GE20372 Factor A and B

New HIV-1 Protease Inhibitors, Produced by *Streptomyces* sp. ATCC 55925

S. STEFANELLI, L. CAVALETTI, E. SARUBBI, E. RAGG†, L. COLOMBO and E. SELVA

Marion Merrell Dow Research Institute,
Lepetit Research Center,
Gerenzano (VA) Italy
† Department of Agri. Food Molecular Sciences,
Università degli Studi,
Via Celoria 2, Milano, Italy

(Received for publication November 21, 1994)

It is widely accepted that specific inhibitors of the HIV-1 aspartic protease might be interesting chemical leads to develop effective therapeutic agents for the treatment of AIDS[1,2].

In the course of a screening program to discover new inhibitors of HIV-1 protease from microbial fermentation broths[3], we have isolated the novel molecules GE20372 factors A (**1**) and B (**2**) shown in Fig. 1. These microbial products are tetrapeptide derivatives structurally related to the previously described microbial alkaline protease inhibitors (MAPI)-α (**3**) and β (**4**)[4~6]. Here we describe the isolation, structure elucidation and inhibitory activity of GE20372 A and B.

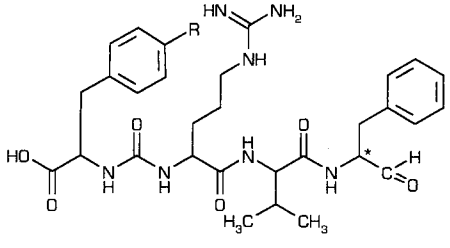
The producing microorganism was isolated from a soil sample collected in Binago (CO), Italy. Its aerial mycelium showed branched hyphae with spirals of non motile spores. LL-diaminopimelic acid[7], and no diagnostic sugars[8] were found in its whole-cell hydrolysates. The strain was thus classified as *Streptomyces* sp. (ATCC 55925).

The strain was grown in 500-ml Erlenmeyer flasks containing 100 ml of a seed medium consisting of glucose 2%, yeast extract 0.2%, soybean meal 0.8%, NaCl 0.1% and $CaCO_3$ 0.4%, pH 7.3. A 25 hours culture was inoculated (5%) into a 9 liters jar fermenter containing 6 liters of the production medium. This medium was obtained by supplementing L-tyrosine (100 μg/ml) to the seed medium. The culture was incubated at 28 °C for 24 hours under 900 rpm stirring and 0.5 vvm aeration and was then inoculated into 200 liters of the production medium. The culture was harvested after 24 hours of incubation at 28 °C under 180 rpm stirring and 0.5 vvm aeration.

The production was monitored with the HIV-1 protease inhibition assay[3] and with the method of 2,4-dinitrophenylhydrazine derivatization and HPLC analysis previously described[5]. The complex of (**1**) and (**2**) was recovered from the broth and was purified according to Scheme 1.

Factor B (**2**) was then purified by preparative HPLC on a 250 × 10 mm Supelcosil SP LC-8 column (Supelco, Inc). The column was eluted at 5 ml/minute flow rate with a 30 minute linear gradient from 20% to 45% of phase B. Phase A and B were 40 mM ammonium formate buffer pH 5.0 - acetonitrile 98 : 2 (v/v) and 40 mM ammonium formate buffer pH 5.0 - acetonitrile - tetrahydrofuran 4 : 3 : 3 (v/v), respectively. UV detection was at 230 nm. Pure (**2**) was obtained after concentration of the eluates and lyophilization. Under these conditions, (**1**) was eluted as a broad peak and its purification was difficult. It was thus derivatized to the $NaHSO_3$ adduct by evaporating

Fig. 1. Structures of α MAPI, β MAPI, GE20372 A and GE20372 B.



Compound		R	Config at*
(**1**)	GE20372 A	OH	*S*
(**2**)	GE20372 B	OH	*R*
(**3**)	α-MAPI	H	*S*
(**4**)	β-MAPI	H	*R*

Scheme 1. Isolation of the GE20372 A and B complex.

Harvested Broth (200 liters)
- filtration

Filtered Broth
- Batch adsorption on S112 6 liters resin (The Dow Chemical Company)

S112 Resin
- washing with water
- elution with acetone - water - *n*-butanol 8 : 1 : 1 (v/v)

Eluates
- concentration under vacuum
- precipitation upon addition of petroleum ether

Crude preparation (21.6 gr)
- chromatography on silanized silica gel (E. Merck, Darmstadt)
- Elution: 20 mM NaH_2PO_4 - acetonitrile 85 : 15 (v/v)

Eluates
- concentration
- Adsorption on S-112 resin (The Dow Chemical Co.)
- washing with distilled water
- Acetonitrile elution

Eluates
- concentration
- Lyophilization

Complex of (**1**) and (**2**) (2.1 g)

to dryness a mixture of 500 mg of the complex in 20 ml of methanol and 10 ml of water saturated with $NaHSO_3$. The adduct gave a good chromatographic separation in the above HPLC system eluted with a 30 minute linear gradient from 20% to 30% of phase B. (**2**) was regenerated from the adduct during the work up after chromatographic separation and eventually recovered by lyophilization.

