PEPTIDE INHIBITORS OF ASPARTIC PROTEINASES WITH HYDROXYETHYLENE ISOSTERE REPLACEMENT OF PEPTIDE BOND. II. PREPARATION OF PSEUDOTETRAPEPTIDES DERIVED FROM DIASTEREOISOMERIC 5-AMINO-2-BENZYL-4-HYDROXY-6-PHENYLHEXANOIC ACIDS

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Received January 29, 1998
Accepted March 18, 1998

Twelve pseudotetrapeptides, Boc-NHCH(CH_2Ph)CH(OH)CH_2CH(CH_2Ph) CO-Xaa-Phe-NH_2 **9–11**, were prepared by [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate-mediated couplings of diastereoisomeric *O*-silylated (2*R* or 2*S*,4*R* or 4*S*,5*S*)-2-benzyl-5-(*tert*-butoxycarbonyl)amino-4-hydroxy-6-phenylhexanoic acids **1** with dipeptides H-Xaa-Phe-NH_2 (Xaa = Gln, Glu(OBzl) or Ile) **3–5**, followed by *O*-deprotection. Pseudotetrapeptides **9–11** were tested for inhibition of aspartic proteinases secreted by *Candida albicans* and *C. tropicalis*. The level of inhibition of both yeast proteinases was very low, contrasting with the nanomolar IC_{50} values obtained for inhibition of HIV-1 proteinase.

Key words: *Candida* sp. proteinases; HIV-1 proteinase; Aspartic proteinase inhibitors; Pseudopeptides; Peptidomimetics.

In the course of our work on *Candida* sp.-secreted aspartic proteinases, SAPs****, (ref.[1] and references cited therein) we sought low-molecular-weight peptide inhibitors

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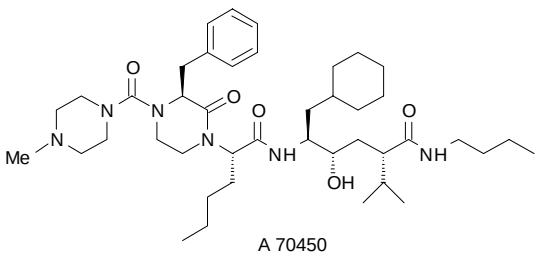
**** Abbreviations: AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; BOP, [(benzotriazol-1-yl)oxy]-tris(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; CAAP, *C. albicans* aspartic proteinase; CTAP, *C. tropicalis* aspartic proteinase; EDTA, ethylenediaminotetraacetic acid; HIV-1 P, human immunodefficiency virus type 1 proteinase; Nph, 4-nitrophenylalanine; SAP, secreted aspartic proteinase; TBDMS, *tert*-butyldimethylsilyl; TFA, trifluoroacetic acid. The nomenclature of amino acids and peptides is in agreement with IUPAC–IUB recomendations (*Eur. J. Biochem*. **1984**, *138*, 9). All amino acids used were of L-configuration.

with small number of peptide bonds (suppression of proteolysis) and adjusted lipophilicity (improved transport over membranes). Recently, we examined[1] the effect of structural changes within a limited series of aminomethylene, hydroxyethylamine, statine and norstatine incorporating pseudopeptides on their potency to inhibit purified SAPs. For hydroxyethylamine, statine and norstatine inhibitors we found pronounced dependence of inhibition on the hydroxy group stereochemistry in P1–P1′ position of an isostere. Similar results were obtained earlier for other proteinases, notably human renin (see, *e.g.*, ref.[2]) and HIV-1 proteinase[3]. One of the most studied groups of proteinase inhibitors is based on the hydroxyethylene isostere (see, *e.g.*, ref.[4]). However, only a single compound of this class, A-70450 (Abbot), was tested[5] for inhibition of *C. albicans* SAP. This compound, though tightly bound to the isolated enzyme, was inactive in *in vivo* experiments. We decided to prepare a more representative set of hydroxyethylene



