

ISOLATION OF α -MAPI FROM
FERMENTATION BROTHS DURING
A SCREENING PROGRAM FOR
HIV-1 PROTEASE INHIBITORS

S. STELLA, G. SADDLER, E. SARUBBI,
L. COLOMBO, S. STEFANELLI,
M. DENARO and E. SELVA

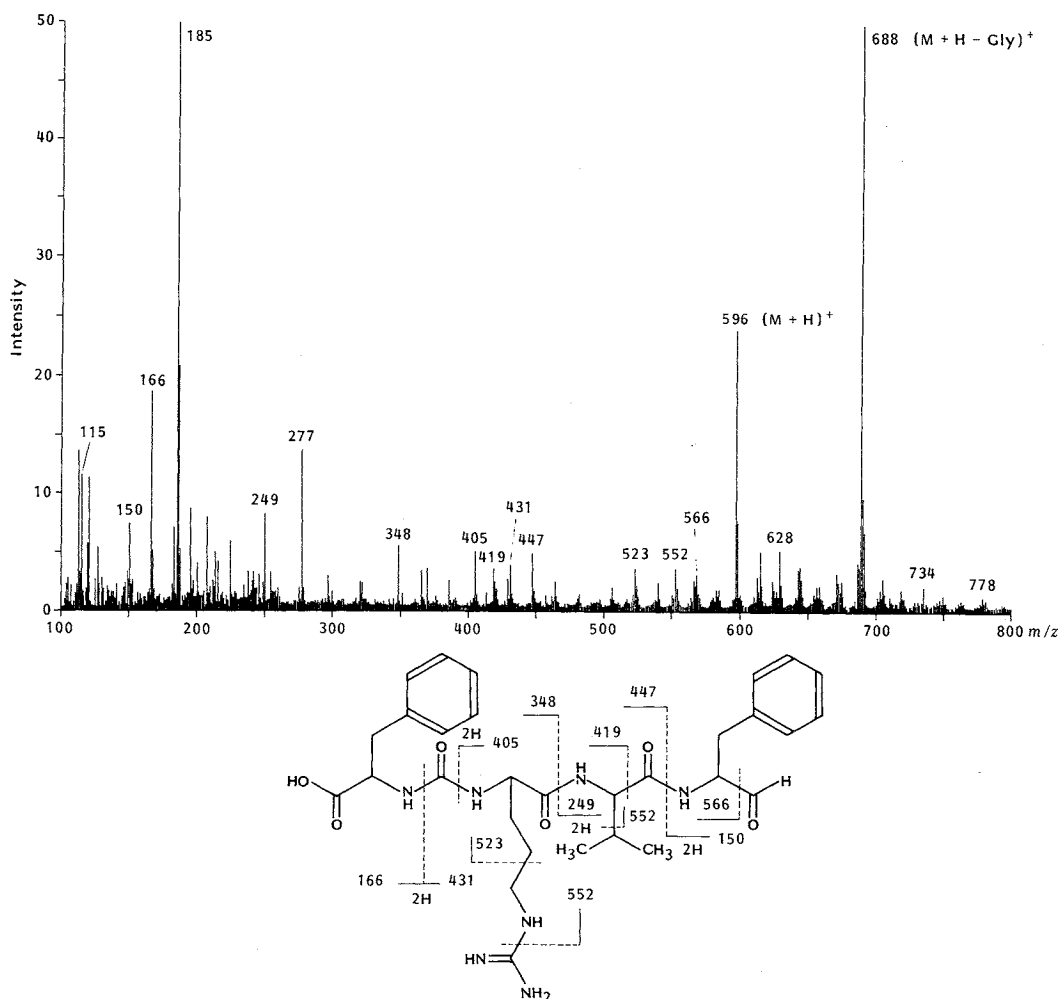
MMDRI, Lepetit Research Center,
Gerenzano (VA), Italy

(Received for publication March 27, 1991)

