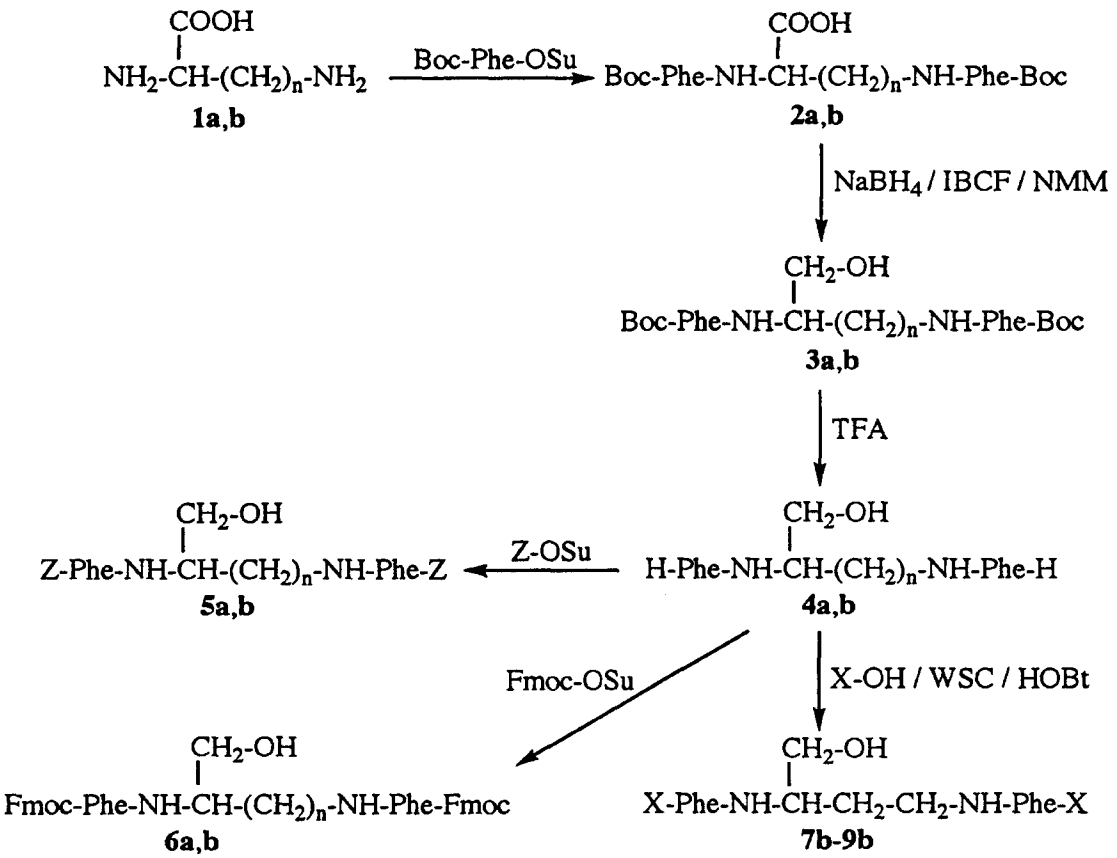


Figure 2. Synthesis of 2,3-diamino-1-hydroxypropane (n = 1) or 2,4-diamino-1-hydroxybutane (n = 2) containing pseudotripeptides. X = indol-2-carbonyl, benzofuran-2-carbonyl, pyridine-2-carbonyl.

preliminary evidence that these designated groups bind in the corresponding subsites of the enzyme [15].