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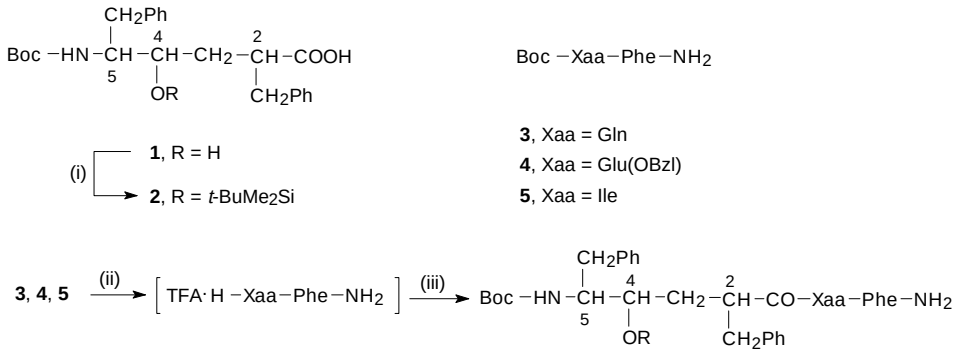
inhibitors and to test them on purified CAAP and CTAP. The selection of the isosteres was based on the observation that *Candida* sp. SAPs prefer peptide substrates with lipophilic side chains in P1–P1′ positions[6] and that amino acid sequences Gln-Phe, Glu-Phe or Ile-Phe formed *C*-termini of short nanomolar inhibitors of HIV-1 and HIV-2 aspartic proteinases[7]. Recently, we described[8] the synthesis of four diastereoisomeric 5-amino-2-benzyl-4-hydroxy-6-phenylhexanoic acids **1a–1d** which we considered as a good replacement for the cleavable Phe-Phe peptide bond in aspartic proteinases inhibitors[9]. The selected *N*-terminal Boc group is usually well accommodated by spatious S2 aspartic proteinases subsites and *C*-terminal amido group offers flexibility for future introduction of divergent nitrogen substituents.

Synthesis of pseudotetrapeptides was accomplished in three steps. Dipeptides **3–5** were prepared by a conventional solution phase method[10] with orthogonal Boc/Bzl protection. Removal of the Boc group was performed by trifluoroacetic acid. Coupling of deprotected peptides with a 2-benzyl-5-[(*tert*-butoxycarbonyl)amino]-4-hydroxy-6-phenylhexanoic acid was reported as troublesome[11]. In our hands, treatment of the acid **1d** with dicyclohexylcarbodiimide or BOP in the presence of H-Ile-Phe-NH_2 in DMF afforded (2*S*,4*R*)-2-benzyl-5-{(1*S*)-1-[(*tert*-butoxycarbonyl)amino]-2-phenylethyl}butyrolactone in quantitative yield, but not the required pseudotetrapeptide **11d**.

The cyclization of hydroxy acids **1** was prevented by protecting the hydroxy groups with *tert*-butyldimethylsilyl function, as described previously[11] (Scheme 1). The silylation reaction was sluggish, even in the presence of a large excess of silylating reagent (*tert*-butyldimethylsilyl chloride and imidazole), but the yields and purity of *O*-protected acids **2a–2d** were excellent. In the next step we coupled silyloxy acids **2a–2d** with *N*-deacylated dipeptides **3–5** to *N*,*O*-protected pseudotetrapeptides **6–8**. Side-chain deprotection of Glu(OBzl)-containing peptides **7a–7d** was achieved by Pd-catalyzed hydrogenolysis. Final removal of silyl groups was performed by treatment with tetrabutylammonium fluoride; this reaction was very slow (3–10 days), controlling, in fact, total yields of coupling reaction, deprotection steps and preparative reversed phase HPLC (see Table I).



