NOVEL RETROVIRUS PROTEASE INHIBITORS, RPI-856 A, B, C AND D, PRODUCED BY *Streptomyces* sp. AL-322

TSUNEO ASANO†, KUNIO MATSUOKA, TSUNEAKI HIDA, MAKOTO KOBAYASHI, YUMIKO KITAMURA, TAKAKI HAYAKAWA††, SHIGEMI IINUMA, ATSUSHI KAKINUMA††,††† and KOICHI KATO

Discovery Research Division and
†† Pharmaceutical Research Division, Pharmaceutical Group, Takeda Chemical Industries, Ltd., 2-17-85 Jusohonmachi, Yodogawa-ku, Osaka 532, Japan

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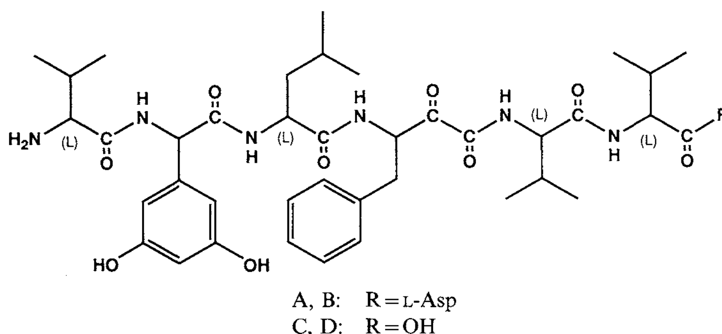
Four kinds of retrovirus protease inhibitors (RPI-856 A, B, C and D) were isolated as white powder from the culture filtrate of a soil isolate, *Streptomyces* sp. AL-322 by column chromatography using Diaion HP-20, Sephadex LH-20, ODS reversed phase HPLC and SP-2SW ion exchange HPLC. The structures of these inhibitors were elucidated by physico-chemical properties, chemical reactions and spectral analyses, as valyl-ADPAA-leucyl-AOPBA-valyl-valyl-aspartic acid (RPI-856 A and B) and valyl-ADPAA-leucyl-AOPBA-valyl-valine (RPI-856 C and D) [ADPAA = 2-amino-2-(3,5-dihydroxyphenyl)acetic acid, AOPBA = 3-amino-2-oxo-4-phenylbutyric acid]. RPI-856 A and B, and RPI-856 C and D were both determined to be diastereomers each other on the asymmetric carbon in AOPBA. These four inhibitors strongly inhibited *in vitro* HIV-1 protease and HTLV-I protease both derived from recombinant *Escherichia coli* with IC_{50} of $10^{-7} \sim 10^{-8}$ M.

The human immunodeficiency virus (HIV) encodes a protease which proteolytically processes the precursor *gag* and *gag-pol* polyprotein to form mature proteins essential for production of infectious viral particles. For this reason, attempts to develop agents that inhibit this protease have been extensively made for the treatment of acquired immunodeficiency syndrome (AIDS) and related diseases[1~9].

In the previous paper, we reported purification of enzymatically active HTLV-I protease expressed in recombinant *Escherichia coli* and charactarization of the purified enzyme[10]. We also expressed HIV-1 protease in recombinant *E. coli*. In the present work we screened various microbial metabolites in search

Fig. 1. Structures of RPI-856 A, B, C and D.



A, B: R = L-Asp
C, D: R = OH

† Corresponding author: T. ASANO, Discovery Research Division, Takeda Chemical Industries, Ltd., 10 Wadai, Tsukuba 300-42, Japan.

††† Present address: Department of Applied Biological Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-01, Japan

of HIV-1 and HTLV-I protease inhibitors and found strong inhibitory activity in the culture medium of a soil isolate, *Streptomyces* sp. AL-322. The active substances isolated and named as RPI-856 A, B, C and D (Fig. 1) were shown to be potent inhibitors of both HIV-1 and HTLV-I proteases *in vitro*.

In this paper, we describe the taxonomy of the producing strain, the purification, the structure and protease inhibitory activities of RPI-856 A, B, C and D.