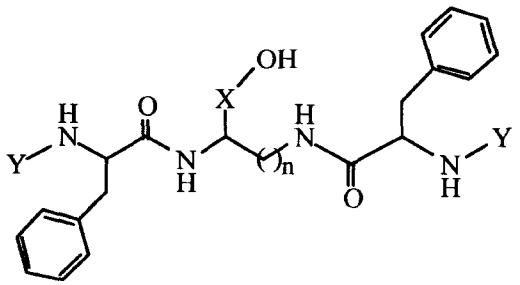
2. Chemistry

The preparation of hydroxyalkyldiamino core structure containing pseudopeptides **3a** and **b**–**6a** and **b** is outlined in *figure 2*. Coupling of two equivalents of Boc-Phe-OSu with L-2,3-diaminopropanoic acid **1a** or L-2,4-diaminobutanoic acid **1b** gave the key pseudotripeptide intermediates **2a** and **2b**. Target alcohols **3a** and **3b** were prepared by chemoselective reduction of the mixed anhydrides of the corresponding acids **2a** and **2b**. As previously observed for the synthesis of homoserine containing peptides [16], we found that a mixed anhydride obtained from **2a** and **2b** by reaction with isobutyl chloroformate, cleanly reacted with 3 M equivalents of sodium borohydride, to lead to the corresponding **3a** and **3b** in very good yields and with retention of stereochemistry at the adjacent stereocenter. Deprotection of **3a** and **3b** followed by coupling of Z-OSu or Fmoc-OSu afforded Z-pseudotripeptides **5a** and **5b** or Fmoc-analogues **6a** and **6b**, respectively. Finally, acylation of **4b** with indol-2-carboxylic acid, benzofuran-2-carboxylic acid or pyperidin-2-carboxylic acid, in the presence of WSC and HOBt, gave analogues **7b**–**9b**.

3. Biological activity

All compounds were tested for their inhibition of purified recombinant HIV-1 Pr using the peptide substrate His-Lys-Ala-Arg-Val-Leu-Phe(p-NO_2)-Glu-Ala-Nle-Ser (Bachem Bioscience) as previously described [14, 15]. The Leu-Phe(p-NO_2) bond of the substrate is cleaved by the enzyme, and substrate and products were separated by reversed-phase HPLC, absorbance being measured at

Table I. Structure and inhibitory potencies of compounds.



Compound	X	(n)	Y	TLC RF (A)	HPLC (t_R)	M.p. [°C]	$[\alpha]_D$[a]	MS[M + H$^+$] Calculated	MS[M + H$^+$] Found	IC_{50} (nM)[b]	ED_{50} (nM)[b]
2a	C=O	1	Boc	0.68	4.35	155–157	−12.3	598	598	> 10 000	ND[c]
2b	C=O	2	Boc	0.59	4.12	163–166	−9.8	612	612	> 10 000	ND
3a	CH_2	1	Boc	0.68	5.52	164–167	−7.1	584	584	9 700	ND
3b	CH_2	2	Boc	0.87	7.65	192–194	−11.6	598	598	4 100	3 600
5a	CH_2	1	Z	0.66	4.68	187–189	−9.9	653	653	5 700	ND
5b	CH_2	2	Z	0.64	5.58	163–165	−12.3	667	667	1 800	2 300
6a	CH_2	1	Fmoc	0.75	7.02	230–233	−18.0	830	830	430	340
6b	CH_2	2	Fmoc	0.70	5.21	151–153	−5.5	844	844	160	110
7b	CH_2	2	Ind	0.57	4.23	201–204	−24.2	686	686	2 900	3 800
8b	CH_2	2	Ben	0.61	4.73	197–199	−21.4	687	687	3 000	ND
9b	CH_2	2	Pyp	0.49	3.98	211–214	−28.3	610	610	3 900	ND
LC										455	290[d]

[a]c 1.0, methanol; temperature 21 °C.
[b]Values are the average of at least two determinations (n = 2) unless otherwise noted.
[c]Not determined.
[d]ED_{50} values ranged from 130–350 nM (n = 8).

220 nm. Inhibitory activities are expressed as IC_{50} (concentration of compound which inhibits 50% cleavage of substrate). Some compounds were also tested for their ability to inhibit the replication of the HIV-1 virus in cell culture (See experimental section).

A potent pseudopeptidic HIV-1 Pr inhibitor, KNI-272 *(figure 1)*, was used as a standard (17), yielding an average IC_{50} = 4.8 nM against the isolated enzyme and an ED_{50} = 50 nM in cell culture.

4. Results and discussion

The HIV-1 Pr inhibition activity of new pseudotripeptides **2a–9b** is reported in *table I*, in comparison with LC. Analysis of the results from the HIV-1 Pr tests showed weak inhibition for almost all compounds, displaying IC_{50} values ≥ 2.8 μM. Only compounds **6a** and **6b** displayed significant activities comparable to aminodiol derivatives [13] even if they are at least 50–100 times less active than structurally more complex molecules such as potent KNI-272 [17] and inhibitors approved for the treatment of AIDS [18–20]. As expected, the presence of the carboxylic group in the core structure of pseudopeptides **2a** and **2b** is not tolerated, suggesting that this functionality is incapable of hydrogen bonding to the catalytic aspartate residues of the enzyme.

