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THE STRUCTURE OF β-MAPI, A NOVEL PROTEINASE INHIBITOR

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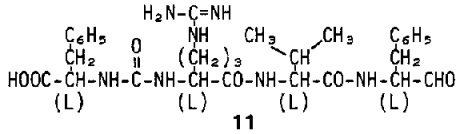
Abstract—Structure of β-MAPI, a novel proteinase inhibitor produced by *Streptomyces nigrescens* WT-27, is determined as 1.

A novel proteinase inhibitor MAPI, produced by *Stm. nigrescens* WT-27, shows a specific inhibitory effect against various microbial alkaline proteinases, α-chymotrypsin and thiol proteinases.[1a,b] MAPI is a mixture of three compounds (designated as α-, β- and γ-MAPI) showing the same inhibitory spectra. The compounds are isolated by preparative high-performance liquid chromatography (hplc) in a reversed-phase partition mode.[1c] The structure of α-MAPI was reported in the previous paper.[2]



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β-MAPI has features common to α-MAPI (**11**), ^{1}H-NMR, ^{13}C-NMR and UV spectra of both compounds are alike. However difference between the two compounds can be noted, β-MAPI shows weaker inhibitory potential against proteinases. In the present report, the structural elucidation of β-MAPI is described.

β-MAPI (**1**) is isolated from MAPI mixture, crystallized from aqueous methanol as colorless needles. The molecular formula was established as $C_{30}H_{41}N_7O_6$ by elemental analysis and mass spectrometry. The UV spectrum [$\lambda_{max}^{HOAc:MeOH(4:1)}$ 268 nm (ε165), 264(318), 258(406), 252(318) and 247(246)] shows the presence of phyenyl group, which is also supported by ^{1}H-NMR and ^{13}C-NMR spectra. IR bands (ν1650 and 1560 cm^{-1}) suggest the presence of an amide bond. The compound **1** gave positive reactions to chlorine–tolidine, Sakaguchi, diacetyl-α-naphtol, Tollens, triphenyltetrazolium chloride reagents but negative to ninhydrin. The ^{1}H-NMR spectrum of **1** in DMF-d_7 shows the presence of one aldehyde group (δ 9.6, d, J = 1 Hz). Treatment of **1** with acidic methanol afforded a product (**2**), the ^{1}H-NMR and ^{13}C-NMR spectra of which were consistent with the structure for dimethyl acetal monomethyl ester of **1**, indicating the presence of an aldehyde group.

Amino acid analysis of the hydrolyzate (6N HCl, 150°, 48h) of **1** showed phenylalanine (0.78 mole), valine (1.00 mole) and arginine (0.79 mole). Hydrazinolysis (100°, 6h) of **1** gave only phenylalanine, suggesting that phenylalanine is the C-terminal residue in 1.

Hydrolysis of **1** with 0.5N HCl in HOAc at 120° for 40 min afforded L-valine, two ninhydrin-negative and Sakaguchi-positive products (**4** and **5**), and a ninhydrin-negative and triphenyltetrazolium chloride-positive product (**6**) (Chart I).

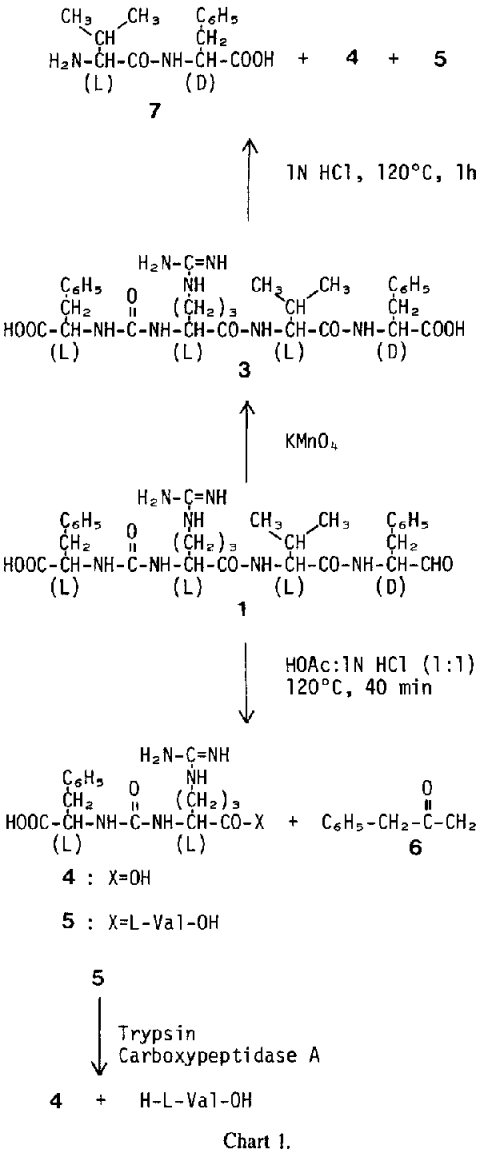

Chart 1.

