Tetrahedron Letters No. 7, pp 625 - 628. 0040-4039/79/0208-0625$02.00/0

STRUCTURAL ELUCIDATION OF ALPHA-MAPI, A NOVEL MICROBIAL ALKALINE PROTEINASE INHIBITOR, PRODUCED BY STREPTOMYCES NIGRESCENS WT-27

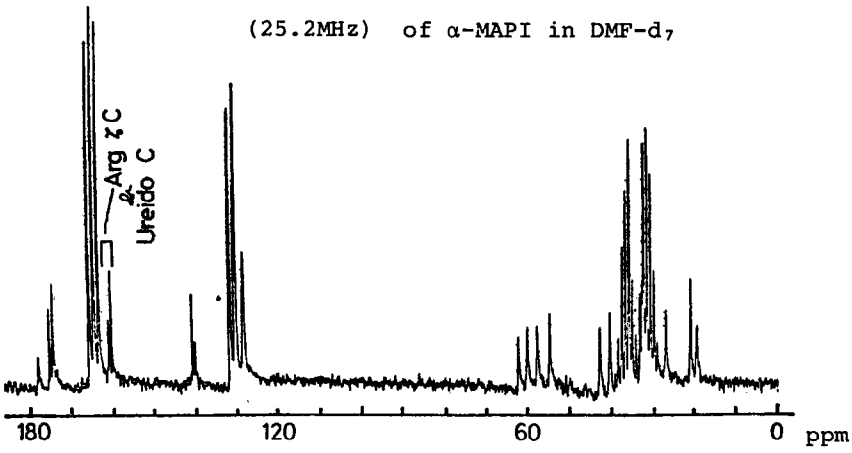
Takashi Watanabe, Kenichi Fukuhara and Sawao Murao*

Laboratory of Applied Microbiology, Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai, Osaka 591, Japan

As reproted in previous papers [1, 2], a novel proteinase inhibitor MAPI, produced by *streptomyces nigrescens* WT-27, showed a specific inhibitory activity against various microbial alkaline proteinases, α-chymotrypsin and thiol proteinases. MAPI in the culture filtrate was purified successively via adsorption on Amberlite XAD-2, extraction with n-butanol, chromatography using Amberlite XAD-7, Dowex 1X2, Amberlite CG-50, alumina and silica gel. MAPI was a mixture of three compounds (designated as α-, β-, and γ-MAPI), which showed the same inhibitory spectra but different inhibitory potentials.

α-MAPI was isolated by preparative high performance liquid chromatography (HPLC) and crystallized from aqueous methanol as needles. Properties of α-MAPI were as follows: mp 204∿205°C(dec.); $[\alpha]_D^{22}$ -18°(c 1.0, acetic acid); Found: C 58.49, H 6.97, N 15.54%; Calcd. for $C_{30}H_{41}N_7O_6 \cdot H_2O$: C 58.71, H 7.06, N 15.98%; IR (KBr): 3400∿3300(NH), 1650∿1640(amide I, guanidinium and ureido), 1550∿1540(amide II), 1460∿1440(phenyl), 1390(COOH), 750, 690cm^{-1}(phenyl); UV: $\lambda_{max}^{80\%aq.MeOH}$(ε) 268 (304), 264(402), 258(509), 252(440), 247nm(360); PMR[90MHz, δ value(ppm) in DMF-d_7]: 0.7∿1.0[6H, $CH(C\underline{H}_3)_2$(in Val)], 1.2∿1.9[4H m, $CH\text{-}C\underline{H}_2\text{-}C\underline{H}_2$-(in Arg)], 1.9∿2.3 [1H m, $C\underline{H}(CH_2)_3$(in Val)], 2.9∿3.4[4H m, two $C\underline{H}_2$(in Phe)], 3.50[2H t, J=6Hz, $-C\underline{H}_2$-NH-(in Arg)], 4.0∿4.7[4H m, four methines], 7.3[10H m, two $C_6\underline{H}_5$(in Phe)], 6.6∿8.6 [m, N$\underline{H}$, N$\underline{H}_2$, O$\underline{H}$], 9.6[1H d, J=1Hz, C$\underline{H}$O]; ^{13}C-NMR[25.2MHz, in DMF-d_7; as shown in Fig.1]; Rf 0.73[cellulose, TLC, $CHCl_3$-n-butanol-ethanol-28%NH_4OH-H_2O (20:40:50: 27:13)]. α-MAPI gave positive reactions to chlorine-tolidine, Sakaguchi, diacetyl-α-naphtol, Tollens and 2,3,5-triphenyltetrazolium chloride reagents, but