The 2,4-diamino-1-hydroxybutane containing compounds (**3b**, **5b** and **6b**) are slightly more active than the corresponding diamino hydroxypropane derivatives (**3a**, **5a** and **6a**) suggesting that the latter spacer can allow less effective interactions between the inhibitors and protease.

Comparison of Boc (**3a** and **3b**), Z (**5a** and **5b**) and Fmoc (**6a** and **6b**) functionalities at P2/P2^1 shows that a bulky tricyclic system is preferred over a single aromatic ring or a branched aliphatic group, confirming the trend previously observed for symmetrical LC related analogues [14, 21]. This preference seems to be supported by the inhibitory potencies of the bis-acyl derivatives **7b–9b** which are at least 15-fold less potent than the Fmoc analogue **6b**. Nevertheless, it should be pointed out that

in comparison to carbamate derivatives (e.g. **5b** and **6b**), in the bis-hetero acyl compounds **7b–9b** the oxy groups have been deleted and this modification might be critical for enzyme-inhibitor interactions.

Five LC analogues were tested for their ability to inhibit the replication of the HIV-1 virus in cell culture. As can be readily observed *(table I)*, the measured ED_{50} values are quite close to the IC_{50} values for enzyme inhibition as had been noted previously for LC [22] and aminodiol inhibitors [13, 23]. Then, in spite of their modest ability to inhibit the enzyme, **6a** and **6b** showed satisfactory inhibition of HIV replication in cell culture (ED_{50} = 340 and 130 nM, respectively), a result that may indicate good cell membrane penetration by this class of compounds.

5. Conclusion

Previous efforts in this laboratory resulted in the synthesis, from amino acid starting materials, of a series of HIV-1 protease inhibitors containing a novel hydroxyalkyl gem-diamino core structure as exemplified by LC [14, 21]. In spite of its low ability to inhibit the isolated enzyme, LC showed satisfactory inhibition of HIV replication in cell culture [22]. This attractive property prompted us to design, through computational studies, LC analogues with improved potency against the isolated protease while maintaining the lipophilic character of the parent molecule. By lengthening, with an ethylene group, the core structure of LC we have been successful in designing the pseudotripeptide **6b** which has increased potency against the enzyme and the virus in cell culture.

6. Experimental protocols

6.1. General

Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin Elmer 141 polarimeter with a 10 cm water jacketed cell. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectra were obtained using the HPG2025A mass spectrometer. HPLC analysis was performed on a Bruker liquid chromatograph LC21-C instrument using a Vydac C 18 218 TP 5415 column (175 × 4.5 mm, 5 mm particle size) equipped with a Bruker LC 313 UV variable wavelength detector; recording and quantification were accomplished with a chromatographic data processor (Epson computer FX80X7). Analytical determinations were carried out by a gradient made up of two solvents: A, 10% (v/v) acetonitrile in water; B, 60% (v/v) acetonitrile in water, both containing 0.1% TFA. The gradient program used was as follows: linear gradient from 0–100% B in 25 min at a flow of 1 mL/min. All analogues showed purity greater than 99% following analytical HPLC monitored at 220 nm. Preparative reversed-Phase HPLC was carried out with a Waters Delta Prep 3 000 using a Delta Pack C 18–300 A (30 mm × 30 cm, 15 mm, spherical). The gradient used was identical to that of analytical determinations. Chromatography was performed at a flow rate of 30 mL/min. ^{1}H-NMR spectra were obtained using a Varian Gemini 300 (Palo Alto, CA, USA). Amino acid analyses were carried out using PITC methodology as the amino acid derivatization reagent (Pico-Tag, Waters-Millipore, Waltham, MA, USA). Lyophilised samples of peptides (50–100 pmol) were placed in heat treated borosilicate tubes (50 × 4 mm), sealed and hydrolysed using 200 mL 6 N HCl containing 1% phenol in the Pico-Tag work station for 1 h at 150 °C. A Hypersil ODS column (250 × 4.6 mm, 5 mm particle size) was employed to separate the PITC-amino acid derivatives. TLC used precoated plates of silica gel F254 (E. Merk, Darmstadt, FRG) in the following solvent system: (A) 1-butanol/acetic acid/H_2O (3:1:1), (B) EtOAc/pyridine/acetic acid/H_2O (12:4:1.2:2.2), (C) CH_2Cl_2/MeOH/toluene (8.5:1:O.5), (D) $CHCl_3$/MeOH/benzene/H_2O (8:8:8:1). Ninhydrin 1%, fluorescamine and chlorine spray reagents were employed to detect the peptides.