(**1**) and (**2**) are isomers with a molecular weight of 611 Da, as determined by FAB-MS. Their ^1H and ^{13}C NMR assignments are reported in Tables 1 and 2, respectively. The ^1H NMR assignments were based on 2D-ROESY and TOCSY experiments and the ^{13}C NMR signals were attributed from the inverse correlated spectra through one-bond and multiple-bond connectivities[10]. The peptide sequence resulted from ROESY spectra (300 ms mixing time) which showed interactions between Phe-NH and Val-NH and between Val-NH and Arg-NH. The aldehydic function of the phenylalaninal moiety was demonstrated by a multiple bond correlation (2 or 3 bonds) between aldehydic carbonyl and Phe-Hα.

To determine the chiralities of the asymmetric centers in (**1**) and (**2**), the complex was reduced with $NaBH_4$ and the reduced factors were separated by preparative HPLC on a 250 × 10 mm Supelcosil SP LC-8 column (Supelco, Inc). The column was eluted at 5 ml/minute flow rate with a 42 minutes linear gradient from 22% to 82% of phase B. Phase A and B were 40 mM ammonium acetate-acetonitrile-tetrahydrofuran 98 : 1 : 1 (v/v) and 3 : 5 : 2 (v/v), respectively. The reduced compounds were hydrolyzed (6 N HCl, 1% phenol, 105°C for three days) and derivatized with 3.5 N HCl in anhydrous methanol (30 minutes at 100°C) and then with trifluoroacetic anhydride (15 minutes at 100°C). GC-MS analyses on an (Helifex Chirasil-Val column[9]) by comparison with authentic standards showed the presence of L-Tyr, L-Arg and L-Val in both factors. L and D phenylalaninol were found as the distinctive components of (**1**) and (**2**), respectively. These results are consistent with the structural assignments reported in Fig. 1.

Compounds (**1**) and (**2**) are similar to the MAPI isomers (**3**) and (**4**), but contain L-tyrosine in place of L-phenylalanine. (**1**) and (**2**) are normally coproduced with MAPI by *Streptomyces* sp. ATCC 55925. The production of the GE20372 complex was significantly increased by supplementing the fermentation medium with an appropriate amount of tyrosine.

The inhibition of HIV-1 protease by the two GE20372 isomers (**1**) and (**2**) is shown in Table 3, together with

Table 1. ^1H and ^{13}C NMR spectral data of GE 20372 factor A.*

δ_H (ppm)	Multiplicity	Assignment	δ_C (ppm)	Assignment
9.44	1H s	Aldehyde	156	Tyr-Arδ
8.48	1H d	Phe-NH	129.9	Tyr-Arγ
7.80	1H d	Val-NH	127.8	Phe-Ar
			~128.8	
7.43	1H t	Arg-NHε	114.7	Tyr-Arβ
7.25	5H m	Phe-Ar	110	Tyr-Arα
~7.20				
6.94	2H	Tyr-Ar	59.3	Phe-Cα
6.64	2H d	Tyr-Ar	56.9	Val-Cα
6.50	1H br s	Ureidic-NH	53.7	Tyr-Cα
6.26	1H br s	Tyr-NH	51.6	Arg-Cα
4.35	1H m	Phe-Hα	40.2	Arg-Cδ
4.25	1H t	Tyr-Hα	36.4	Tyr-Cβ
4.20	1H dd	Arg-Hα	33.0	Phe-Cβ
4.18	1H m	Val-Hα	30.1	Val-Cβ
3.15	m	Phe-Hβ	29.8	Arg-Cβ
3.08	m	Arg-H$\delta+\delta'$	24.8	Arg-Cγ
2.80	m	Phe-Hβ'	17.3	Val-Me
2.85	m	Tyr-Hβ		
2.75	m	Tyr-Hβ'		
1.92	1H m	Val-Hβ		
1.55	4H m	Arg-H$\beta+\beta'$		
~1.42		Arg-H$\gamma+\gamma'$		
0.80	6H m	Val-Me$\gamma+\gamma'$		

* ^1H NMR spectra were recorded on a Bruker AM-500 at 30°C, about 1 mg dissolved in DMSO-d_6 + vapours of TFA. Chemical shifts are given in δ (ppm) using as reference standard DMSO residual peak set at 2.5 ppm for 1H and set at 39.7 ppm for ^{13}C.