Inhibitors of the HIV-1 aspartyl protease may

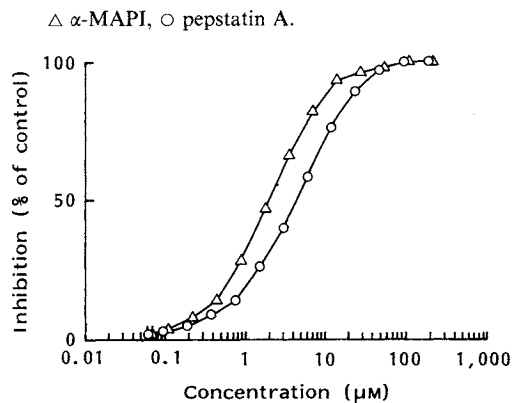
provide novel therapeutic agents in the treatment of AIDS^{1,2}). Rational design based on structural data of this viral protease has led to the synthesis of molecules with specific inhibitory activity³⁻⁷). There are, however, few reports of HIV-1 protease inhibitors from natural sources^{8,9}). Production of protease inhibitors, in the wider sense, by microorganisms is well known¹⁰). We have recorded in literature 188 published compounds; three quarters being produced by actinomycetes, the remainder evenly divided between other bacteria and fungi. Consequently, we decided to develop a solid-phase immunoassay designed to detect the presence of HIV-1 protease inhibitors in fermenta-

Fig. 1. Positive ion FAB-MS: proposed mass spectral fragmentation pattern for MAPI.



FAB mass spectra from a Kratos MS50 instrument, fitted with an Ion Tech saddle field atom gun. Xe gas was typically used at 6 kV voltage and 1 mA discharge current. Samples were dissolved in DMSO-glycerol mixture (1:1).

Fig. 2. Activity of α -MAPI and pepstatin A in the solid phase assay for HIV-1 protease.

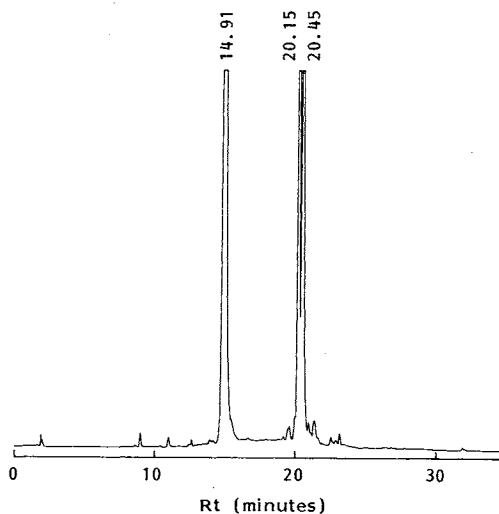


Experimental conditions: 5 μ l of filtered broths were mixed (10 minutes; room temperature) in round bottom microtiter wells with 45 μ l of 0.25 M MES-NaOH buffer, pH 6.0, 0.1 M NaCl, 0.6% w/v BSA, 0.025% Tween 20, 1 mM sodium EDTA, 5 μ g/ml leupeptin, 1 mM PMSF and 1% *Escherichia coli* extract containing recombinant HIV-1 protease. Samples were then incubated at 37°C for 40 minutes with 25 μ l/well of gal-gag110 fusion protein (50 μ g/ml). 50 μ l of reaction solutions were then transferred into wells of a flat-bottom microtitre plate pre-coated with monoclonal antibody 1G12. The plates were incubated for 2 hours at room temperature to allow the selective binding of the uncleaved gal-gag110 to the antibody. The solutions were then discarded and the plates washed 4~5 times with PBS containing 0.05% Tween 20. The uncleaved gal-gag110 was evaluated after addition of 240 μ l/well of 1 mg/ml PNPG, a β -galactosidase-specific chromogenic substrate, in 50 mM sodium phosphate buffer, pH 7.8, 50 mM NaCl, 1 mM MgCl₂ and 70 mM 2-ME and 1 hour incubation at room temperature. The reaction was quenched with 60 μ l of 1.5 M Na₂CO₃ and absorbance at 405 nm was determined.

tion broths¹¹). The assay is based on a recombinant protein consisting of *Escherichia coli* β -galactosidase fused to a portion of the HIV-1 gag polyprotein containing the p17~p24 cleavage site. This fusion protein acts as a specific substrate for HIV-1 protease. The proteolytic activity is detected using a very specific monoclonal antibody immobilized on standard 96-well microtiter plates. This antibody binds the fusion protein in the gag region only if this has not been cleaved by the HIV-1 protease.

More than 12,000 strains have been screened using this method, 70% of which were actinomycetes, the remainder being fungi. After retesting and eliminating replicate strains, 31 positive activities have been

Fig. 3. HPLC profile of MAPI dinitrophenylhydrazones.



