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des-Asp[4]-Amastatin, MRK-22 as an Inhibitor of Aminopeptidase M produced by *Streptomyces* sp. SL20209

KO, HACK-RYONG, HYO-KON CHUN, SEUNG-HO KIM[1], NACK-KIE SUNG[2] AND YUNG-HEE KHO*

Microbial Chemistry R.G. and [1]Protein Engineering R.G., Korea Research Institute of Bioscience and Biotechnology, KIST, P.O. Box 115, Yusung, Taejeon 305-600, Korea
[2]Department of Food Science and Technology, Gyeongsang National University, Chinju 660-701, Korea

MRK-22, an inhibitor of aminopeptidase M was isolated from the culture broth of *Streptomyces* sp. SL20209. The structure of MRK-22 was defined to be 3-amino-2-hydroxy-5-methylhexanoyl-valyl-valine, des-Asp[4]-amastatin, by spectroscopic analysis and this was also confirmed by solid phase synthesis of the inhibitor. The molecular formula and weight of MRK-22 were $C_{17}H_{33}N_3O_5$ and MW 359(M^+), respectively, and its IC_{50} value against hog kidney AP-M was 0.79 μg/ml.

Aminopeptidase M (AP-M; EC 3.4.11.2) is a membrane-bound metallo exopeptidase and participates in various cellular phenomena (6). Its inhibitors have been reported to have many kinds of therapeutical effects including immunopotentiation, antitumour activity and analgesia (3, 4, 7).

In the course of the screening of AP-M inhibitors, we isolated *Streptomyces* sp. SL20209 from a soil sample as a strain producing the inhibitor. In the preceeding paper (5), we reported the isolation and structural determination of valistatin, a new inhibitor of aminopeptidase M, produced by *Streptomyces* sp. SL20209. Valistatin is a tripeptide, having the structure of 3-amino-2-hydroxy-4-phenylbutanoyl-valyl-valine. Additionally, it was found that another inhibitor of AP-M named MRK-22 was also present in the culture broth of *Streptomyces* sp. SL20209. Accordingly, in this paper we report the structural determination of MRK-22.

MATERIALS AND METHODS

Enzyme and Substrate

AP-M (EC 3.4.11.2) from hog kidney and synthetic L-leucine-p-nitroanilide were purchased from Sigma Co. (USA) and used as enzyme and substrate for the assay for AP-M inhibitory activity, respectively.

Microorganism

Streptomyces sp. SL20209 was isolated from a soil sample by the Screening Room of the Korea Research Institute of Bioscience and Biotechnology and its taxonomic studies will be published elsewhere.

Assay for AP-M Inhibitory Activity

The assay for AP-M inhibitory activity was performed as previously reported (5), and the activity was described as percent inhibition.

Production and Isolation of MRK-22

MRK-22 was co-produced with valistatin in the culture broth of *Streptomyces* sp. SL20209. Therefore the procedure for isolation of MRK-22 was identical with that of valistatin as previously reported (5), but two inhibitors were finally seperated by a reverse phase HPLC using a Phenomenex $5C_{18}$ column(ϕ 10.6 × 250 mm, flow rate 2.0 ml/min, UV 210 nm) with a 35 : 65 mixture of methanol and water. Thus, a peak with 22 minutes of retention time was collected as an active compound and named MRK-22.

Instrumental Analysis

Analysis for amino acid and spectroscopic studies were performed as described previously (5).

Solid Phase Synthesis of MRK-22

MRK-22 was automatically synthesized by the solid phase method (8) using an Applied Biosystems (Model 431A) peptide synthesizer on HMP-resin (4-hydroxymethylphenoxy methyl copolystyrene-1% divinylbenzene). The *t*-BOC-(2*S*, 3*R*)-3-amino-2-hydroxy-5-methylhexanoic acid(*t*-BOC-AHMHA) used in peptide synthesis was purchased from Sigma (USA). Purification of the inhibitor from a cocktail of synthesis reactions was carried out by HPLC on a Phenomenex ODS column ($5C_{18}$ HS,

*Corresponding author
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10.6 × 250 mm, 35% MeOH). Active fractions were combined and concentrated under reduced pressure in a small volume of water, then lyophilized under a freeze dryer to give an amorphous whitish powder.