In formulae **1**, **2**, **6-11**:

	Configuration		
	C-2	C-4	C-5
a	*S*	*S*	*S*
b	*R*	*R*	*S*
c	*S*	*R*	*S*
d	*R*	*S*	*S*

	R	Xaa
6	*t*-$BuMe_2Si$	Gln
7	*t*-$BuMe_2Si$	Glu(OBzl)
8	*t*-$BuMe_2Si$	Ile

	R	Xaa
9	H	Gln
10	H	Glu
11	H	Ile

(i) *t*-$BuMe_2SiCl$, imidazole, DMF; (ii) TFA, CH_2Cl_2; (iii) **2**, BOP, *N*-methylmorpholine; (iv) for **6** only: H_2, Pd, MeOH; (v) $Bu_4N^+F^-$

SCHEME 1

Synthesized pseudotetrapeptides were examined for *C. albicans* and *C. tropicalis* aspartic proteinases inhibition and, in a preliminary test, for their ability to inhibit proteinase from human immunodeficiency virus-1. In a spectrophotometric assay, these compounds inhibit both *Candida* proteinases only weakly, exhibiting IC_{50} in the micromolar range. On the other hand, they are potent inhibitors of the HIV-1 proteinase[9] (for

original IC_{50} values see Table II). It is difficult to speculate on the reason for this discrepancy without additional structural information. However, the results shown in Table II indicate that the active cleft specificity requirements of both *Candida* proteases differ significantly from those of the HIV-1 P. Introduction of Ile into the the P2′ position of the inhibitors **11a–11d** (compound **11d** was described previously by a Merck

TABLE I
Analytical data for inhibitors Boc-NHCH(CH_2Ph)CH(OH)CH_2CH(CH_2Ph)CO-Xaa-Phe-NH_2 **9–11**

Compound	Configuration			Xaa	Yield[a] %	R_t, min[b]	HR-FAB MS, *m/z* calculated (found)[c]
	C-2	C-4	C-5				
9a	*S*	*S*	*S*	Gln	57	15.8	687.3636 (688.3790)
9b	*R*	*R*	*S*	Gln	23	15.7	687.3636 (688.3627)
9c	*S*	*R*	*S*	Gln	53	15.5	687.3636 (688.3434)
9d	*R*	*S*	*S*	Gln	38	16.0	687.3636 (688.3515)
10a	*S*	*S*	*S*	Glu	27	16.5	688.3472 (689.3552)
10b	*R*	*R*	*S*	Glu	43	16.4	688.3472 (689.3691)
10c	*S*	*R*	*S*	Glu	36	16.2	688.3472 (689.3673)
10d	*R*	*S*	*S*	Glu	29	15.9	688.3472 (689.3647)
11a	*S*	*S*	*S*	Ile	23	20.0	672.3887 (673.3995)
11b	*R*	*R*	*S*	Ile	9	20.0	672.3887 (673.3974)
11c	*S*	*R*	*S*	Ile	18	21.1	672.3887 (673.3907)
11d	*R*	*S*	*S*	Ile	76	20.4	672.3887 (673.3989)

[a] Total yield for following steps: coupling, deprotection and HPLC purification. [b] Retention time on a Vydac analytical column (see Experimental). [c] [M + H].

TABLE II
CAAP, CTAP and HIV-1 P inhibition by pseudotetrapeptides **9–11**

Enzyme	IC_{50}, nM											
	9a	**9b**	**9c**	**9d**	**10a**	**10b**	**10c**	**10d**	**11a**	**11b**	**11c**	**11d**
CAAP	[a]	[a]	$28 . 10^3$	[a]	[a]	[a]	$14 . 10^3$	[a]	[a]	[a]	[a]	[a]
CTAP	[a]	[a]	$39 . 10^3$	[a]	$41 . 10^3$	[a]	$15 . 10^3$	[a]	[a]	[a]	[a]	[a]
HIV-1 P	0.8	54	36	<0.1	0.3	1.1	0.4	<0.1	1.4	2.7	3.5	0.4

[a] >100 000.

group[12]) leads to inactive compounds. For Gln or Glu in the same position, a weak activity was observed for compounds **9c** and **10c**. Interestingly, both of them have *S*,*R*,*S* configurations of asymmetric carbons C-2, C-4 and C-5, respectively (Table I). These combinations of configurations have been shown to yield the weakest inhibitors of HIV-1 P of the whole presented series (Table II).