Amino acid analysis of the hydrolyzate (6N HCl, 150°, 48h) of **4** gave phenylalanine (0.57 mole) and arginine (0.61 mole). The ^{1}H-NMR spectrum of **4** in ND_4OD/D_2O also indicates that **4** is composed of L- or D-phenylalanine and L- or D-arginine. Treatment of **4** with

methanolic HCl afforded its dimethyl ester (**8**). Two OMe signals (δ3.63, 6H) in ^{1}H-NMR spectrum and OMe signals in ^{13}C-NMR spectrum (Table 1) indicate the presence of two methoxy-CO groups in **8**, introduced by esterification. This result, together with three pKa' values (~3, 4.2, >11), leads to the presence of two carboxyl groups in **4**. The ^{13}C-NMR spectrum of **4** in ND_4OD/D_2O, resolves 16 C signals; nine C for phenylalanine residue and 6 for arginine. The residual one signal (δ156.9 or 159.0) could be assigned to ureido CO carbon, on the basis of its chemical shift. Thus, **4** is deduced to be an ureido-type derivative composed of phenylalanine and arginine.

The stereochemistry of phenylalanine and arginine in **4** is disclosed from the following evidence (Chart 2).

Table 1. Chemical shifts of carbon signals of **4**, **8**, **9** and **10** in DMSO-d_6

Carbons	4	8	9	10
Arg γC	25.2	25.1	25.3	25.3
Arg βC	31.1	29.1	29.0	31.0
Arg αC	53.2	52.1	52.3	54.0
Phe αC	55.4	54.2	55.4	54.5
Phe C4	126.2	126.7	125.7	126.6
Phe C3,5	128.1	128.4	127.8	128.4
Phe C2,6	129.5	129.3	129.7	129.3
Phe C1	138.6	137.0	139.2	137.4
Arg δC	157.4	157.1	157.5	157.2
Ureido C	157.7			157.4
Phe C=O	175.4	172.5	175.7	173.4
Arg C=O	175.9	173.3	173.8	176.4
OMe	-	51.9	51.8	51.6

The chemical shifts are given as δc-values in ppm from internal TMS. Carbon signals of arginine δC and phenylalanine βC overlap with that of solvent.

Hydrolysis of dimethyl ester **8** with α-chymotrypsin, which specifically hydrolyzes ester bond adjacent to the CO of L-phenylalanine residue in peptides, afforded quantitatively **9**. The ^{1}H-NMR spectrum of **9** in DMSO-d_6 shows only one OMe proton signal (δ3.62, 3H). In the ^{13}C-NMR spectra of **9** and **8** in DMSO-d_6 (Table 1), five of these 13 signals, having identical δc-values (difference in 0.1~0.5 ppm), are assigned for arginine moiety, and the other seven assigned for phenylalanine (difference in 0.6~3.2 ppm). These results indicate that the ester bond of phenylalanine moiety in **8** is hydrolyzed by the enzyme. Therefore, phenylalanine residue in **8** or **4** is deduced to be L-isomer and the two possible structure for **4**, containing D-phenylalanine, could be ruled out. Hydrolysis of monomethyl ester **9** with trypsin, which specifically hydrolyzes ester bond adjacent to the CO of arginine, afforded a product, which is identified as the starting compound **4** by ^{1}H-NMR, ^{13}C-NMR, IR spectra and hplc. Thus the arginine residue in **9** or **4** is deduced to be L-isomer. Hydrolysis of dimethyl ester **8** with trypsin gave another product **10**, which is assumed to be monomethyl ester derivative, because the ^{1}H-NMR spectrum of **10** in DMSO-d_6 shows one OMe proton signal (δ3.56, 3H). Comparing the ^{13}C-NMR spectra of **8** and **10** in DMSO-d_6 (Table 1), the signals showing different chemical shift are five carbons of arginine residue. These results are rationalized as trypsin catalyzed cleavage of the ester bond of arginine in **8**. It is confirmed that arginine in **8** or **4** is L-isomer. The ester bond of phenylalanine in **10** was hydrolyzed by α-chymotrypsin to regenerate the starting compound **4**, which is identified by ^{1}H-NMR, ^{13}C-NMR, IR spectra and hplc. Thus phenylalanine in **10** or **4** is confirmed to be L-isomer. Accordingly the structure of **4** is established as N-[(S)-1-carboxy-2-phenylethyl]carbamoyl-L-arginine.

The other product **5** gave positive reactions to chlorine-tolidine, Sakaguchi and diacetyl-α-naphtol reagents but negative to ninhydrin. Amino acid analysis of the hydrolyzate (6N HCl, 150°, 44h) gave phenylalanine (0.53 mole), arginine (0.54 mole) and valine (1.00 mole). The ^{1}H-NMR spectrum of **5** in ND_4OD/D_2O also indicates that **5** is composed of phenylalanine, valine and arginine. Hydrolysis of **5** with carboxypeptidase A, which specifically hydrolyzes amido bond of C-terminal

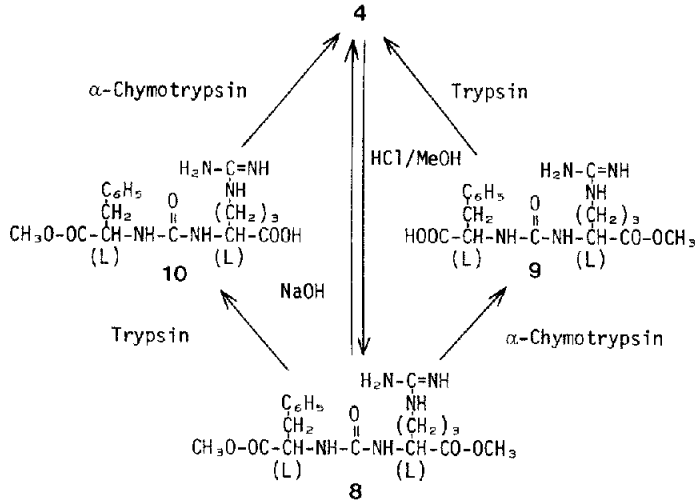

Chart 2.