Table 2. ^1H and ^{13}C NMR spectral data of GE 20372 factor B.*

δ_H (ppm)	Multiplicity	Assignment	δ_C (ppm)	Assignment
9.48	1H s	Aldehyde	156	Tyr-Arδ
8.45	1H d	Phe-NH	129.9	Tyr-Arγ
7.73	1H d	Val-NH	128.9	Phe-Ar
			128.3	
			126.0	
7.42	1H t	Arg-NHε	114.7	Tyr-Arβ
7.29	5H m	Phe-Ar	110	Tyr-Arα
~7.14				
6.94	2H d	Tyr-Ar	59.4	Phe-Cα
6.59	2H d	Tyr-Ar	57.1	Val-Cα
6.49	1H br s	Ureidic-NH	53.9	Tyr-Cα
6.23	1H br s	Tyr-NH	52.0	Arg-Cα
4.37	1H m	Phe-Hα	40.3	Arg-Cδ
4.27	1H t	Tyr-Hα	36.4	Tyr-Cβ
4.22	1H dd	Arg-Hα	33.0	Phe-Cβ
4.17	1H m	Val-Hα	29.8	Arg-Cβ
3.25	3H m	Phe-Hβ +	29.8	Val-Cβ
~3.00		Arg-H$\delta+\delta'$		
2.94	3H m	Phe-Hβ' +	24.6	Arg-Cγ
~2.68		Tyr-H$\beta+\beta'$		
1.84	1H m	Val-Hβ	17.5	Val-Me
1.68	4H m	Arg-H$\beta+\beta'$		
~1.35		Arg-H$\gamma+\gamma'$		
0.68	6H m	Val-Me$\gamma+\gamma'$		

* ^1H NMR spectra were recorded on a Bruker AM-500 at 30°C, about 1 mg dissolved in DMSO-d_6 + vapours of TFA. Chemical shifts are given in δ (ppm) using as reference standard DMSO residual peak set at 2.5 ppm for 1H and set at 39.7 ppm for ^{13}C.

Table 3. Inhibition of HIV-1 protease (pH 6.0).

Compound	IC_{50} (μM)
α-MAPI	2
β-MAPI	32
GE20372 A	18
GE20372 B	58

the activity of the MAPI isomers (α and β). All inhibitory activities were determined as previously described.[3] These data indicate that (**1**) and (**2**) are somewhat less potent inhibitors of the viral enzyme when compared with the corresponding isomers of MAPI, (**3**) and (**4**). Evidently the incorporation of hydroxylic function into the aromatic ring of the phenylalanine moiety of MAPI has a slight negative effect on its inhibitory activity on HIV-1 protease.

References

1) MEEK, T. D.: Inhibitors of HIV-1 protease. J. Enzyme Inhib. 6: 65~98, 1992
2) WLODAWER, A. & J. W. ERICKSON: Structure-based inhibitors of HIV-1 protease. Ann. Rev. Biochem. 62: 543~585, 1993
3) SARUBBI, E.; M. L. NOLLI, F. ANDRONICO, S. STELLA, G. S. SADDLER., E. SELVA, A. SICCARDI & M. DENARO: A high throughput assay for inhibitors of HIV-1 protease. Screening of microbial metabolites. FEBS Letters 279: 265~269, 1991
4) 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
5) STELLA S.; G. S. 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
6) KANETO, R.; H. CHIBA, K. DOBASHI, I. KOJIMA, K. SAKAI, N. SHIBAMOTO, H. NISSHIDA, R. OKAMOTO, H. AKAGAWA & S. MITZUNO: Mer-N5075A, a potential HIV-1 protease inhibitor, produced by *Streptomyces chromofuscus*. J. Antibiotics 46: 1622~1624, 1993
7) STANECK, J. L. & G. D. ROBERTS: Simplified Approach to Identification of Aerobic Actinomycetes by Thin Layer Chromatography. Appl. Microbiol. 28: 226~231, 1974
8) SADDLER, G. S.; P. TAVECCHIA, S. LOCIURO, M. ZANOL, L. COLOMBO & E. SELVA: Analysis of madurose and other actinomycete whole cell sugars by gas chromatography. J. Microbiol. Methods 14: 185~191, 1991
9) ABE, I.; S. KURAMOTO & S. MUSHA: Helifex Chirasil-Val; GC of amino acids enantiomers. J. High Resolut. Chromatogr. 7: 366~370, 1983
10) BAX, A. & M. F. SUMMERS: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple-quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986