Fig.1. Proton noise-decoupled ^{13}C FT-NMR spectrum (25.2MHz) of α-MAPI in DMF-d_7

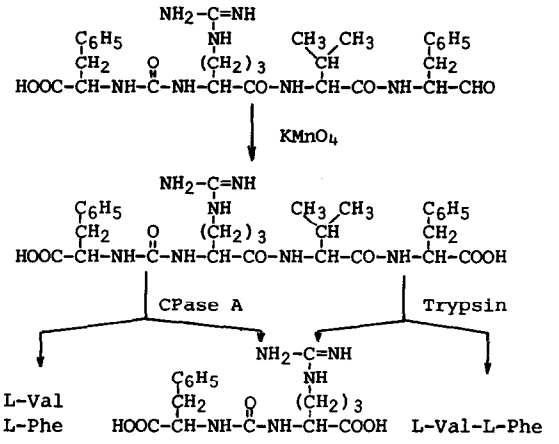


negative to ninhydrin reagent. α-MAPI was sparingly soluble in all solvents except acetic acid, dimethylsulfoxide and dimethylformamide.

On hydrolysis with 6N HCl (in evacuated tube, 150°C, 48 hours), α-MAPI gave phenylalanine (0.79mole), valine (1.00mole) and arginine (0.84mole). On hydrolysis at 110°C for 48 hours, it gave phenylalanine (0.10mole), valine (1.00mole) and arginine (0.09mole), suggesting the presence of acid resistant bond such as ureido bond (-NH-C(=O)-NH), which was also assumed by its ^{13}C-NMR spectrum (Fig.1, Two signals having δ value of 160ppm may be assigned to ureido carbon and guanidinium carbon of arginine residue). On hydrazinolysis (in evacuated tube, 100°C, 6 hours), α-MAPI gave only phenylalanine.

Oxidation of α-MAPI with potassium permanganate gave an oxidation product (α-MAPI-O), which was crystallized from aqueous methanol. Found: C 58.55, H 6.63, N 15.81%; Calcd. for $C_{30}H_{41}N_7O_7$: C 58.91, H, 6.76, N 16.03%; mp 230∿231°C(dec.); IR(KBr): ∿3300(NH), 2950(CH), ∿1720(sh. COOH), 1680∿1620(amide I, guanidinium and ureido), 1550(amide II), 1440∿1460(phenyl), 1390(COOH), 750(phenyl), 700cm^{-1} (phenyl); UV: $\lambda_{max}^{H_2O}(\varepsilon)$ 268(218), 264(316), 258(409), 252(343), 247nm(270); $[\alpha]_D^{22}$ -10°(c 0.5, H_2O); PMR[90MHz, δ value(ppm) in D_2O]: 0.9[6H d, J=7Hz, $CH(C\underline{H}_3)_2$(in Val)], 1.3∿1.8[4H m, $CH\text{-}C\underline{H}_2\text{-}C\underline{H}_2\text{-}$(in Arg)], 1.8∿2.3[1H m, $-C\underline{H}(CH_3)_2$(in Val)], 2.8∿3.3[6H m, two $C\underline{H}_2$(in Phe) and $-C\underline{H}_2\text{-}NH\text{-}$(in Arg)], 4.0∿4.6[4H m, four α-methines], 7.35[10H m, two $C_6\underline{H}_5$(in Phe)]; RF 0.57 (TLC mentioned above). α-MAPI-O gave positive reactions to chlorine-tolidine, Sakaguchi and diacetyl-α-naphtol

reagents, but negative to Tollens, 2,3,5-triphenyltetrazolium chloride and ninhydrin reagents.



On hydrolysis with 6N HCl, α-MAPI-O gave additional one mole of phenylalanine, suggesting that the aldehyde moiety in α-MAPI was converted to phenylalanine by oxidation.

α-MAPI-O, containing arginine residue, was completely digested into two fragments by trypsin (pH 8.0, 37°C, 6 hours, ratio 1:25), suggesting that the arginine was L-isomer. Two fragments were isolated by a preparative HPLC using LiChroprep RP-8 (E. Merck).