L-amino acid residue in peptides, gave quantitatively valine and **4**. This indicates that valine residue in **5** is L-isomer and is located at C-terminal. Hydrolysis of **5** with trypsin also gave quantitatively valine and **4**, implying that amino group of L-valine moiety in **5** is joined to the CO group of arginine in **4** and that arginine residue in **5** or **4** is L-isomer. The ^{13}C-NMR spectrum of **5** in ND_4OD/D_2O resolves all carbon signals for the proposed structure of **5**. Thus the structure of **5** is established as N-[(S)-1-carboxy-2-phenylethyl] carbamoyl-L-arginyl-L-valine.

As mentioned above, hydrazinolysis of β-MAPI (**1**) afforded only phenylalanine as its C-terminal residue, indicating that the carbonyl group of L-valine moiety in **1** is joined to another constituent.

The product **6**, isolated from etheral extract of hydrolyzate of **1**, gave positive reaction to triphenyltetrazolium chloride reagent. The molecular formula of **6** is established as $C_9H_{10}O_2$: M^+ *m/z* 150(150.067; calc. for $C_9H_{10}O_2$, 150.068). The ^{1}H-NMR signals of **6** in $CDCl_3$ [δ2.8~3.1(1H, m, disappeared upon deutration), 3.70(2H, s), 4.25(2H, s), 7.1~7.4(5H, m)], ^{13}C-NMR signals [δ45.2, 67.1, 126.8, 128.3, 128.6, 132.7 and 206.6] and IR bands [ν3450(alcoholic OH), 1720(ketone CO) and 1050 cm^{-1}(alcoholic C–O)] show presence of two methylene, one alcoholic OH, one ketone CO and one phenyl groups. The mass spectrum of **6**, besides molecular ion peak at *m/z* 150, shows a peak at *m/z* 119 (M-31, 119.049: calc. for C_8H_7O, 119.050), the formation of which is rationalized as α-cleavage of **6** with loss of hydroxymethylene cations. Successive fragmentation yielded the most abondant peak at *m/z* 91 (M-59, 91.056; calc. for C_7H_7, 91.055) corresponding to tropylium ions, which is interpreted as cleavage of *m/z* 119 ions with loss of CO. The mass spectral analysis of **6** indicates that the structure of **6** is 1-hydroxy-3-phenyl-2-propanone. Finally the structure is unambiguously established by direct comparison with an authentic sample prepared from phenylacetic acid.[3]

Treatment of β-MAPI (**1**) with potassium permanganate in aqueous t-BuOH affordered its oxidation product **3**. The 400 MHz ^{1}H-NMR spectrum of **3** in DMSO-d_6 is presented in Fig. 1. Assignment of individual resonances to proton of constituent amino acid was achieved in decoupling experiments, which allows unambiguous confirmation that **3** is composed of two phenylalanine, valine and arginine (Table 2). Amino acid analysis of the hydrolyzate (6N HCl, 150°, 48h) of **3** showed phenylalanine (1.91 mole), valine (1.00 mole) and arginine (0.81 mole). The compound **3** is resistant to

Table 2. Chemical shifts and coupling constants of proton signals of **3** in DMSO-d_6

Protons	δ value
Hγ in Val	0.79 d (6.9)
Hγ' in Val	0.81 d (6.9)
Hγγ' in Arg	1.31 m
Hβ in Arg	1.44 m
Hβ' in Arg	1.53 m
Hβ in Val	2.06 qqd (6.9/6.9/6.9)
Hδ in Arg	2.79 m
Hδ' in Arg	ca 3.0 m
Hβ-A in Phe I	2.86 dd (13.7/8.1)
Hβ-B in Phe I	3.01 dd (13.7/5.3)
Hβ-A in Phe II	2.89 dd (13.4/6.9)
Hβ-B in Phe II	3.11 dd (13.4/4.6)
Hα in Phe II	4.09 dd (5.0/6.9)
Hα in Val	4.16 dd (9.2/6.4)
Hα in Phe I & Arg	4.30 m
Amido H in Phe I*	6.25 d (7.6)
Amido H in Arg*	6.26 d (8.0)
C_6H_5 in Phe I & II	7.13 s?
	7.19 d (7.5)
	7.26 t (6.7)
NH-C(=NH)NH_2 in Arg	7.1~7.2 m br
Amido H in Phe II	7.35 d (6.9)
Amido H in Val	7.86 d (9.2)
COOH	9.12 s br

The chemical shifts are given as δ-values in ppm from internal TMS. Assignments denoted with * may be interchanged. Coupling constants in Hz are given in parentheses after the multiplicities.

