Mer-N5075A, A POTENTIAL HIV-1 PROTEASE INHIBITOR, PRODUCED BY *Streptomyces chromofuscus*

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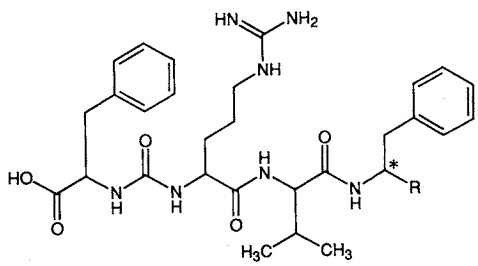
Human immunodeficiency virus type 1 protease (HIV-1 protease) is essential for replication of HIV-1 and has been identified as a potential target for AIDS[1]. During the course of screening for HIV-1 protease inhibitors, Mer-N5075A was discovered in the culture filtrate of a soil-isolated *Streptomyces* and turned out to belong to the MAPI group of compounds (Fig. 1)[2~4]. In this paper, we report the production, isolation, structure determination of Mer-N5075A, and its biological activities.

Mer-N5075A producing organism was isolated from a soil sample collected in Okinawa Prefecture, Japan and was classified as *Streptomyces chromofuscus* Mer-N5075 (FERM P-13134) according to the method of SHIRLING *et al.*[5].

Screening of HIV-1 protease inhibitors was carried out in the following process: HIV-1 protease expressed in *Escherichia coli* KP3998[6] harboring pMAK105 was partially purified with a modification of the method by STRICKLER *et al.*[7]. Enzyme reaction was achieved using a heptapeptide (Ser-Gln-Asn-Tyr-Pro-Ile-Val) as a substrate, which was monitored by HPLC. The details of this method will be reported elsewhere.

The strain Mer-N5075 was inoculated into 100 ml of a seed medium consisting of: potato starch 2%, glucose 1%, soybean meal 2%, KH_2PO_4 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.05% in tap water in a 500-ml Erlenmeyer flask and cultivated at 28°C for 3 days on a rotary shaker (200 rpm). The seed culture was transferred into a 15-liter jar fermentor containing 10 liters of a production medium consisting of: glycerol 2.5%, meat extract 0.5%, Polypepton (Nippon Seiyaku) 0.5%, yeast extract (Oriental Koubo)1%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05%, $CaCO_3$ 0.32%, and antifoam 0.05% (pH 7.4 before sterilization). The aeration and the agitation of the fermentation were carried out at 1.0 v/v/minute and 300 rpm, respectively and the temperature was kept constantly at 28°C during the cultivation. The supernatant from 10-liter broth was extracted with the same volume of *n*-BuOH. The resulting extract was concentrated *in vacuo* to give the oily residue (7.5 g). After washing the oily residue with *n*-hexane, the crude complex was dissolved into 5 ml of MeOH and applied to a column of Sephadex LH-20 (Pharmacia, 4 cm i.d. × 45 cm) packed with MeOH. After the elution with the same solvent, the active fractions were collected and concentrated *in vacuo*. The following column chromatography was performed with a column of silica gel (Merck 60 F_{254}, 4 cm i.d. × 45 cm) equilibrated with *n*-BuOH - AcOH (5 : 1) and eluted with the same solvent system. The crude powder (120 mg) containing Mer-N5075A was obtained from the active fractions, dissolved in 2 ml of MeOH - AcOH (100 : 1), and subjected to preparative TLC. The chromatography was developed with a solvent consisting of *n*-BuOH - AcOH (5 : 1). Finally, Sephadex LH-20 column chromatography (1.1 cm i.d. × 90 cm) packed with MeOH was achieved to yield Mer-N5075A (24.2 mg) as a white powder.

Fig. 1. Structures of Mer-N5075A and the MAPI group of compounds.



Compound	R	Config. at*
Mer-N5075A	CH_2OH	*S*
α-MAPI	CHO	*S*
β-MAPI	CHO	*R*

Table 1. ^1H and ^{13}C NMR spectral data of Mer-N5075A.