Materials and Methods

Materials

Recombinant HIV-1 protease was expressed as a larger precursor in *E. coli* MM294 (DE3)/pHIV7004 and autoprocessed to form a mature molecule. The protease was partially purified on S-Sepharose FF and Sephacryl S-100 HR columns as described by STRICKLER *et al.*[11]. Recombinant HTLV-I protease was expressed and partially purified from *E. coli* MM294 (DE3)/pHTI7707 as described[10].

Commercially available reagents and their suppliers were; pepsin (porcine), trypsin (bovine), chymotrypsin (bovine), bromelain (pineapple) and carboxypeptidase A (bovine), Boehringer Mannheim; papain (papaya) and carboxypeptidase B (porcine), Worthington Biochemical Co.; cathepsin D (bovine), hippuryl-L-Phe and hippuryl-L-Arg, Sigma Chemical Co.; prolyl endopeptidase (*Flavobacterium meningosepticum*) and Z-Gly-Pro-pNA, Seikagaku Corporation; thermolysin (*Bacillus thermoproteolyticus*) and hemoglobin (bovine), Wako Pure Chemical Industries, Ltd.; chymosin (calf), ICN Chemicals; carboxypeptidase P (*Penicillium janthinellum*), Takara Shuzo Co., Ltd.; $NaBH_3CN$, Aldrich Chemical Co.; casein, E. Merck & Co., Inc.; Diaion HP-20, Mitsubishi Chemical Industries, Ltd.; Sephadex LH-20, Pharmacia LKB Biotechnology; ODS-120T and SP-2SW column, Tosoh Corporation; YMC-Pack FL-ODS column, YMC Co., Ltd.

Taxonomic Studies

The media and procedures used for cultural and physiological characterization of strain AL-322 were those described by SHIRLING and GOTTLIEB[12]. The color names used in these studies were determined by comparing with the color chips of the "Color Harmony Manual 4th Ed. Container Corporation of America, Chicago, 1958". Utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB[13]. The chemical composition of the cell wall was analyzed by the method of BECKER *et al.*[14]. Sugars in the whole cells were analyzed by the method of MIKAMI and ISHIDA[15].

Fermentation

For the production of inhibitors the following media were used: the seed medium consisting of soluble starch 3%, glucose 2%, soy bean flour 1%, corn steep liquor 1%, Polypepton 0.5%, NaCl 0.3% and $CaCO_3$ 0.5%, and the main medium consisting of glucose 0.5%, dextrin 5%, soy bean meal 3.5% and $CaCO_3$ 0.7%. The microorganisms were inoculated to 40 ml of the seed medium contained in a 200-ml shake flask and cultivated for 3 days at 28°C on a rotary shaker. Fifteen milliliters of the culture fluid were inoculated to 500 ml of the seed medium contained in a 2-liter Sakaguchi flask and the flask was shaken for 3 days at 28°C on a reciprocal shaker at 82 rpm. One liter of the resulting culture fluid was inoculated to 120 liters of the main medium contained in a 200-liter fermentor and fermentation was carried out at 28°C for 4 days under agitation of 120 rpm and aeration of 100% v/v/minute.

Isolation of RPI-856 A, B, C and D

The culture filtrate (100 liters) was passed through a column of Diaion HP-20 (15 cm × 60 cm). The column was washed first with water and then with 30% MeOH. Active substances were eluted with 90% MeOH. The active fractions were pooled and concentrated. The concentrate was applied onto a Sephadex LH-20 column (6 × 107 cm) and the column was developed with 90% MeOH. After concentration of the active fraction, the concentrate was applied onto an ODS-120T column equilibrated at pH 7.2. The column was developed with a linear gradient of 18 ~ 39% CH_3CN. Two active fractions, one containing RPI-856 A and B and the other containing RPI-856 C and D, were each desalted on an ODS-120T column. The

fraction containing RPI-856 A and B was passed through an SP-2SW column at pH 2.5, and the column was developed with a linear gradient of 3~50 mM Na_2SO_4. This enabled the separation of RPI-856 A and B. The fraction containing RPI-856 C and D was passed through an SP-2SW column at pH 4.0, and the column was developed with a linear gradient of 8~50 mM Na_2SO_4. This enabled the separation of RPI-856 C and D. Finally, each pure fraction was desalted on an ODS-120T column to give RPI-856 A, B, C and D each as white powder.