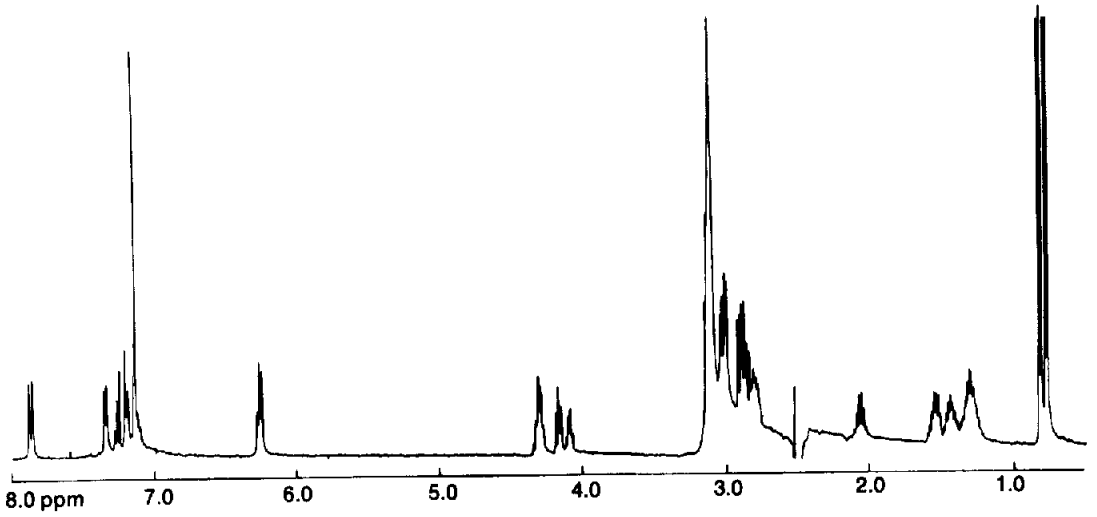

Fig. 1. Partially relaxed FT ^{1}H-NMR spectrum at 400MHz of **3** in DMSO-d_6.

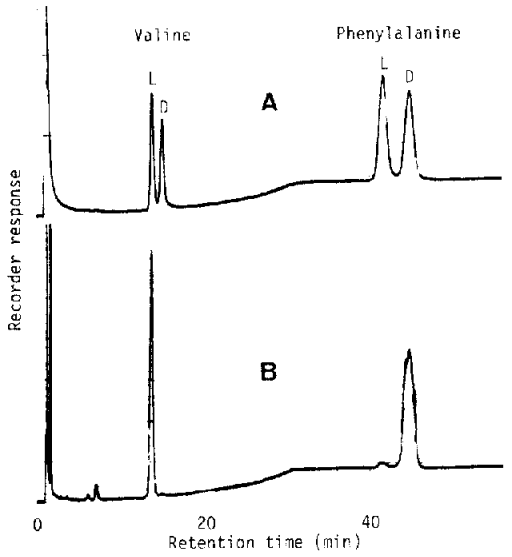

Fig. 2. Determination of the steoreochemistry of 7 by glc. A; authentic enantiomers. B; hydrolyzate of 7.

hydrolysis with proteases (carboxypeptidase or trypsin) in contrast to the oxidation product of α-MAPI (11)[2]. Inhibitory activity of 1 was abolished by oxidation.

Partial hydrolysis of 3 with 1N HCl at 120°C for 1h afforded two new products; 7 and D-phenylalanine, together with 4, 5 and L-valine.

The product 6, isolated from the hydrolyzate of β-MAPI (1), is absolutely absent in the hydrolyzate of 3, indicating that the moiety corresponding to the product 6 is changed by oxidation. This result, together with absolute absence of D-phenylalanine in the hydrolyzate of β-MAPI (1), suggests that 1 contains D-phenylalaninal, which would give the product 6 by acid hydrolysis.

The new product 7 gave positive ninhydrin reaction. On hydrolysis with 6N HCl (110°, 48h), 7 gave an equimolar amount of phenylalanine and valine. N-terminal residue in 7 was identified as valine by Edman degradation.[4] These results indicate that the structure of 7 is assumed to be valylphenylalanine. The absolute structure of 7 is established as L-valyl-D-phenylalanine by glc analysis (Fig. 2), optical rotation, IR, ^{1}H-NMR, ^{13}C-NMR spectra and hplc comparison with authentic samples.

From the results described above, it is concluded that the structure of β-MAPI (1) is N-[(S)-1-carboxy-2-phenylethyl]carbamoyl-L-arginyl-L-valyl-D-phenylalaninal.

It should be noted that β-MAPI (1) is a novel inhibitor different from other proteinase inhibitors,[5] and an optical isomer of α-MAPI (11),[2] with different configuration in the C-terminal phenylalaninal moiety. And it may be worth noting as the first example that optically isomeric peptide aldehyde compounds were isolated in pure state.