6.2. Chemistry

6.2.1. Coupling and deprotection procedures

The main procedures used for the preparation and deprotection of the pseudopeptides of *figure 2* varied little in the individual steps and are therefore summarised in the following general form:

A. Active ester coupling procedure. The activated carboxy component (OSu) (2 mmol) and the protonated amino component (1 mmol) were dissolved in DMF (10 mL) containing N-methylmorpholine (2 mmol). The mixture was allowed to react at room temperature for 8 h and then diluted with EtOAc (100 mL). The solution was washed consecutively with brine, 0.5 N $KHSO_4$, and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was crystallised from appropriate solvents or purified by HPLC.

B. WSC/HOBt coupling procedure. To a solution of the carboxy component (2 mmol) in DMF (10 mL) were added the amino acid component (1 mmol), NMM (2 mmol), HOBt (2 equiv.) and WSC (2.1 mmol) in the above order at 0 °C. The reaction mixture was stirred for

1 h at 0 °C and 14 h at room temperature; then the solution was diluted with EtOAc (100 mL) and worked up as described in (B).

C. Trifluoroacetic acid deprotection. Boc group was removed by treating the peptide with aqueous 90% TFA (1:10, w/v) containing anisole (0.2 mL) for 30–40 min. The solvent was evaporated in vacuo at 0 °C and the residue was triturated with Et_2O; the resulting solid peptide was collected and dried.

6.2.2. Preparation of tripeptides **2a** *and* **2b**

According to the general coupling procedure (A), L-2,3-diamino propanoic acid or L-2,4-diamino butanoic acid (2 mmol) was treated with Boc-Phe-OSu (4 mmol). Crude compounds **2a** and **2b** were crystallised from MeOH-Et_2O (70–75%). The tripeptides gave correct amino acid analysis and their physicochemical properties are reported in *table I.*

6.2.3. Preparation of pseudotripeptides **3a** *and* **3b**

To a stirred solution of tripeptide **2a** or **2b** (1 mmol) in THF (10 mL, distilled over CaH_2) at −15 °C, NMM (0.11 mL, 1 mmol) was added, followed by isobutylchloroformate (0.136 mL, 1 mmol). After 20 min the precipitated NMM hydrochloride was removed by filtration, washed with cold THF (3 × 0.5 mL) and the filtrate was added dropwise to a cold (−10 °C) solution of $NaBH_4$ (0.113 g, 3 mmol) in EtOH (2 mL). The solution was stirred for 10 min at 0 °C and for an additional 15 min at room temperature and then evaporated under reduced pressure. The residue was extracted with EtOAc (35 mL) and washed with 0.5 N $KHSO_4$, brine, 5% $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated. The resulting crude alcohols were purified by HPLC (80–83%).

3a: ^{1}H-NMR ($CDCl_3$) δ 8.41 (d, 2H, J = 8.18 Hz), 7.48–7.24 (m, 10H), 5.91 (d, 1H, J = 7.81 Hz), 5.87 (d, 1H), 5.69 (t, 1H), 4.67 (m, 2H), 3.88–3.72 (m, 2H), 3.61 (m, 1H), 3.43 (m, 2H), 3.31–3.22 (m, 4H), 1.48 (s, 9H), 1.45 (s, 9H). Anal. $C_{31}H_{44}N_4O_7$: C, 63.68; H, 7.58; N, 9.58. Found: C, 63.51; H, 7.62; N, 9.37.