Assay for Enzyme Inhibitory Activity

The assays of inhibition of HIV-1 protease and HTLV-I protease were based on hydrolysis of peptide substrates. HIV-1 protease inhibition assay was performed in a total volume of 30 μl. The reaction mixture consisted of 3 μg substrate gag-11 (SQVSQNYPIVQNL) having the *gag* p17/p24 cleavage site, 1.0 M NaCl-10% glycerol-0.175 M sodium phosphate buffer (pH 6.5), partially purified HIV-1 protease and a test sample. Incubation was carried out at 37°C for 3 hours. Peptide cleavage was analyzed by HPLC with a linear gradient of 0~55% CH_3CN on a reversed phase C-18 column (YMC-Pack, FL-ODS, 4.6×30 mm) and the areas of the peaks corresponding to the cleavage products (SQVSQNY) and the residual substrate were used to calculate the percentage of cleavage. HTLV-I protease inhibitory assay was performed similary with a substrate peptide gag-1 (YVEPTAPQVLPVMHP) as described in the previous paper[10]. The inhibitory activities toward cathepsin D, pepsin and chymosin were assayed using hemoglobin[16] as substrate. The inhibitory activity toward prolyl endopeptidase was assayed using *Z*-Gly-Pro-pNA[17]. The inhibitory activity toward other proteases were measured as described in the previous paper[18].

Analytical Instruments

UV and IR spectra were recorded on a Hitachi 220A spectrophotometer and a Hitachi 285 infrared spectrophotometer, respectively. Amino acid analysis was carried out with a Waters PICO TAG amino acid analyzer. SI-MS spectra were measured with a Hitachi M-80A mass spectrometer. NMR spectra were recorded on a Bruker AC-300 instrument (^{1}H, 300 MHz; ^{13}C, 75 MHz). Automatic Edman degradation was carried out with an Applied Biosystems 470A sequencer.

Reduction of RPI-856 A

RPI-856 A (37.5 mg) was dissolved in 15 ml of 50% CH_3CN-0.1% TFA and $NaBH_3CN$ (150 mg) was added, and the mixture was stirred for 90 minutes at 0°C. The reaction mixture was diluted with water and applied onto an ODS-120T column. Two peak fractions were pooled, concentrated and freeze-dried to give **H-1** (9.8 mg) and **H-2** (23.0 mg) each as white powder.

Results and Discussion

Taxonomic Characterization of the Producing Strain

The producing organism, strain AL-322, was isolated from a soil sample collected in Otsu City, Shiga Prefecture, Japan. It produced aerial mycelium, monopodially branched from well-developed substrate mycelia, with *Rectiflexibiles* spore-bearing hyphae. The mature spore chains were generally long with more than 30 spores per chain. The spores were cylindrical (0.6×1.7~2.0 μm) and their surfaces were smooth. Sporangia, zoospores and sclerotia were not observed. Cultural and physiological characteristics of strain AL-322 are shown in Tables 1 and 2, respectively. The aerial mycelium was white to ivory tint and melanoid pigment was not formed on either tyrosine agar or peptone-yeast extract iron agar. The hydrolysate of cell walls of strain AL-322 contained both LL- and *meso*-diaminopimelic acids. Galactose and mannose were detected in the whole cells, but arabinose, xylose and madurose were absent. MK-9(H_6) and MK-9(H_8) as predominant menaquinones were detected in the cell extract. Based on these taxonomic characteristics, strain AL-322 was considered to belong to the genus *Streptomyces*[19,20]. Strain AL-322 has been deposited to Institute for Fermentation, Osaka under the accession No. IFO 15088.