EXPERIMENTAL

M.ps were measured with a Kofler hot stage apparatus and are uncorrected. The spectra were recorded on the following instruments: IR spectra, JASCO IRA-20; UV spectra, Hitachi 200-10; Mass spectra, Hitachi RMU-6MG and Hitachi RMU-7M spectrometers; ^{1}H-NMR spectra, Varian EM-390 (90 MHz); ^{13}C-NMR spectra, Varian XL-100 (25.2 MHz). Chemical shift (δ) are presented in ppm from TMS or DSS. The 400 MHz ^{1}H-NMR spectra were recorded on a Bruker WH-400 spectrometer (courtesy of Laboratories of Natural Products Chemistry, Otsuka Pharmaceutical Co., Ltd.). Amino acid analysis was carried out with Hitachi KLA-5 automatic amino acid analyzer. Analytical and preparative hplc was carried out on Hitachi 635 apparatus with following packing materials: LiChrosorb RP-18 for analytical and LiChroprep RP-8 for preparative (Merck). Eluents used in hplc are (A) MeOH:H_2O(8:1); (B) MeOH:0.2 M phosphate buffer(pH 6.8):H_2O(6:1:3); (C), (4:1:5); (D), (3:1:6); (E) MeOH:0.05 M NaOAc(pH5.3): H_2O(44:10:46). Solvents used in tlc are (A) $CHCl_3$: n-BuOH:EtOH:28% aq ammonia: H_2O(20:40:50:27:13); (B) $CHCl_3$: n-BuOH: EtOH:H_2O(1:1:1:0.35); (C) $CHCl_3$:ether(9:1); (D) n-BuOH: EtOH:H_2O(4:1:2).

β-MAPI (1). MAPI in the culture filtrate was isolated according to the method described previously.[1c] β-MAPI was isolated from MAPI mixture by prep hplc with eluent B. Crystallization from aq MeOH gave needles: m.p. 211~212° (dec); $[\alpha]_D^{22.0}$°(Cl, HOAc); ν_{max}^{KBr} 3400sbr, 2950w, 1650sbr, 1560sbr, 1500w, 1450w, 1390mbr, 1240w, 1050w, 750w, 690m cm^{-1}; UV in text; ^{1}H-NMR $\delta_{TMS}^{DMF-d_7}$ 0.7~1.0 [6H, q, J = 7Hz, CH(CH_3)$_2$(in val)], 2.9~1.9[4H, m, CH–CH_2–CH_2 (in arg)], 1.9~2.3[1H, m, $CH(CH_3)_2$(in val)], 2.9~3.4[4H, m, two CH_2 (in phenylalanine and phenylalaninal)], 3.5[2H, t, J = 6Hz, CH_2–NH(in arg)], 4.0~4.7[4H, m, four αCH], 7.3[*ca.* 10H, m, two C_6H_5(in phenylalanine and phenylalaninal)], 6.6~8.6[NH, NH_2, COOH], 9.6[1H, d, J = 1Hz, CHO(in phenylalaninal)]: MS m/z 596(MH^+), 560, 446; R_f on cellulose tlc (solvent A): 0.73; capacity factor (k') on hplc (eluent B): 3.90. (Found: C, 56.46; H, 7.11; N, 15.40. $C_{30}H_{41}N_7O_6 \cdot 2H_2O$ requires: C, 57.04; H, 7.18; N, 15.52%).

β-MAPI Dimethyl acetal monomethyl ester (2). A sol of 1 (100 mg) in 0.1 N HCl/MeOH (4 ml) was allowed to stand at room temp overnight. The reaction was followed by hplc (eluent B), which indicated that the starting material (k' = 3.90) was completely converted to a product (k' = ~11). After removal of solvent *in vacuo*, the residue was precipitated from MeOH–ether to give 2 as homogeneous powder (130 mg): m.p. 154~155° (dec); $[\alpha]_D^{28}$ + 0.8°(Cl, MeOH); λ_{max}^{MeOH} 286nm(ε216), 264(300), 258(380), 252(315), 247(246), 242sh(226); ν_{max}^{KBr} 3300sbr, 2950m, 1730m, 1660s, 1650s, 1560s, 1545s, 1500w, 1440m, 1370mbr, 1220m, 1130m, 1080mbr, 750w, 690m cm^{-1}; ^{1}H-NMR $\delta_{TMS}^{DMSO-D_6}$ 0.55 and 0.59[6H, q, J = 7Hz, CH(CH_3)$_2$(in val)], 1.3~1.6[4H, m, CH–CH_2–CH_2(in arg)], 1.7~1.9[1H, m, $CH(CH_3)_2$(in val)], 2.8~3.2[6H, m, two CH_2(in phenylalanine and phenylalaninal dimethyl acetal) and CH_2–NH(in arg)], 3.36 and 3.31[6H, unresolved singlet, two –OCH_3 (in dimethyl acetal)], 3.59[3H, s, –OCH_3(ester)], 3.9~4.6[5H, m, four αCH and –$CH\langle^{OCH_3}_{OCH_3}$], 6.54 and 6.58[total 2h, d, J = 8Hz, two imino protons], ~7.2[13H, m, two C_6H_5 (in phenylalanine and phenylalaninal dimethyl acetal) and guanidinium protons], 7.6~7.8[2H, m], 7.98[1H, d. J = 8Hz, imino proton]; ^{13}C-NMR $\delta_{TMS}^{DMSO-d_6}$ 17.6 and 19.2(val γγ'C), 24.9(arg γC), 30.2(arg βC), 30.7(val βC), 34.2 and 37.1(phenylalanine and phenylalaninal dimethyl acetal βC), 42.1(arg δC), 51.8 and 52.3(two αC and ester OCH_3), 54.3 (acetal OCH_3), 55.8 and 57.7 (αC), 105.3 (–$CH\langle^{OCH_3}_{OCH_3}$), 125.9 and 126.6(phenyl ring C4), 128.3 (phenyl ring C2, 6), 129.2(phenyl ring C3, 5), 137.0 and 138.9(phenyl ring C1), 157.1 and 157.3(guanidinium and ureido carbons), 170.7, 172.0 and 172.8(carbonyl carbons). (Found: C, 54.65; H, 6.91; N, 13.69. $C_{33}H_{49}N_7O_7 \cdot HCl \cdot 2H_2O$ requires: C, 54.42; H, 7.47; N, 13.46%).