^1H[a]				^{13}C[b]		
δ_H (ppm)	m[c]		Assignment	δ_C (ppm)	m[c]	Assignment
0.77	3-H d	J=7.0 Hz	Val-Me	17.9	q	Val-Me
0.78	3-H d	J=6.6 Hz	Val-Me	19.1	q	Val-Me
1.43	3-H m		Arg-β,γ,γ'	24.7	t	Arg-γ
1.69	1-H br s		Arg-β'	29.1	t	Arg-β
1.90	1-H sext.	J=6.6 Hz	Val-β	30.5	d	Val-β
2.63	1-H dd	J=13.9, 8.1 Hz	PheOH-3-H	36.2	t	PheOH-3-C
2.82	1-H m		Phe-2-H	38.3	t	Phe-β
2.84	1-H dd	J=13.9, 5.9 Hz	PheOH-3'-H	40.2	t	Arg-δ
2.96	2-H br dd	J=13.2, 5.1 Hz	Arg-δ, Phe-β'	52.2	d	PheOH-2-C
3.07	1-H m		Arg-δ'	52.6	d	Arg-α
ca. 3.3	2-H m		PheOH-1,1'-H	56.5	d	Phe-α
3.92	1-H m		PheOH-2'-H	57.7	d	Val-α
4.00	1-H m		Phe-α	62.2	t	PheOH-1-C
4.07	1-H dd	J=8.8, 7.0 Hz	Val-α	125.4	d	Phe-Ar(1), PheOH-Ar(1)
4.14	1-H m		Arg-α	125.7	d	
4.83	1-H br s		PheOH-OH	127.6	d×2	Phe-Ar(2,3,5,6), PheOH-Ar(2,3,5,6)
6.08	1-H br d	J=7.0 Hz	Phe-NH	127.9	d×2	
6.66	1-H br s		Arg-NH	128.9	d×2	
7.18	10-H m		Phe-Ar, PheOH-Ar	129.4	d×2	
7.46	3-H br s		Arg-C(=NH)-NH_2	138.9	s	Phe-Ar(4), PheOH-Ar(4)
7.64	1-H br d	J=8.4 Hz	Val-NH	139.3	s	
7.84	1-H br d	J=6.6 Hz	PheOH-NH	157.2	s	Arg-ζ, Ureido C
8.84	1-H br s		Arg-ε	157.9	s	
				170.3	s	Val-CO, Arg-CO
				172.0	s	
				175.6	s	Phe-COOH

[a] DMSO-d_6, 400 MHz.
[b] DMSO-d_6, 100 MHz.
[c] m: Multiplicity.

Mer-N5075A is soluble in dimethyl sulfoxide and AcOH; slightly soluble in MeOH: and insoluble in water. The other physico-chemical properties are as follows: UV λ_{max} (MeOH) nm (ε) 268.3 (231), 264.6 (282), 258.7 (328), 253.4 (289), 248.1 (245); IR ν_{max} (KBr) cm^{-1} 3382 (br), 2963, 2934, 2874, 1642, 1611, 1553, 1499, 1470, 1454, 1395, 1250, 1107, 1044, 700; FAB-MS (m/z) positive; 598 $(M+H)^+$, negative; 596 $(M-H)^-$; HRFAB-MS obsd m/z of $(M+H)^+$, 598.3345, calcd for $C_{30}H_{44}N_7O_6$, 598.3353; Optical rotation $[\alpha]_D^{28}$ $-27.6°$ (c 0.11, AcOH).

The molecular formula of Mer-N5075A was established as $C_{30}H_{43}N_7O_6$ from the results of the HRFAB-MS and the NMR spectral analyses. The ^1H NMR and ^{13}C NMR spectral data in DMSO-d_6 are shown in Table 1.

To determine the stereochemistry of Mer-N5075A, the compound was hydrolyzed according to the method of SHIN-WATANABE *et al.*[2] and the hydrolyzates of the Mer-N5075A were prepared by TLC (Merck 60 F_{254}) with a solvent system consisting of *n*-BuOH - AcOH - H_2O (4:1:2). The configuration of the phenylalaninol was determined using HPLC under the following conditions: Column, Crownpak CR(+)column (Daicel, 6 mm i.d. × 150 mm); mobile phase, $HClO_4$ solution (pH 1.0); flow rate, 0.4 ml/minute; detection, 200 nm; and temperature, 4 °C. Normally, D-phenylalaninol (*R*) and L-phenylalaninol (*S*) were eluted at 41.2 and 44.1 minutes, respectively. The configurations of the other hydrolyzed components were also analyzed with Crownpak CR(+) column (Daicel) or HPTLC CHIR (Merck, HPTLC CHIR) with a solvent system consisting of MeOH - H_2O - Acetonitrile (1:1:4).