Table 1. Cultural characteristics of strain AL-322.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose - nitrate agar	Poor	None	Light ivory (2ca)	None
Yeast extract - malt extract agar	Moderate, wrinkled, bamboo (2gc) to honey gold (2ic)	None	Light wheat (2ea)	None
Oatmeal agar	Moderate, light ivory (2ca) to pastel orange (4ic)	Poor, white to pearl (3ba)	Light ivory (2ca) to pastel orange (4ic)	None
Inorganic salts - starch agar	Moderate, light ivory (2ca)	Good, white to ivory tint (2cb)	Light wheat (2ea) to honey gold (2ic)	None
Glycerol - asparagine agar	Moderate, light ivory (2ca)	Poor, white to pearl (3ba)	Light ivory (2ca) to honey gold (2ic)	None
Glucose - asparagine agar	Moderate, light ivory (2ca)	Poor, white to pearl (3ba)	Light ivory (2ca) to honey gold (2ic)	None
Peptone - yeast extract - iron agar	Poor, light wheat (2ea)	None	Light wheat (2ea)	None
Tyrosine agar	Moderate, bamboo (2gc)	None	Bright yellow (2la) to honey gold (2ic)	None
Nutrient agar	Poor, light ivory (2ca)	None	Light ivory (2ca)	None

Characterization of the producer was carried out according to the methods described by SHIRLING and GOTTLIEB[12]. Unless otherwise stated, results after cultivation at 28°C for 14 days are described.

Table 2. Physiological characteristics of AL-322.

Range of growth temperature	8～35°C	Utilization of: L-Arabinose	+ +
Range of optimum temperature	25～29°C	D-Xylose	+
Reduction of nitrate	−	D-Glucose	+ +
Liquefaction of gelatin	−	D-Fructose	±
Hydrolysis of starch	+	Sucrose	+
Coagulation of milk	−	Inositol	−
Peptonization of milk	−	L-Rhamnose	−
Production of melanoid pigment	−	Raffinose	+
		D-Mannitol	−

Abbreviations: + +, positive; +, weekly positive; ±, doubtful; −, negative.

Table 3. Physico-chemical properties of RPI-856 A, B, C and D.

	A	B	C	D
Nature	White powder	White powder	White powder	White powder
SI-MS m/z $(M+H)^+$	884	884	769	769
Molecular formula	$C_{43}H_{61}N_7O_{13}$	$C_{43}H_{61}N_7O_{13}$	$C_{39}H_{56}N_6O_{10}$	$C_{39}H_{56}N_6O_{10}$
UV λ_{max} (H_2O)	278 nm	278 nm	278 nm	278 nm
IR (KBr)	1645, 1520 cm^{-1}	1650, 1520 cm^{-1}	1670, 1515 cm^{-1}	1670, 1520 cm^{-1}
Amino acid composition	L-Val, L-Leu, L-Asp	L-Val, L-Leu, L-Asp	L-Val, L-Leu	L-Val, L-Leu
N-terminal amino acid	Val	Val	Val	Val
C-terminal amino acid	Asp	Asp	Val	Val
Rf[a]	0.31	0.31	0.55	0.51

[a] *n*-Butanol - Acetic acid - Water - *n*-Butylacetate, 4 : 1 : 1 : 4. Spots were detected by Fluram and RYDON-SMITH[21] reagents.

Fermentation

The total amount of RPI-856 A, B, C and D produced in the culture filtrate reached about 100 μg/ml as RPI-856 A at day 4.

Isolation and Purification of RPI-856 A, B, C and D

Each RPI-856 component was purified by chromatography using Diaion HP-20 and Sephadex LH-20, ODS reversed phase HPLC and SP-2SW ion exchange HPLC to give white powder.

RPI-856 A and B thus obtained were interconvertible in alkaline solutions. When both the solutions of RPI-856 A and B (pH 9.0) were allowed to stand for 24 hours at 37°C, they reached an equilibrium state (A : B = 4 : 6). The same relationship was also observed between RPI-856 C and D.

Table 4. ^{1}H NMR data for RPI-856 A, B, C and D.