Partial hydrolysis of β-MAPI (1). β-MAPI (2.0 g) was dissolved in HOAc (30 ml) and the sol was mixed with 1N HCl (30 ml). The mixture was heated at 120° for 40 min in a sealed tube. The resulting mixture was extracted with ether (180 ml), the organic layer showed the presence of a product: K' = 3.47 on hplc (eluent D). The aq layer showed the presence of one ninhydrin positive product (R_f 0.45 on cellulose tlc with solvent A), two chlorine-tolidine positive products (k' = 0.23, 2.29 on hplc with eluent D), and the starting material.

Isolation of product 6. The etheral layer was washed twice with water (90 ml), dried over Na_2SO_4. Removal of dried solvent produced a syrup, which was chromatographed on a silica gel column using $CHCl_3$-ether(19:1) to give a residue(100 mg). The residue was purified by Sephadex LH-20 using $CHCl_3$-

MeOH(1 : 1) and prep hplc (eluent A) to give homogeneous **6**. Recrystallization from aq MeOH yielded plates (49 mg); m.p. 47, 48°; $[\alpha]_D^{20}$ 0°(Cl, MeOH); λ_{max}^{Hexane} 300 nm sh(ε76.6), 290(162), 280(216), 273(224), 266(287), 259(300), 254(240), 248 sh(178); ν_{max}^{KBr} 3420s, 3360s, 3020w, 2900w, 1720s, 1500w, 1450m, 1410m, 1320w, 1290m, 1250w, 1135m, 1080m, 1050s, 1000w, 760m, 700s cm^{-1}; ^{1}H-NMR, ^{13}C-NMR and high resolution mass spectra: in text.

The product **6** was identified as 1-hydroxy-3-phenyl-2-propanone by UV, IR, mass, ^{1}H-NMR spectra and hplc by comparison with authentic compound, which was prepared from phenylacetic acid.[3]

Isolation of L-*valine.* The aq layer of etheral extraction of β-MAPI hydrolyzate was neutralized with dil ammonia, and adsorbed into a column of Amberlite XAD-2, eluting with water and aq MeOH to give following compounds; valine was eluted with water, the second product (k' = 0.23 on hplc with eluent D) was eluted with 20% aq MeOH, which was attributed to product **4**. The last compound (k' = 2.29 on hplc with eluent D) was eluted with 60% aq MeOH to give **5**. The product **4** and **5** will be described later. Eluant containing valine was adsorbed into a column of Amberlite IR-120, eluted with 0.5N ammonia to give L-valine which was crystallized from aq EtOH to yield plates (54 mg): m.p. > 260° (sublimation); $[\alpha]_D^{21}$ + 5.3°(CO.3, H_2O), $[\alpha]_D^{20}$ + 60.2°(CO.44, HOAc); reported for L-valine[6a]: $[\alpha]_D^{25}$ + 5.6°(Cl ~ 2, H_2O), $[\alpha]_D^{25}$ + 62.0°(Cl ~ 2, HOAc); which was identified by IR, ^{1}H-NMR spectral comparisons with an authentic sample. (Found; C, 51.10; H, 9.41; N, 11.69. $C_5H_{11}NO_2$ requires: C, 51.26; H, 9.46; N, 11.96%).

Isolation of product **4**. Eluant from Amberlite XAD-2 column with 20% aq MeOH was evaporated to dryness and **4** in the residue was isolated by prep hplc (eluent D) and Amberlite XAD-2 as crystalline powder (309 mg): m.p. 202 ~ 203° (dec); $[\alpha]_D^{27}$ + 18.6° (C1.7, MeOH); $\lambda_{max}^{H_2O}$ 268nm(ε103), 264(142), 258(180), 252(153), 247(121); ν_{max}^{KBr} 3350sbr, 3200mbr, 2950w, 1730mbr, 1660sbr, 1650sbr, 1500w, 1450w, 1400mbr, 1220mbr, 1120wbr, 760w, 690m cm^{-1}; ^{1}H-NMR $\delta_{DSS}^{6\%ND_4OD/D_2O}$ 1.3 ~ 1.8[4H, m, CH–CH$_2$–CH$_2$(in arg)], 2.82 and 3.06[2H, J_{AB} = 13Hz, J_{AX} = 8Hz, J_{BX} = 5Hz, CH$_2$(in phe)], 3.06 [2H, t, J = 6Hz, CH$_2$–CH$_2$–NH(in arg)], 3.79 ~ 4.00[1H, m, CH(in arg)], 4.20[1H, J_{AX} = 8Hz, J_{BX} = 5Hz, CH(in phe)], 7.25[5H, m, C_6H_5(in phe)]; $\delta_{TMS}^{DMSO\text{-}d_6}$ 1.2 ~ 1.8[4H, m, CH–CH$_2$–CH$_2$(in arg)], 2.8 ~ 3.2 [4H, m, CH$_2$(in phe) and CH$_2$–CH$_2$–NH(in arg)], 3.9 ~ 4.3[2H, m, αCH (in arg and phe)], 6.2 ~ 6.5[2H, m, NH(in arg and phe)], 7.2[5H, m, C_6H_5(in phe)], 7.4 ~ 7.8[4H, m, CH$_2$–NH–C(=NH)NH$_2$(in arg)], 8.53[2H, m, carboxyl protons in arg and phe]; ^{13}C-NMR in Table 1; pKa' in H_2O ~ 3, 4.2, > 11. (Found: C, 50.31; H, 6.28; N, 17.94. $C_{16}H_{23}N_5O_5 \cdot H_2O$ requires: C, 50.12; H, 6.57; N, 18.27%).