RESULTS AND DISCUSSION

The active peak with 22 minutes of retention time through a reverse phase HPLC was collected and concentrated to dryness yielding a whitish powder and this compound, showing the inhibitory activity against AP-M, was designated as MRK-22. MRK-22 has no characteristic ultraviolet absorption (data not shown). The IR spectrum (KBr) of the inhibitor showed absorption bands at 3200~3400 cm^{-1} and 1520~1660 cm^{-1} indicating the presence of peptide bonds (Fig. 1). Acid hydrolysis of MRK-22 with HCl at 105℃ for 20 hours yielded two ninhydrin positive products on TLC chromatography. Amino acid analysis of the hydrolysates indicated that MRK-22 contained one mole of an unknown amino acid and two moles of valine(Fig. 2). The retention time of an unknown amino acid in MRK-22 was similar to that of leucine. The ^{1}H-NMR data and spectrum are shown in Table 1 and Fig. 3, respectively, and the assignments defined by ^{1}H-^{1}H COSY NMR (data not shown).

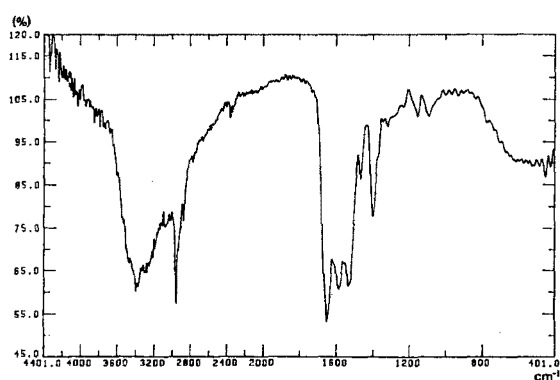

Fig. 1. IR spectrum of MRK-22.

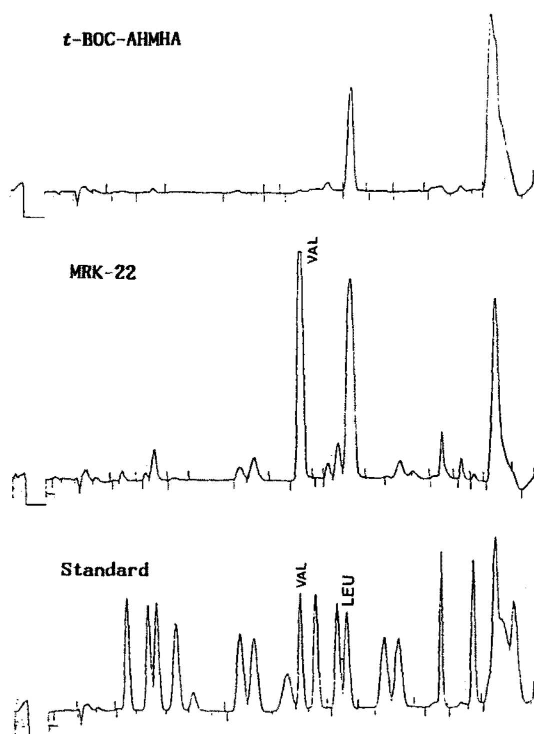

Fig. 2. Amino acids analysis of the acid-hydrolysate of MRK-22 and (2*S*, 3*R*)-AHMHA.