Residue	Position	Chemical shift (δ ppm)			
		A	B	C	D
Val-1	NH_2	—	8.00	—	—
	α	3.75	3.76	3.73	3.69
ADPAA	NH	8.77	8.68	8.77	8.76
	α	5.36	5.36	5.35	5.35
	Ar (2H)	6.32	6.34	6.31	6.31
	Ar	6.18	6.19	6.17	6.16
	OH (2H)	9.31	—	9.27	9.26
Leu	NH	8.33	8.07	8.31	8.26
	α	4.46	4.38	4.45	4.39
	β	1.42	1.26	1.41	1.23
	γ	1.62	1.44	1.61	1.14, 1.39
AOPBA	NH	8.30	8.10	8.30	8.37
	β	5.22	5.17	5.23	5.14
	γ	3.02	3.08	3.01	3.08
		2.84	2.66	2.84	2.60
	Ar (2H)	7.19	7.19	7.19	7.20
	Ar (2H)	7.13	7.12	7.13	7.13
	Ar	7.16	7.17	7.16	7.20
Val-2	NH	8.21	8.16	8.12	8.27
	α	4.24	4.25	4.28	4.26
Val-3	NH	8.13	7.91	8.21	8.15
	α	4.21	4.22	4.11	4.14
Asp	NH	8.23	8.06	—	—
	α	4.49	4.54	—	—
	β	2.66	2.67	—	—
		2.55	2.57	—	—
Val	β	2.00	2.08	2.04	2.05
		2.00	2.02	2.04	2.05
		2.00	2.06	2.04	2.05
Val, Leu	CH_3	0.87	0.77	0.87	0.75
		0.84	0.76	0.83	0.74
		0.81	0.87	0.83	0.89
		0.86	0.87	0.88	0.89
		0.94	0.83	0.90	0.84
		0.84	0.95	0.93	0.92
		0.76	0.85	0.78	0.90
		0.95	0.95	0.94	0.92

^{1}H NMR spectrum recorded at 300 MHz in DMSO-d_6. Chemical shifts measured from TMS as internal reference. ADPAA; 2-amino-2-(3,5-dihydroxyphenyl)acetic acid. AOPBA; 3-amino-2-oxo-4-phenylbutyric acid. Ar; aromatic signal.

Table 5. ^{13}C NMR data for RPI-856 A, B, C and D.

Residue	Position	Chemical shift (δ ppm)			
		A	B	C	D
Val-1	CO	167.1	167.2	167.1	167.3
	α	56.8	57.1	56.8	56.8
ADPAA	CO	168.9	168.9	168.9	168.8
	α	56.1	56.5	56.1	56.2
	Ar (×2)	158.2	158.3	158.2	158.1
	Ar	139.1	139.1	139.1	139.1
	Ar (×2)	105.8	106.1	105.8	105.8
	Ar	101.9	102.3	101.9	101.9
Leu	CO	171.7	171.5	171.7	171.5
	α	50.3	51.0	50.3	50.6
	β	41.4	41.7	41.3	41.6
	γ	23.9	23.9	23.8	23.8
AOPBA	CO	159.7	160.4	159.7	160.1
	α-CO	195.8	195.6	195.8	195.6
	β	54.7	55.3	54.6	55.4
	γ	35.2	35.5	35.2	35.3
	Ar	136.6	137.2	136.5	137.2
	Ar (×2)	128.8	129.0	128.8	129.0
	Ar (×2)	128.2	128.1	128.2	128.1
	Ar	126.5	126.3	126.5	126.3
Val-2	CO	169.7	169.8	170.1	170.1
	α	57.6	58.2	57.3	58.0
Val-3	CO	170.3	170.4	172.5	172.6
	α	57.4	57.6	57.3	57.2
Asp	COOH	172.2	171.9	—	—
	COOH	171.5	171.3	—	—
	α	48.5	48.6	—	—
	β	36.2	36.0	—	—
Val	β	30.8	30.6	30.9	30.6
		30.5	30.6	30.0	30.0
		30.0	30.0	29.6	29.7
Val, Leu	CH_3	23.0	22.8	23.0	22.9
		21.5	21.6	21.5	21.5
		19.1	19.1	19.0	19.0
		19.0	19.0	18.9	19.0
		17.9	18.2	18.0	18.2
		17.9	18.0	17.9	18.0
		17.9	17.9	17.9	17.9
		17.6	17.5	17.6	17.5