A sol of **4**(100 mg) in 1N HCl/MeOH (3.5 ml) was heated at 70° for 1 hr. The reaction was followed by hplc (eluent C) which indicated that the starting material (k' = ~0) was completely converted to a product (k' = 4.46). Removal of solvent *in vacuo* gave methylated product **8** as oil (110 mg) which was subjected to the following series of experiment without isolation; λ_{max}^{film} 3300sbr, 3050w, 2950w, 1740s, 1660s, 1560s, 1500w, 1460m, 1440m, 1360mbr, 1220s, 1180w, 1130w, 760w, 700m cm^{-1}; ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$ 1.3 ~ 1.8[4H, m, CH–CH$_2$–CH$_2$(in arg)], 2.85 ~ 3.0[2H, m, CH$_2$(in phe)], 3.1 ~ 3.3[2H, m, CH$_2$–CH$_2$–NH(in arg)], 3.63[6H, s, OCH$_3$], 4.0 ~ 4.3 [1H, m, CH(in arg)], 4.41[1H, dd, J = 7Hz, CH(in phe)], 6.50[1H, d, J = 8.4Hz, NH(in phe)], 6.76[1H, d, J = 8.1Hz, NH(in arg)], 7.1 ~ 7.4 [8H, m, C_6H_5(in phe) and CH$_2$–NH–C(=NH)NH$_2$(in arg)], 7.84[1H, m, CH$_2$–NH–C(=NH)NH$_2$(in arg)]; ^{13}C–NMR in Table 1. The compound **8** was easily hydrolyzed to give **4** in alkaline MeOH sol.

The oil **8**(50 mg) in MeOH(0.5 ml) and 0.05M phosphate buffer pH 6.5 (5 ml) was hydrolyzed with α-chymotrypsin (2 mg, 0.2 ml of 1% sol in 0.0001N HCl) at 37°. The reaction was followed by hplc (eluent C). After 2 hr incubation, **8** was completely converted to a single product (k' = 1.02) which was isolated by prep hplc (eluent C) and Amberlite XAD-2 to give **9** as white powder (37 mg): m.p. 157 ~ 159° (dec); $[\alpha]_D^{27}$ + 21.4°(C1.9, MeOH); λ_{max}^{MeOH} 268nm(ε103), 264(158), 258(207), 252(177), 247(147), 242 sh(134); ν_{max}^{KBr} 3300sbr, 2950w, 1735m, 1660sbr, 1560sbr, 1500w, 1450w, 1440w, 1400m, 1370wbr, 1220w, 1180w, 1130wbr, 760w, 700m cm^{-1}; ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$ 1.3 ~ 1.8[4H, m, CH–CH$_2$–CH$_2$(in arg)], 2.8 ~ 3.2[4H, m, CH$_2$–CH$_2$–NH(in arg) and CH$_2$(in phe)], 3.62[3H, s, OCH$_3$], 3.8 ~ 4.3[2H, m, CH(in arg) and CH(in phe)], 6.02[1H, d, J = 6.6Hz, NH(in phe)], 6.75[1H, d, J = 7.5Hz, NH(in arg)], 7.18 [5H, m, C_6H_5(in phe)], 7.67[4H, m, CH$_2$–NH–C(=NH)NH$_2$(in arg)], 8.72[1H, m, COOH(in phe)]; ^{13}C-NMR in Table 1. (Found: C, 51.24; H, 6.53; N, 17.56. $C_{17}H_{25}N_5O_5 \cdot H_2O$ requires: C, 51.38; H, 6.85; N, 17.62%).

The ester **9**(50.5 mg) in MeOH (0.5 ml) and 0.05 M phosphate buffer pH 6.5 (5 ml) was treated with trypsin (1.4 mg) at 37° for 1.5 hr. The compound **9** was completely converted to a single product, which was isolated by Amberlite XAD-2 column to give **4**(36.1 mg), which was identified as the starting material **4** by IR, ^{1}H-NMR and ^{13}C-NMR spectra. (Found: C, 50.72; H, 6.26; N, 18.44. $C_{16}H_{23}N_5O_5 \cdot H_2O$ requires: C, 50.12; H, 6.57; N, 18.27%).