The ^{1}H-NMR spectrum in deutero methanol indicated the presence of six methyl groups (at 1.00, 1.00, 0.95, 0.91, 1.02, and 1.00 ppm, respectively), one methylene group (at 1.64 and 1.48 ppm) and seven methine groups (at 4.24, 3.54, 1.77, 4.19, 2.16, 4.06 and 2.29 ppm, respectively). The existence of a $(CH_3)_2$-CH-CH_2-CH and two of $(CH_3)_2$-CH-CH- was confirmed by the ^{1}H-^{1}H COSY NMR spectrum. The FAB-MS spectrum of MRK-22 showed significant peaks at *m/z* 360(M+H), 314, 275(+2H), 215, 131(+H), 116 and 86 (Fig. 4). Among these fragment ions *m/z* $360(M+H)^+$ was determined to be the molecular ion peak. From the fragmentation patterns, it was expected that MRK-22 contained AHMHA (3-amino-2-hydroxy-5-methylhexanoic acid), an N-terminal amino acid of amastatin known as AP-A inhibitor (9). Therefore, the peaks at *m/z* 275 (+2H), 215 and 116 were derived from the elimination of H_2N-CH-$CH_2CH(CH_3)_2$ in AHMHA, AHMHA and Val_1-Val_2-C=O from the parent peak (m/z 360, M+H), respectively. The significant peak at *m/z* 86 was derived from elimination of HO-CH-C=O from AHMHA. Also, this fragmentation pattern (i.e. fragment ion peaks such as *m/z* 215 and 116) indicated that the sequence of amino acid components in MRK-22 should be AHMHA-Val-Val in order.

Table 1. ^{1}H-and ^{1}H-^{1}H COSY NMR data for MRK-22(δ CD_3OD, 300 MHz).

Assignment		MRK-22
AHMHA	2-CH	4.24(d, *J*=2.4[a])
	3-CH	3.54(m)
	4-CH_2	1.64(m), 1.48(m)
	5-CH	1.77(m)
	CH_3	1.00(d, *J*=6.6)
	CH_3	1.00(d, *J*=6.6)
Val_1	α-CH	4.19(d, *J*=3.9)
	β-CH	2.16(m)
	CH_3	0.95(d, *J*=6.6)
	CH_3	0.91(d, *J*=6.6)
Val_2	α-CH	4.06(d, *J*=7.5)
	β-CH	2.29(m)
	CH_3	1.02(d, *J*=6.6)
	CH_3	1.00(d, *J*=6.6)

[a]Coupling constant in Hz.

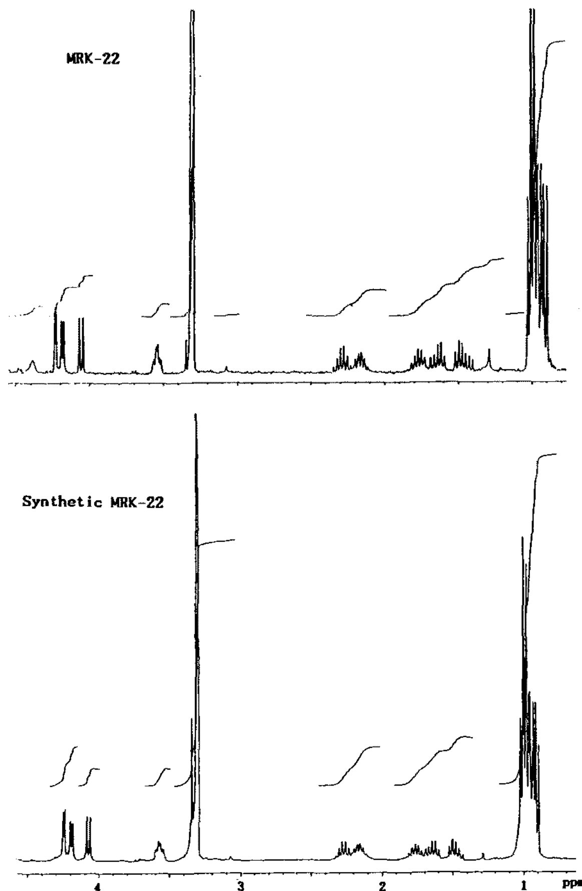

Fig. 3. ¹H-NMR spectrum of MRK-22 in CD_3OD.

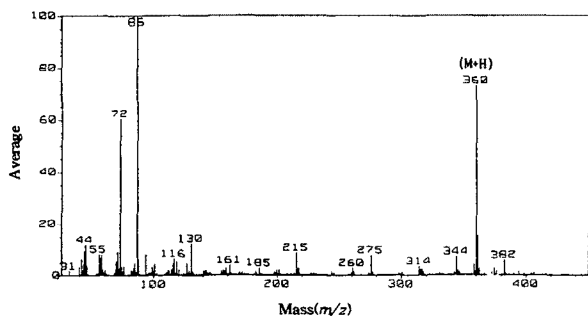

Fig. 4. FAB-MS spectrum of MRK-22.