Experimental conditions: filtered broths were treated with one-tenth volume of S112 resin. The ethanol eluates of the resin were incubated with one volume of 2,4-dinitrophenylhydrazine (5 mM in CH₃CN) and one volume of 0.1 M H₃PO₄.

Column: Altex Ultrasphere (5 μ m) ODS 4.6 \times 250 mm; flow rate: 1.5 ml/minute; elution: linear gradient from 10% to 70% of phase B in 30 minutes; phase A: CH₃CN 20 mM ammonium phosphate (10:90); phase B: CH₃CN, 20 mM ammonium phosphate (70:30); detection: UV at 350 nm.

found. 24 activities were found in the broths of strains belonging to the genus *Streptomyces*; 4 *Micromonospora*, 2 *Nocardia* and 1 fungal producer were also found.

The activity from strain GE16457 was isolated and characterized. The producer strain after morphological and chemotaxonomic evaluation was found to be a member of the genus *Streptomyces*. Production of the inhibitor was obtained by growing the producer strain for 72 hours in a medium composed of: glucose 2%, soybean meal 0.8%, yeast extract 0.2%, calcium carbonate 0.4%, sodium chloride 0.1%, pH 7.4. The protease inhibitor was adsorbed from the filtered broth on the polystyrenic resin S112 (Dow Chemical Co.) and was subsequently extracted from the concentrated acetonitrile eluates with butanol. This crude preparation was purified by normal pressure reverse-phase chromatography and subsequently preparative HPLC on a 250 \times 25 mm column packed with Lichrosorb Merck RP-18 (7 μ m). The separation was accomplished with a linear gradient of acetonitrile (25 to 50%)

and phosphate buffer (0.02 M, pH 6.0) with UV detection at 220 nm. The purified inhibitor was found structurally belonging to the MAPI complex of α and β epimers which was previously described as microbial protease inhibitor¹²⁻¹⁴. The identification was reached as a result of FAB-MS analysis (Fig. 1). ¹H and ¹³C NMR of the isolated product confirmed the attribution.

The GE16457 product was treated with KMnO₄ and the oxidation product was hydrolyzed (6 N HCl with 1% phenol; 105°C for 24 hours) and derivatized with pentafluoropropionic anhydride and with 2-propanol-HCl. The analysis by HR-GC using a Heliflex Chirasil-Val (Alltech) capillary column¹⁵ found L-phenylalanine along with L-valine and L-arginine. This is consistent with the structure of the α -MAPI epimer, as the hydrolysis derivative of α -MAPI contains two L-phenylalanine residues¹³. β -MAPI is the epimer at the carbon atom adjacent to the aldehydic function and would be characterized by the presence of D-phenylalanine¹⁴. β -MAPI was also isolated from the crude butanol extract, as D-phenylalanine was found in the hydrolysates after oxidation. This product showed no activity against HIV-1 protease. The oxidation product of the active α -MAPI was also found to be inactive. These data indicate that the aldehydic function and the configuration of its adjacent atom is crucial for the activity of these molecules on the HIV-1 protease.

Pepstatin A is a known inhibitor of aspartic proteases including the HIV-1 protease^{8,9}. α -MAPI has been previously characterized as an inhibitor of alkaline proteases, with no activity on pepsin or other aspartic proteases¹². Nevertheless, in our HIV-1 protease assay (Fig. 2) α -MAPI isolated from strain GE16457 and pepstatin A show similar inhibition profiles.

A high sensitivity HPLC method was set up for the rapid detection of the MAPI molecules in the positive broths (Fig. 3). Of the 24 positive activities detected from strains belonging to the genus *Streptomyces*, the MAPI complex was found ten times, thus appearing a common secondary metabolite of the *Streptomyces* strains isolated for activity against HIV-1 protease.

Acknowledgments

We gratefully acknowledge P. FERRARI for the NMR analyses, G. BERETTA, N. MONTANINI and G. ZAFFARONI for the isolation of microorganisms tested during the research program and A. GUINDANI, E. CORTI, G. ROMANO and A. RIUNNO for the technical support.