EXPERIMENTAL

Methods and Materials

Melting points were determined on a micro melting point apparatus Boetius. The temperature data are uncorrected. ^{1}H NMR spectra (δ, ppm; *J*, Hz) were measured on a Varian Unity 500 spectrometer at 499.8 MHz, ^{13}C NMR spectra at 125.7 MHz, both in deuteriochloroform. Tetramethylsilane was used as an internal standard. Optical rotations were determined on a Perkin–Elmer 141 MC or 241 polarimeter at 20–25 °C in a 10 cm cell. IR spectra were measured in chloroform (unless stated otherwise) on a Bruker IFS 88 spectrometer (wavenumbers in cm^{-1}). Mass spectra were recorded on a ZAB-EQ (VG Analytical) instrument using the FAB (Xe, 8 kV) techniques. Enzyme kinetics were measured on an Aminco 2000 DW spectrophotometer.

Thin-layer chromatography was performed on silica gel plates (Silica gel 60 G*, Merck) in chloroform–methanol (19 : 1). Spots were detected by spraying with 2% solution of $Ce(SO_4)_2$ in 1 M sulfuric acid, followed by pyrolysis or with 1% ninhydrine in ethanol and subsequent heating to 110 °C.

Analytical HPLC was performed on a Spectra Physics instrument with a Vydac 2128TP54 column, 250 × 0.46 cm, flow rate 1 ml/min, linear gradient from 100% phase A (0.05% TFA in 50% aqueous MeOH) to 100% phase B (0.05% TFA in MeOH) in 30 min, with UV detector setting at 222 nm. Preparative HPLC was performed on a Vydac 218TP510 column, 25 × 1.0 cm, flow rate 3 ml/min with a linear gradient from 0.05% TFA in H_2O to 0.05% TFA in 70% aqueous MeOH in 70 min with UV detector setting at 225 nm.

Preparation of Silylated Hexanoic Acids **2**. General Procedure

Hexanoic acid **1** (0.30 g, 0.73 mmol) and imidazole (1.84g, 27.01 mmol) were dissolved in dry DMF (5 ml) under argon. *tert*-Butyl(dimethyl)silyl chloride (1.97 g, 13.14 mmol) was added and the mixture was kept at room temperature for 4 days. Excess of the silyl chloride and silyl ester of acid **2** were solvolyzed by 1 h treatment with methanol (4 ml) at ambient temperature. Aqueous citric acid (50 ml) was added and the product was extracted with diethyl ether (3 × 25 ml). Combined extracts were washed with water and once with brine, dried ($MgSO_4$) and concentrated. Crude product (1.13 g) was purified by flash silica gel chromatography (0 to 3 % of methanol in dichloromethane).

(2S,4S,5S)-2-Benzyl-5-[(tert-butoxycarbonyl)amino]-4-tert-butyldimethylsilyloxy-6-phenylhexanoic acid **2a** was obtained in 90% yield as an oil, R_F 0.49. FAB MS, *m/z* (rel.%): 550 (M + Na, 8), 528 (M + H, 8), 428 (79), 278 (100). IR spectrum: 1 457, 1 600 (arom.), 1 707 (C=O carbamate and COOH dimer), 1 745 (COOH monomer), 3 442 (NH), 3 523 (OH, COOH monomer).