To confirm the presence of AHMHA in the MRK-22 molecule, amino acid analysis of the MRK-22 and standard compound hydrolyzed under the same condition was reperformed. As shown in Fig. 2, the retention time of the other amino acid (other than valine) was identical with that of authentic (2*S*, 3*R*)-AHMHA. This was supported by HPLC and TLC chromatographies shown in Table 2. Thus, the structure of MRK-22 was determined to be 3-amino-2-hydroxy-5-methylhexanoyl-valyl-valine (Fig. 5). 3-Amino-2-hydroxy-5-methyl-hexanoyl-valyl-valine was synthesized by solid phase method. The retention time of the synthesized compound was 22 minutes in the HPLC system as described in Materials and Methods, and was identical with that of the microbial product.. The ¹H-NMR spectrum of synthesized compound was also the same as that of microbial product (Fig. 3).

Table 2. Identification data for (2*S*, 3*R*)-AHMHA and L-valine composed MRK-22 by HPLC and TLC analysis.

Compounds	Retention time (min) on HPLC[a]	Rf value on TLC[b]
(2*S*, 3*R*)-AHMHA	11.28	0.40
L-Valine	16.22	0.39
Hydrolysate of MRK-22		
AHMHA	11.34	0.42
Valine	16.25	0.39

[a]On the reverse column of Lichrosorb RP-18(4.6×250 mm), flow rate 1.0 ml/min, eluant MeCN-0.1% TFA(12:88). [b]On the silica gel TLC plate 60 F_{254}(Merck), mobile phase BuOH-AcOH-Water(4:1:1).

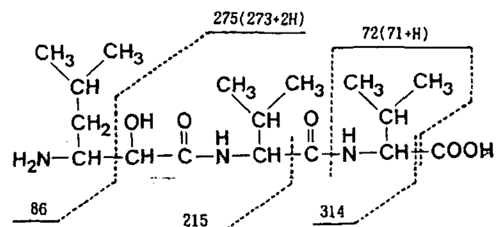

Fig. 5. Structure of MRK-22.

On the other hand, Aoyagi *et al.* (1) reported amastatin, an inhibitor of AP-A, produced by actinomycetes, thereafter the structure of amastatin was determined to be [(2*S*, 3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-L-valyl-L-valyl-L-aspartic acid by Tobe *et al.* (9). Therefore, MRK-22 is des-Asp⁴-amastatin. Tobe *et al.* (10) chemically synthesized the stereoisomers and analogues of amastatin to test their structure-activity relationship. Of them, a compound having the identical structure with that of MRK-22 was synthesized. However, the microbial production of 3-amino-2-hydroxy-5-methylhexanoyl-valyl-valine is reported for the first time in this paper. The presence of des-Asp⁴-amastatin may not be due to the degradation of amastatin in the course of isolation since the active retention time of the *n*-butanol extract of the culture broth is the same as that of MRK-22. To confirm whether MRK-22 is the biosynthetic intermediate or enzymatic hydrolysis product of amastatin, further research will be required.

MRK-22 produced by *Streptomyces* sp. SL20209 was soluble in water and methanol but not in chloroform, ethyl acetate or *n*-hexane. The molecular weight and formula were proposed as $C_{17}H_{33}N_3O_5$ (MW 359) from FAB-MS and ¹H-NMR spectra. Thin layer chromatography

on silica gel 60 F_{254} (Merck), MRK-22 gave a single spot at Rf 0.40 developing with *n*-butanol-acetic acid-water (4:1:1). The IC_{50} value of MRK-22 against hog kidney AP-M was 0.79 μg/ml while the value of amastatin was 0.56 μg/ml. Tobe *et al.* (10) reported that in a study of the relationship of the lenght of the peptide chain and activity, a tetrapeptide such as amastatin has the strongest activity towards AP-A. Therefore, the lower activity of MRK-22 than amastatin against AP-M may be due to a shortage of peptide length.

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