The ester **8**(50 mg) in MeOH (0.5 ml) and 0.05 M phosphate buffer pH 6.5 (5 ml) was hydrolyzed with trypsin (1 mg) at 37° for 2 hr. A product (k' = 1.63 on hplc with eluent C) was isolated by prep hplc (eluent C) to give **10** (28 mg); m.p. 147 ~ 148°(dec); $[\alpha]_D^{26}$ + 10.5°(CO.5, MeOH); λ_{max}^{MeOH} 268nm(ε101), 264(168), 258(215), 252(174), 247(136), 242 sh(88); ν_{max}^{KBr} resemble to **9** but not identical; ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$ 1.2 ~ 1.9[4H, m, CH–CH$_2$–CH$_2$(in arg)], 2.8 ~ 3.2[4H, m, CH$_2$–CH$_2$–NH(in arg) and CH$_2$ (in phe)], 3.56[3H, s, OCH$_3$], 3.6 ~ 3.9[1H, m, CH(in arg)], 4.33[1H, dd, J = 7Hz, CH(in phe)], 6.19[1H, d, J = 6.9Hz, NH(in arg)], 6.67[1H, d, J = 7.5Hz, NH(in phe)], 7.25[5H, m, C_6H_5(in phe)], 7.5 ~ 7.8[4H, m, CH$_2$–NH–C(=NH)NH$_2$(in arg)], 9.06[1H, m, COOH(in arg)]; ^{13}C-NMR in Table 1.

The ester **10**(25 mg) was converted to a product by α-chymotrypsin. The product was isolated as homogeneous powder (18 mg) by the method described above, and was also identified as the starting material **4** by IR, ^{1}H-NMR and ^{13}C-NMR spectra.

Isolation of product **5**. The other product in the eluent from Amberlite XAD-2 column with 60% aq MeOH described above, was isolated by prep hplc (eluent C) and Amberlite XAD-2 column to give **5** as crystalline powder (135 mg): m.p. 216 ~ 220°(dec); $[\alpha]_D^{19}$ + 9.8°(C1.02, H_2O); $\lambda_{max}^{H_2O}$ 268nm(ε93.4), 264(139), 258(181), 252(150), 247(118); ν_{max}^{KBr} 3300sbr, 2950w, 1730mbr, 1660sbr, 1560sbr, 1500w, 1470w, 1450w, 1400mbr, 1230mbr, 1100mbr, 760w, 700m cm^{-1}; ^{1}H-NMR $\delta_{DSS}^{1\%ND_4OD/D_2O}$ 0.82 and 0.89[each 3H, d, J = 7Hz, CH(CH$_3$)$_2$ (in val)], 1.5 ~ 1.7[4H, m, CH–CH$_2$–CH$_2$(in arg)], 1.9 ~ 2.3[1H, m, CH(CH$_3$)$_2$(in val)], 2.88 and 3.12[2H, J_{AB} = 13Hz, J_{AX} = 8Hz, J_{BX} = 6Hz, CH$_2$(in phe)], 3.05[2H, t, J = 6Hz, CH$_2$–CH$_2$–NH(in arg)], 4.02[1H, d, J = 6 Hz, αCH(in val)], 4.2[1H, m, αCH(in arg)], 4.28[1H, J_{AX} = 8Hz, J_{BX} = 6Hz, αCH(in phe)], 7.3[5H, m, C_6H_5(in phe)]; ^{13}C-NMR $\delta_{DSS}^{1N\,ND_4OD/D_2O}$ 20.0 and 21.5(val γγ'C), 26.9(arg γC), 31.4(arg βC), 33.2(val βC), 40.9(phe βC), 43.2(arg δC), 56.1, 59.3 and 63.2(αC), 129.3(phenyl ring C4), 131.2(phenyl ring C2, 6), 132.0(phenyl ring C3, 5), 140.4(phenyl ring C1), 159.4 and 161.2(guanidinium and ureido carbons), 176.6, 180.8 and 181.6(carbonyl carbons).

The compound **5**(20 mg) in 0.2M N-ethylmorpholine-HOAc buffer pH 8.5(2 ml) was treated with carboxypeptidase A(0.1 mg, 0.1 ml of 0.1% in the same buffer) at 37° and the reaction was monitored by cellulose tlc (solvent A) and hplc (eluent D). After 30 min incubation, **5** was completely hydrolyzed to give valine (R_f 0.45) and a product (R_f 0.25) which was identified as **4** by comparison with tlc and hplc.

Hydrolysis of **5** with trypsin also gave valine and **4**.

Oxidation of β-MAPI (**1**). To a stirred sol of **1**(400 mg) in t-BuOH (130 ml) and H_2O(70 ml) was added pulverized $KMnO_4$ (130 mg) at room temp. Cellulose tlc (solvent A) showed the appearance of a product (R_f 0.57) after 20 min reaction. After 30 min stirring, the excess $KMnO_4$ was decomposed by addition of MeOH. The mixture was filtrated and evaporated to dryness. The residue was suspended in H_2O, the suspension was acidified with dil HCl and extracted with n-BuOH. The organic layer was evaporated to dryness and the residue (320 mg) was subjected to prep hplc (eluent B) to afforded **3**, which was crystallized from aq MeOH to give needles (175 mg): m.p. 279 ~ 281° (dec); $[\alpha]_D^{21}$ + 8°(CO.5, 1N NH_4OH); $\lambda_{max}^{0.2N\,NH_4OH}$ 268nm (ε278), 264(409), 258(535), 252(440), 247(330); ν_{max}^{KBr} 3300s, 3050w, 2950w, 1710m, 1650msh, 1620s, 1550s, 1500w, 1440wbr, 1390mbr, 1220m, 1100w, 760w, 720w, 690w cm^{-1}; ^{1}H-NMR in Fig. 1 and Table 2. The compound showed no inhibitory effect on various proteinases