(2R,4S,5S)-2-Benzyl-5-[(tert-butoxycarbonyl)amino]-4-tert-butyldimethylsilyloxy-6-phenylhexanoic acid **2d** was obtained in 87% yield as an oil, R_F 0.43. FAB MS, *m/z* (rel.%): 528 (M + H, 16), 472 (6), 428 (100), 414 (4), 340 (8), 296 (12), 279 (100). IR spectrum: 1 455, 1 604 (arom.), 1 710 (C=O carbamate and COOH dimer), 1 746 (COOH monomer), 3 445 (NH), 3 512 (OH, COOH monomer).

(2S,4R,5S)-2-Benzyl-5-[(tert-butoxycarbonyl)amino]-4-tert-butyldimethylsilyloxy-6-phenylhexanoic acid **2c** was obtained in 91% yield as an oil, R_F 0.43. FAB MS, *m/z* (rel.%): 550 (M + Na, 10), 528 (M + H, 40), 472 (10), 428 (100), 414 (12), 340 (29), 296 (24), 278 (100). IR spectrum: 1 455, 1 603 (arom.), 1 708 (C=O carbamate and COOH dimer), 1 750 (COOH monomer), 2 856 (OH, COOH dimer), 3 444 (NH), 3 520 (OH, COOH monomer). ^{1}H NMR spectrum: –0.01 s, 6 H $(CH_3)_2Si$); 0.87 s, 9 H $(CH_3)_3CSi$); 1.32 s, 9 H $(CH_3)_3CO$; 1.60 ddd, 1 H, *J*(3a,2) = 3.7, *J*(3a,4) = 7.7, *J*(3a,3b) = 13.8 (H-3a); 1.96 ddd, 1 H, *J*(3b,4) = 4.4, *J*(3b,2) = 10.0, *J*(3a,3b) = 13.8 (H-3b); 2.63 dd, 1 H, *J*(6a,5) = 8.8, *J*(6a,6b) = 13.3 (H-6a); 2.83 dd, 1 H, *J*(6b,5) = 5.1, *J*(6a,6b) = 13.3 (H-6b); 2.80 bdd, 1 H, *J*(11a,2) = 7.0, *J*(11a,11b) = 13.5 (H-11a); 2.92 ddt, 1 H, *J*(2,3a) = 3.7, *J*(2,11a) = 7.0, *J*(2,11b) = 7.4, *J*(2,3b) = 10.0 (H-2); 3.03 dd, 1 H, *J*(11b,2) = 7.4, *J*(11a,11b) = 13.5 (H-11b); 3.80 m, 1 H (H-4); 3.86 m, 1 H (H-5); 4.45 bd, 1 H, *J*(NH,5) = 8.0 (NH); 7.14–7.30 m, 10 H (2 × arom.). ^{13}C NMR spectrum: –4.59 q ($(CH_3)_2Si$); 18.07 s ($(CH_3)_3$**C**Si); 25.89 q (($\mathbf{C}H_3)_3CSi$); 28.26 q (($\mathbf{C}H_3)_3CO$); 29.69 t (C-3); 35.40 t (C-6); 38.97 t (C-11); 43.12 d (C-2); (C-5); 72.20 d (C-4); 79.04 s ($(CH_3)_3$**C**O); 126.22 d (arom.); 126.60 d (arom.); 128.39 d (arom.); 128.52 d (arom.); 129.00 d (arom.); 129.21 d (arom.); 138.50 s (arom.); 138.50 s (arom.); ($(CH_3)_3$CO**C**=O) and (C-1) signals disappeared in noise.

(2R,4R,5S)-2-Benzyl-5-[(tert-butoxycarbonyl)amino]-4-tert-butyldimethylsilyloxy-6-phenylhexanoic acid **2b** was obtained in 92% yield as an oil, R_F 0.39. FAB MS, *m/z* (rel.%): 528 (M + H, 16), 472 (6), 428 (77), 340 (26), 296 (16), 278 (13). IR spectrum: 1 454, 1 603 (arom.), 1 709 (C=O carbamate and COOH dimer), 1 760 (COOH monomer), 3 450 (NH), 3 520 (OH, COOH monomer).