References

- 1) JOHNSTON, M. I.; H. S. ALLAUDEEN & N. SARVER: HIV proteinase as a target for drug action. *Trends Pharmacol. Sci.* 10: 305~307, 1989
- 2) DUNN, B. M. & J. KAY: Targets for antiviral chemotherapy: HIV-proteinase. *Antivir. Chem. Chemother.* 1: 3~8, 1990
- 3) MILLER, M.; J. SCHNEIDER, B. K. SATHYANARAYANA, M. V. TOTH, G. R. MARSHALL, L. CLAWSON, L. SELK, S. B. H. KENT & A. WLODAWER: Structure of complex of synthetic HIV-1 protease with a substrate-based inhibitor at 2.3 Å resolution. *Science* 246: 1149~1152, 1989
- 4) DREYER, G. B.; B. W. METCALF, T. A. TOMASZEK, T. J. CARR, A. C. CHANDLER, III, L. HYLAND, S. A. FAKHOURY, V. W. MAGAARD, M. L. MOORE, J. E. STRICKLER, C. DEBOUCK & T. D. MEEK: Inhibition of human immunodeficiency virus 1 protease *in vitro*: Rational design of substrate analogue inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 86: 9752~9756, 1989
- 5) TOMASSELLI, A. G.; M. K. OLSEN, J. O. HUI, D. J. STAPLES, T. K. SAWYER, R. L. HEINRIKSON & C.-S. C. TOMICH: Substrate analogue inhibition and active site titration of purified recombinant HIV-1 protease. *Biochemistry* 29: 264~269, 1990
- 6) ROBERTS, N. A.; J. A. MARTIN, D. KINCHINGTON, A. V. BROADHURST, J. C. CRAIG, I. B. DUNCAN, S. A. GALPIN, B. K. HANDA, J. KAY, A. KRÖHN, R. W. LAMBERT, J. H. MERRETT, J. S. MILLS, K. E. B. PARKES, S. REDSHAW, A. J. RITCHIE, D. L. TAYLOR, G. J. THOMAS & P. J. MACHIN: Rational design of peptide-based HIV proteinase inhibitors. *Science* 248: 358~361, 1990
- 7) ERICKSON, J.; D. J. NEIDHART, J. VANDRIE, D. J. KEMPF, X. C. WANG, D. W. NORBECK, J. J. PLATTNER, J. W. RITTENHOUSE, M. TURON, N. WIDEBURG, W. E. KOHLBRENNER, R. SIMMER, R. HELFRICH, D. A. PAUL & M. KNIGGE: Design, activity, and 2.8 Å crystal structure of a C₂ symmetric inhibitor complexed to HIV-1 protease. *Science* 249: 527~533, 1990
- 8) SEELMEIER, S.; H. SCHMIDT, V. TURK & K. VON DER HELM: Human immunodeficiency virus has an aspartic-type protease that can be inhibited by pepstatin A. *Proc. Natl. Acad. Sci. U.S.A.* 85: 6612~6616, 1988
- 9) RICHARDS, A. D.; R. ROBERTS, B. M. DUNN, M. C. GRAVES & J. KAY: Effective blocking of HIV-1 proteinase activity by characteristic inhibitors of aspartic proteinases. *FEBS Lett.* 247: 113~117, 1989
- 10) UMEZAWA, H.: Low-molecular-weight enzyme inhibitors of microbial origin. *Annu. Rev. Microbiol.* 36: 75~99, 1982
- 11) SARUBBI, E.; M. L. NOLLI, F. ANDRONICO, S. STELLA, G. SADDLER, E. SELVA, A. SICCARDI & M. DENARO: A high throughput assay for inhibitors of HIV-1 protease: screening of microbial metabolites. *FEBS*

- Lett. 279: 265~269, 1991
- 12) 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
- 13) WATANABE, T.; K. FUKUHARA & S. MURAO: Structural elucidation of alpha-MAPI, a novel microbial alkaline proteinase inhibitor produced by *Streptomyces nigrescens* WT-27. Tetrahedron Lett. 1979: 625~628, 1979
- 14) SHIN-WATANABE, T.; K. FUKUHARA & S. MURAO: The structure of β -MAPI, a novel proteinase inhibitor. Tetrahedron 38: 1775~1780, 1982
- 15) ABE, I.; S. KURAMOTO & S. MURAO: Heliflex Chirasil-Val; GC of amino acid enantiomers. J. High Resolut. Chromatogr. 7: 366~370, 1983