One fragment (designated as T-1) obtained as needles gave positive chlorine-tolidine, Sakaguchi and diacetyl-α-naphtol but negative ninhydrin reactions. IR (KBr): ∿3300(NH), 1650(amide I, guanidinium and ureido), 1550(amide II), 1400 (COOH), 700cm^{-1}(phenyl); UV: $\lambda_{max}^{H_2O}$(ε) 268(103), 258(180), 252(153), 247(120); PMR [90MHz, δ value(ppm) in D_2O]: 1.2∿1.8[4H m, CH-C$\underline{H}_2$-C$\underline{H}_2$-(in Arg)], 2.90 and 3.15 [2H, J_{AB}=13Hz, J_{AX}=8Hz, J_{BX}=5Hz, C$\underline{H}_2$(in Phe)], 3.15[2H t, J=6Hz, -CH$_2$-C$\underline{H}_2$-NH-(in Arg)], 3.85∿4.05[1H m, C$\underline{H}$(in Arg)], 4.25[1H, J_{AX}=8Hz, J_{BX}=5Hz, C$\underline{H}$(in Phe)], 7.35 [5H m, $C_6\underline{H}_5$(in Phe)]; Rf 0.25 (TLC mentioned above). On hydrolysis with 6N HCl (110°C, 44 hours), T-1 gave an equimolar amount of phenylalanine and arginine. This fragment was relatively resistant to acid hydrolysis. On hydrazinolysis, T-1 gave two C-terminal amino acids, phenylalanine and arginine, the latter being detected as ornithine. Its ^{13}C-NMR spectrum indicated the presence of ureido bond (two signals at about 160ppm could be assigned to ureido carbon and guanidinium carbon of arginine residue). Thus the fragment T-1 was deduced to be an ureido-type derivative, N-(1-carboxy-2-phenylethyl)carbamoyl-L-arginine.

Anonther fragment (designated as T-2) also obtained as crystals gave positive chlorine-tolidine and ninhydrin reactions, but negative Sakaguchi and diacetyl-α-naphytol reactions. IR(KBr): ∿3300(NH), 2950(CH), ∿1680(amide I), 1620(COOH), 1580∿1520(NH, amide II), 1400(COOH), 700cm^{-1}(phenyl); UV:$\lambda_{max}^{H_2O}$(ε)

268(96), 264(143), 258(188), 252(153), 247nm(113); PMR[90MHz, δ value(ppm) in D_2O]: 0.97[6 H d, J=7Hz, $CH(C\underline{H}_3)_2$(in Val)], 1.95∿2.45[1H m, $C\underline{H}(CH_3)_2$(in Val)], 2.95 and 3.20[2H, J_{AB}=15Hz, J_{AX}=8Hz, J_{BX}=6Hz, $C\underline{H}_2$(in Phe)], 3.71[1H d, J=6Hz, α-C$\underline{H}$(in Val)], 4.47[1H, J_{AX}=8Hz, J_{BX}=6Hz, α-C$\underline{H}$(in Phe)], 7.3[5H m, $C_6\underline{H}_5$(in Phe)]; Rf 0.75 (TLC mentioned above).

On hydrolysis with 6N HCl (110°C, 48 hours), T-2 gave an equimolar amount of L-phenylalanine and L-valine. N-terminus of T-2 was identified as valine by Edman degradation, and C-terminus as phenylalanine by hydrazinolysis. PMR spectrum, TLC and HPLC of T-2 were exactly identical to those of the authentic L-valyl-L-phenylalanine.

α-MAPI-O was also digested by carboxypeptidase A (pH 8.0, 4°C, ratio 1:600) to liberate phenylalanine in the first period. After 8 hours, α-MAPI was converted to an equimolar amount of phenylalanine, valine and a compound with positive Sakaguchi reaction, which identified as T-1 by TLC and HPLC. From the results described above, it is concluded that the structure of α-MAPI is N-(1-carboxy-2-phenylethyl)carbamoyl-L-arginyl-L-valyl-L-phenylalaninal. Elucidation of configuration of phenylalanine is in progress.

α-MAPI has different proteinase inhibitory spectra compared with other peptidic proteinase inhibitors with aldehyde and ureido bond, such as antipain [3] and chymostains [4]. The structure of β-MAPI will be described elsewhere.

References

1. S. Murao and T. Watanabe, Agric. Biol. Chem., 41 1313 (1977).
2. S. Murao and T. Watanabe, *ibid.*, 42(12), in press (1978).
3. S. Umezawa, K. Tatsuta, K. Fujimoto, T. Tsuchiya, H. Umezawa, H. Naganawa J. Antibiotics, 25, 267 (1972).
4. K. Tatsuta, N. Mikami, K. Fujimoto, S. Umezawa, H. Umezawa, T. Aoyagi, *ibid.*, 26, 625 (1973).

(Received in Japan 25 November 1978)