Synthesis of Boc-Dipeptides **3–5**. General Procedure

Phenylalanine amide (0.93 g, 5.67 mmol), dissolved in DMF (5 ml), was added at room temperature to a solution of appropriate Boc-amino acid (5.15 mmol), BOP (2.50 g, 5.67 mmol) and *N*-methylmorpholine (0.70 ml, 6.24 mmol) in DMF (10 ml). After 3 h the solvent was evaporated (30 °C, 13 Pa), the residue was dissolved in ethyl acetate, extracted with cold 1 M $KHSO_4$, 1 M $NaHCO_3$ and brine. Ethyl acetate solution was dried ($MgSO_4$) and evaporated. For analysis, the dipeptide was crystallized from ethyl acetate–hexane.

Boc-Gln-Phe-NH$_2$ (**3**): Yield 66%, m.p. 183–187 °C, $[\alpha]_D$ –24.3° (*c* 1.14, MeOH). FAB MS, *m/z*: 393 [M + H]. For $C_{19}H_{28}N_4O_5$ (392.5) calculated: 58.14% C, 7.19%, 14.27% N; found: 57.90% C, 7.19% H, 13.82% N.

Boc-Glu(OBzl)-Phe-NH$_2$ (**4**): Yield 91%, m.p. 135–138 °C, $[\alpha]_D$ –20.8° (*c* 0.85, MeOH). FAB MS, *m/z*: 506 [M + Na], 484 [M + H]. For $C_{26}H_{33}N_3O_6$ (483.6) calculated: 64.58% C, 6.88% H, 8.69% N; found: 63.97% C, 6.55% H, 8.63% N.

Boc-Ile-Phe-NH$_2$ (**5**): Yield 94%, m.p. 179–183 °C, $[\alpha]_D$ –38.6° (*c* 1.1, MeOH). FAB MS, *m/z*: 378 [M + H]. For $C_{20}H_{31}N_3O_4$ (377.5) calculated: 63.64% C, 8.27% H, 11.13% N; found: 63.94% C, 8.29% H, 11.10% N.

Synthesis of Silylated Diastereoisomeric Pseudotetrapeptides **6–8**. General Procedure

A) *Boc-Deprotection of dipeptides* **3–5**. A Boc-dipeptide **7** (0.1 mmol) was dissolved in dichloromethane (1 ml) and TFA (3 ml) was added at room temperature. After 30 min dichloromethane and TFA were evaporated *in vacuo*, the residue was again dissolved in dichloromethane and evaporated. White semicrystalline solid was triturated with dry diethyl ether, filtered and dried over KOH in a desiccator. Yields of dipeptide trifluotoacetate salts were 86–92%.

B) Coupling of silyloxy acids **2** *with TFA·dipeptides*. Silyloxy acid **2** (21.3 mg, 0.04 mmol) was dissolved in DMF (2 ml) and BOP was added (20 mg, 0.044 mmol). Trifluoroacetate salt of H-Gln-Phe-NH_2 (0.06 mmol) (or TFA·Glu(OBzl)-Phe-NH_2 or TFA·Ile-Phe-NH_2) was dissolved in a mixture

of DMF (2 ml) and *N*-methylmorpholine (72 ml, 0.07 mmol). Both solutions were combined and the reaction mixture was kept at ambient temperature for 3 h. The solvents were evaporated to dryness in high vacuum, the residue was dissolved in ethyl acetate (5 ml) and washed with 10% aqueous citric acid, 10% $NaHCO_3$ and brine. The solution was dried ($MgSO_4$) and evaporated. All silylated pseudotetrapeptides were oils, with the exception of **6a** which crystallized on standing. Yield 87%, m.p. 109–113 °C. FAB MS, *m/z* (rel.%): 824 (22) [M + Na], 802 (70) [M + H], 702 (100), 655 (22).

Crude compounds **6–8**, obtained in 84 to 99% yields were used in the next step without any purification.