As shown in Fig. 2, the configuration of the phenylalaninol in Mer-N5075A was turned out to be L-form (*S*). In addition, all the other components possess the L-form: Therefore, the stereochemistry of Mer-N5075A is as same as that of α-MAPI.

The IC_{50} values against HIV-1 protease of

Fig. 2. Configuration determination of the phenylalaninol in Mer-N5075A.

(A) Mer-N5075A, (B) standard samples.

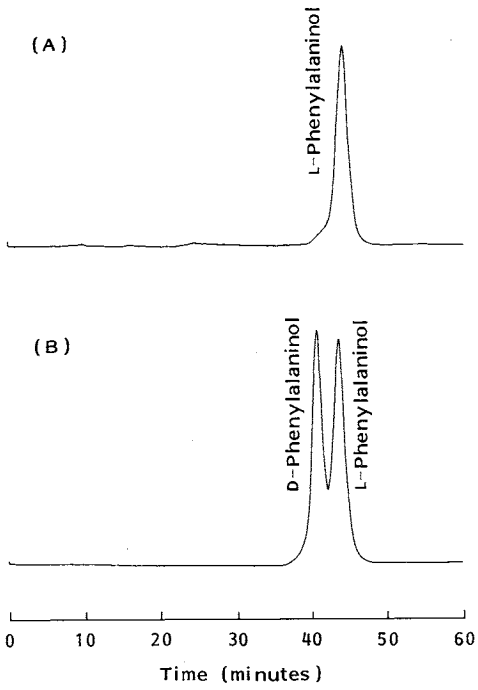


Table 2. HIV-1 protease inhibitory activity of Mer-N5075A and MAPIs.

Compound	IC_{50} (μM)
Mer-N5075A	17.8
α-MAPI	1.3
β-MAPI	18.3

Mer-N5075A and the MAPI group of compounds were summarized in Table 2.

Our data showed that the aldehydic function is more attributable to the HIV-1 protease inhibitory action than the alcoholic function and confirmed that the *S*-configuration at the carbon adjacent to the aldehyde function is crucial to the inhibition[8].

Although anti HIV-1 activity was not detected when Mer-N5075A and α-MAPI were subjected at doses of 100 μg/ml to an *in vitro* system using MT-4 cell and HIV-1, Mer-N5075A and the MAPI group of compounds gave a new insight for designing HIV-1 protease inhibitors.

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References

1) DEBOUCK, C.: The HIV-1 protease as a therapeutic target for AIDS. AIDS Res. Hum. Retroviruses 2: 153~164, 1992
2) SHIN-WATANABE, T.; K. FUKUHARA & S. MURAO: The structure of β-MAPI, a novel proteinase inhibitor. Tetrahedron 38: 1775~1780, 1982
3) WATANABE, T. & S. MURAO: Purification and characterization of crystalline microbial alkaline proteinase inhibitors (MAPI) produced by *Streptomyces nigrescens* WT-27. Agric. Biol. Chem. 43: 243~250, 1979
4) WATANABE, T.; K. FUKUHARA & S. MURAO: Structural elucidation of alpha-MAPI, a novel microbial alkaline proteinase inhibitor, produced by *S. nigrescens* WT-27. Tetrahedron Lett. 1979: 625~628, 1979
5) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
6) MIKI, T.; T. YASUKOUCHI, H. NAGATANI, M. FURUO, T. ORITA, H. YAMADA, T. IMOTO & T. HORIUCHI: Construction of a plasmid vector for the regulatable high level expression of eukaryotic genes in *Escherichia coli*: an application to overproduction of chicken lysozyme. Protein Engineering 4: 327~332, 1987
7) STRICKLER, J. E.; J. GORNIAK, B. DAYTON, T. MEEK, M. MOORE, V. MAGGARD, J. MALINOWSKI & C. DEBOUCK: Characterization and autoprocessing of precursor and mature forms of human immunodeficiency virus type 1 (HIV-1) protease purified from *Escherichia coli*. Proteins Struct. Funct. Genet. 6: 139~154, 1989
8) STELLA, S.; G. SADDLER, E. SARUBBI, L. COLOMBO, S. STEFANELLI, M. DENARO & E. SELVA: Isolation of α-MAPI from fermentation broths during a screening program for HIV-1 protease inhibitors. J. Antibiotics 44: 1019~1022, 1991