^{13}C NMR spectrum recorded at 75 MHz in DMSO-d_6. Chemical shifts measured from TMS as internal reference. ADPAA; 2-amino-2-(3,5-dihydroxyphenyl)-acetic acid. AOPBA; 3-amino-2-oxo-4-phenylbutyric acid. Ar; aromatic signal.

Structural Determination of RPI-856 A, B, C and D

The physico-chemical properties of RPI-856 A, B, C and D are summarized in Table 3. The UV spectra of RPI-856 A, B, C and D showed a strong absorption at 278 nm in water. Their IR spectra showed absorption bands at 1645~1670 and 1515~1520 cm^{-1}, suggesting the presence of peptide bonds. The amino acid analysis indicated the presence of valine, leucine and aspartic acid (3 : 1 : 1) in RPI-856 A and B, and valine and leucine (3 : 1) in RPI-856 C and D. Edman degradation of RPI-856 A, B, C and D all gave valine as N-terminus. Carboxypeptidase P digestion of RPI-856 A and B at 37°C for 4 hours liberated 0.8 mol aspartic acid and 0.05 mol of valine. Digestion of RPI-856 A and B each gave a new peak on SP-2SW ion exchange HPLC. The peak derived from A was identical to RPI-856 C and the peak derived from B was identical to RPI-856 D. Carboxypeptidase P digestion at 37°C for 20 hours of RPI-856 C and D liberated 0.2 mol of valine. These results suggested that the C-terminal amino acids of RPI-856 C and D were both valine and that RPI-856 A and B had an additional aspartic acid residue at the C-terminus of RPI-856 C and D, respectively.

The ^{1}H NMR and ^{13}C NMR spectra of RPI-856 A, B, C and D are shown in Tables 4 and 5. ^{1}H NMR spectra indicated the presence of the 4 partial structures (I~IV) shown below, in addition to the usual amino acid residues. The COLOC spectrum of RPI-856 A gave information on the linkages of these partial structures and the amino acid sequence (Fig. 2). The structures I and II were linked to constitute a 2-amino-2-(3,5-dihydroxyphenyl)acetic acid (ADPAA) residue. On the other hand, the structures III and IV were supposed to constitute a 3-amino-2-oxo-4-phenylbutyric acid (AOPBA) residue with the carbonyl group observed at 195.8 ppm. The presence of this residue was confirmed by reduction of the carbonyl group to yield dihydro compounds (**H-1** and **H-2**), as shown in Fig. 3.

Deletion of the C-terminal aspartic acid residue of RPI-856 A resulted in formation of RPI-856 C. RPI-856 B and D were diastereomers of RPI-856 A and C, respectively, at the β-methine carbon of AOPBA residue.

Some protease inhibitors which contain an unusual amino acid residue having an α-ketone group have been reported. Poststatin produced by a *Streptomyces* sp. inhibits prolyl endopeptidase and contains 3-amino-2-oxo-valeric acid (postin)[22]. A synthetic inhibitor of cysteine protease and serine protease was reported to contain AOPBA[23]. An amino peptidase inhibitor, bestatin, contains dihydro-AOPBA[24] which is produced by reduction of the ketone group of AOPBA. It is interesting that the unusual amino acid, AOPBA, and its derivatives are found in the structures of several protease inhibitors as described

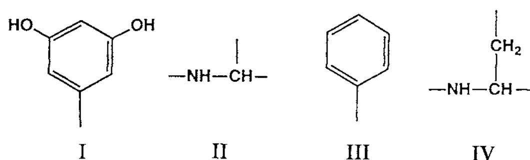


Fig. 2. COLOC experiment of RPI-856 A.