Preparation of Diastereoisomeric Tetrapeptides **9–11**. General Procedure

A) O-Debenzylation of peptides **7**. Protected isosteric tetrapeptide Boc-Phe[CH(OTBDMS)-CH_2]Phe-Glu(OBzl)-Phe-NH_2 **7** (69 mg, 0.08 mmol) was dissolved in MeOH (1 ml) and added to a stirred suspension of Pd-sponge (≈50 mg) in methanol (2 ml). The mixture was deoxygenated with a stream of nitrogen (15 min) and hydrogen was then passed through the suspension. When deprotection was complete (HPLC), Pd was filtered off and the filtrate was evaporated *in vacuo*. Debenzylated silylated pseudopeptides were used in the following step without any purification.

B) O-Desilylation of peptides **6–8**. To a silylated pseudotetrapeptide (25 mg, 0.034 mmol) dissolved in THF (3 ml), 0.5 M tetrabutylammonium fluoride in tetrahydrofurane (1.7 ml, 0.85 mmol) was added at room temperature. Progress of desilylation was followed by TLC (12% MeOH in $CHCl_3$). After the silylated peptide disappeared (3–10 days), THF was evaporated and the residue was mixed with 10% aqueous citric acid (2 ml). The yellowish precipitate was centrifuged, washed twice with water, dissolved in a small volume of aqueous methanol and purified by preparative HPLC. Fractions containing the pseudopeptide were combined, the solvents were removed *in vacuo*, the residue was dissolved in 10% AcOH and lyophilized. Purity of all pseudopeptides was checked by analytical HPLC and their identity was confirmed by HR-FAB MS (see Table I).

Cultivation

C. albicans and *C. tropicalis* were grown in 1.2% yeast carbon base medium containing 0.4% bovine serum albumin. pH of the medium was adjusted to 4.0. After three days of cultivation at 30 °C with shaking at 200 min^{-1} cells were harvested and supernatant containing SAP was used for isolation of CAAP and CTAP.

Isolation of Proteinases

A) CAAP and CTAP were isolated from above supernatant using an anion exchange chromatography on DEAE-Sephadex A25 as described by Fusek *et al.*[6].

B) Recombinant HIV-1 P was obtained in an adapted T7 RNA polymerase/promoter system as described previously[13]. Briefly, HIV-1 P was isolated from inclusion bodies formed in the host *Escherichia coli* strain by solubilization in urea followed by chromatography on QAE-Sephadex and SP-Sephadex.

Inhibition Studies

A) IC_{50} values for inhibition of CAAP and CTAP were determined by a spectrophotometric assay at 305 nm with a chromogenic substrate[14], Lys-Pro-Ala-Glu-Phe-Nph-Ala-Leu. In a standard experiment, 1.5 nmol of SAP was incubated in 0.1 M sodium acetate buffer (pH 3.3) with 40 μmol of

substrate and various concentrations of an inhibitor dissolved in DMSO. Final concentration of DMSO in the assay was lower than 2.5%. IC_{50} values were obtained from Dixon plots.

B) IC_{50} values for inhibition of HIV-1 P were obtained as described[9]. Typically, 8 pmol of HIV-1 P was added to 20 nmol of chromogenic substrate Lys-Ala-Arg-Val-Ahx-Nph-Glu-Ala-Ahx-NH_2 in a buffer (pH 4.7) which was 0.1 M in sodium acetate, 0.3 M in NaCl and 4 mM in EDTA and the substrate cleavage was followed spectrophotometrically in the presence of various concentrations of inhibitors. IC_{50} values were obtained from Dixon plots.

Financial support of this research by Grant Agency of the Czech Republic (Grants No. 205/95/1028 and No. 303/96/1235) and by International Scholar's Award, Howard Hughes Medical Institute (HHMI 75195-540801 to J. K.) is gratefully acknowledged. The authors thank Dr K. Ubik for mass spectral measurements and Analytical Department of this Institute for elemental analyses.

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