3b: ^{1}H-NMR ($CDCl_3$) δ 8.51–8.30 (dd, 2H), 7.57–7.26 (m, 10H), 6.01 (d, 1H, J = 7.78 Hz), 5.85 (d, 1H), 5.61 (t, 1H), 4.75 (m, 2H), 3.91–3.80 (m, 2H), 3.60 (m, 1H), 3.48 (m, 2H), 3.20–3.04 (m, 4H), 1.52 (s, 9H), 1.47 (s, 9H), 1.28 (m, 2H). Anal. $C_{32}H_{46}N_4O_7$: C, 64.19; H, 7.74; N, 9.36. Found: C, 63.98; H, 7.86; N, 9.41.

Characterisation of **3a** and **3b** are summarised in *table I.*

6.2.4. General preparation of pseudotripeptides **5a** *and* **5b**, **6a** *and* **6b**

According to deprotection procedure C, **3a** and **3b** were treated with TFA. The resulting deprotected **4a** and **4b** were recrystallised from MeOH-Et_2O (89%) and, according to coupling procedure A, were treated with Z-OSu or Fmoc-OSu (two-fold excess) to lead to the corresponding Z- or Fmoc-title pseudopeptides (65–70%). All compounds gave correct elemental analysis. Characterisation of **5a** and **5b**, **6a** and **6b** are summarised in *table I.*

6.2.5. Preparation of pseudotripeptides **7a–9a**

According to general procedure B, **4a** (1 mmol) was treated with the appropriate arylcarboxylic acid (2 mmol). Crude bis-acyl derivatives **7a–9a** were purified by HPLC (70–75%). All pseudopeptides gave correct elemental analysis and expected ^{1}H-NMR spectra. The physicochemical properties of **7a–9a** are reported in *table I.*

6.2.6. Test for the inhibition of HIV-1 protease

For determination of IC_{50} values, affinity-purified HIV-1 protease (Bachem Bioscience), 1.1 nM final concentration, was added to a solution (100 mL final volume) containing inhibitor, 4 mM peptide substrate (His-Lys-Ala-Arg-Val-Leu-p-nitro-Phe-Glu-Ala-Nle-Ser, Bachem Bioscience), and 1.0% DMSO in assay buffer: 1.0 mM dithiothreitol, 0.1% glycerol, 80 mM sodium acetate, 160 mM sodium chloride, 1.0 mM EDTA, all at pH 4.7. The solution was mixed and incubated for 25 min at 37 °C and the reaction quenched by the addition of trifluoroacetic acid, 2% final concentration. The Leu-Phe(p-NO_2) bond of the substrate was cleaved by the enzyme. The cleavage products and substrate were separated by reversed-phase HPLC. Absorbance was measured at 220 nm, peak areas were determined, and percent conversion to product was calculated using relative peak areas. The data were plotted as percent control (the ratio of percent conversion in the presence and absence of inhibitor) vs. inhibitor concentration and fit with the equation $Y = 100/1 + (x/IC_{50})^A$, where IC_{50} is the inhibitor concentration at 50% inhibition and A is the slope of the inhibition curve. A norstatin-peptidic HIV-1 protease inhibitor (KNI-272) [17] was used as a standard, yielding an average IC_{50} = 4.8 nM in this assay. Inhibitor concentrations of 0.1, 1 and 10 mM were initially evaluated to aid in choosing the concentrations of inhibitor used for determination of the IC_{50}.

6.2.7. Cell culture activity against HIV-1 IIIB

HIV-1 IIIB was obtained from HIV-1 IIIB chronically infected Molt-4 cells as a supernatant fluid. The 50% tissue culture infection dose (TC ID_{50}) was determined by an endpoint titration procedure [24]. CEM cells (5 000/mL) were exposed to HIV-1 IIIB fluid at a multiplicity of infection (m.o.i.) 0.001 TC ID_{50} (mL).

Aliquots (0.2 mL) of cells were placed in 96 well microtitre plates with 2 mL of the appropriate concentrations of inhibitors dissolved in DMSO. After incubation for 6 d in RPMI-1640 medium containing 10% foetal calf serum, the p24 antigen of HIV in the supernatant was determined by an ELISA assay kit (RETRO-TEK, Cellular Products Inc., Buffalo, USA). The ED_{50} values were calculated as the dose of the inhibitor that resulted in a 50% reduction in p24 levels as compared to those in control wells.

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