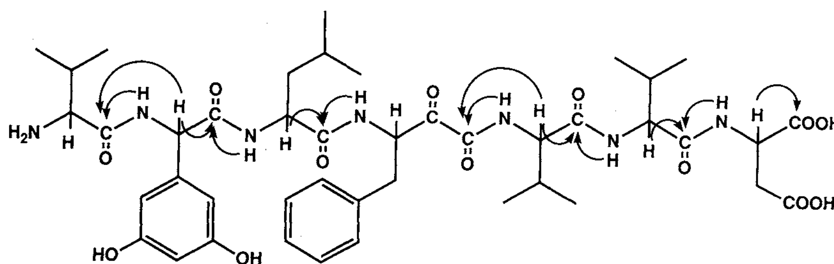
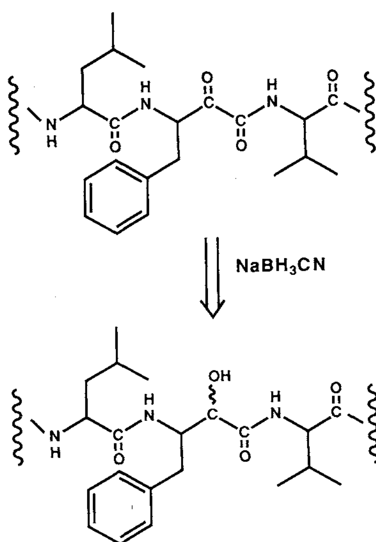


Table 6. Protease inhibition spectra of RPI-856 A, B, C and D.

Protease	(substrate)	IC_{50} (M)			
		A	B	C	D
HIV-1 protease	(gag-11)[a]	3.7×10^{-8}	2.6×10^{-7}	5.5×10^{-8}	2.8×10^{-7}
HTLV-I protease	(gag-1)[b]	2.7×10^{-8}	8.1×10^{-8}	9.2×10^{-8}	2.0×10^{-7}
Cathepsin D	(hemoglobin)	8.8×10^{-7}	8.6×10^{-6}	2.0×10^{-6}	$>10^{-5}$
Pepsin	(hemoglobin)	1.9×10^{-6}	$>10^{-5}$	4.8×10^{-6}	$>10^{-5}$
Chymosin	(hemoglobin)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Trypsin	(casein)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Chymotrypsin	(casein)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Papain	(casein)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Bromelain	(casein)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Thermolysin	(casein)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Prolyl endopeptidase	(Z-Gly-Pro-pNA)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Carboxypeptidase A	(hippuryl-L-Phe)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
Carboxypeptidase B	(hippuryl-L-Arg)	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$

[a] gag-11; a synthetic peptide(SQVSQNYPIVQNL).
[b] gag-1; a synthetic peptide(YVEPTAPQVLPVMHP).

Fig. 3. Reduction of RPI-856 A.



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Protease Inhibition Spectra of RPI-856 A, B, C and D

The effect of RPI-856 A, B, C and D on the proteolytic activity of HIV-1 protease, HTLV-I protease, cathepsin D, pepsin, chymosin, trypsin, chymotrypsin, papain, bromelain, thermolysin, prolyl endopeptidase, carboxypeptidases A and B are summarized in Table 6. RPI-856 A, B, C and D inhibited both HIV-1 protease and HTLV-I protease. It also inhibited, to a lesser extent, aspartic proteases such as cathepsin D and pepsin but did not inhibit other proteases such as trypsin, papain and prolyl endopeptidase. RPI-856 A competitively inhibited both HIV-1 protease and HTLV-I protease and the *Ki* values of RPI-856 A for HIV-1 protease and HTLV-I protease were 1.3×10^{-8} M and 1×10^{-9} M, respectively. These results indicate that RPI-856 A, B, C and D are specific inhibitors for HIV-1 protease and HTLV-I protease.

The dihydro derivatives of RPI-856 A, **H-1** and **H-2**, showed very weak inhibitory activity. Therefore, the α-ketone group of AOPBA in RPI-856 seems to have a critical role for the inhibitory activity.

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