Notes

AM4299 A AND B, NOVEL THIOL PROTEASE INHIBITORS

ATSUKI MORISHITA, SATORU ISHIKAWA, YASUNOBU ITO, KIYOSHI OGAWA, MASAKI TAKADA, SATOSHI YAGINUMA and SHUJI YAMAMOTO

Institute for Life Science Research, Asahi Chemical Industry Co., Ltd., Ohito, Shizuoka 410-23, Japan

(Received for publication May 11, 1994)

Low molecular weight protease inhibitors are useful as medicines or reagents for research. During our screening for thiol protease-specific inhibitors from culture filtrates of microorganisms, we have succeeded in the isolation of new compounds, estatins A and B[1]. We have further screened for inhibitors against cathepsin B, and discovered additional new inhibitors named AM4299 A and B from the culture filtrate of fungi.

The producing culture was isolated from a soil sample collected in Iriomote island, Okinawa Prefecture, Japan. This strain was identified as *Chromelosporium fulvum* on the basis of its morphological properties[2,3], and was assigned *C. fulvum* M4299 (FERM P-13825).

In this paper, we report the isolations, physicochemical properties and inhibitory activities of AM4299 A and B.

Inhibitory activities of compounds against cathepsin B (from bovine spleen, Sigma) and cathepsin L (from human kidney, Nova Biochem) were measured acccording to the method of BAJOKOVSKI[4] using α-*N*-benzyloxycarbonyl-*L*-lysine *p*-nitrophenyl ester (Sigma) as a substrate. The activities of papain (from *Carica papaya*, Boehringer), trypsin (from bovine pancreas, Sigma) and α-chymotrypsin (from bovine pancreas, P-L Biochemicals) were measured according to the method reported previously[1]. To determined the concentration of an inhibitior required for 50% inhibition (IC_{50}), the percent inhibition was calculated as follows: % inhibition = 100(A − B)/A; where A is the optical absorbance at 280 nm of the reaction mixture without inhibitor and B is the absorbance with inhibitor.

For the production of AM4299 A and B, *C. fulvum* M4299 was inoculated into a 500-ml Erlenmyer flask containing 100 ml of seed medium composed of glucose 1%, dextrin 1%, yeast extract 0.5%, casein hydrolysate 0.5%, $CaCO_3$ 0.1% and Celite 1% (pH 7.0). The seed culture was incubated for 3 days at 26°C on a rotary shaker (200 rpm). One hundred ml of the culture were then transferred to a 30-liter jar fermentor containing 20 liters of fermentation medium having the following composition: glucose 2%, peptone 1%, corn steep liquor 1%, KH_2PO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1% and FS-antifoam (Dow Corning K. K.) 0.02% (pH 6.5). The fermentation was carried out for 4 days at 26°C with agitation at 200 rpm and air flow at 20 liters/minute.

To isolate AM4299 A and B, the fermentation broth (20 liters × 4) was filtered, and the filtrate was adsorbed on a carbon column (5 liters). The isolation procedure of AM4299 is shown in Fig. 1. These inhibitors were isolated by column chromatography using carbon, cation-exchange and anion-exchange resins and were successively separated into two components by silica gel chromatography. Purification of each component was achieved by CM-Sephadex and Sephadex G-15 chromatography. AM4299 A and B were isolated as white powder. The purity of each preparation was examined by TLC (plate; Kieselgel 60 F_{254} (Merck), solvent system; $CHCl_3$ - MeOH - acetic acid (5 : 5 : 0.5)).

AM4299 A and B were stable at pH 2.0, 7.0 and 9.0 at 60°C for 30 minutes. They were soluble in water, acetic acid, dimethyl sulfoxide and pyridine, and insoluble in most organic solvents such as ethyl acetate, chloroform and benzene. Positive Feigl and thiosulfate tests[5] indicate the existence of 1,2-dicarboxylic acid and epoxide groups in AM4299 A and B. However, positive reaction for ninhydrin with AM4299 B indicates the existence of an amino group in the molecule. The IR spectra of AM4299 A and B suggested the existence of amide I, carbonyl (1640 cm^{-1}), amide II (1560 cm^{-1}), epoxide (900 cm^{-1}) and amine groups (3400 cm^{-1}). Other physico-chemical properties are listed in Table 1. Elemental analysis and FAB-MS established the molecular formulas of $C_{15}H_{26}O_6N_2$ for AM4299 A and $C_{16}H_{27}O_7N_3$ for AM4299 B.

When AM4299 A and B were hydrolyzed with 6 N HCl (105°C, 18 hours) and the hydrolysates analyzed for the presence amino acid, leucine was detected in AM4299 A and leucine and lysine were

Fig. 1. Isolation and purification of AM4299 A and B.

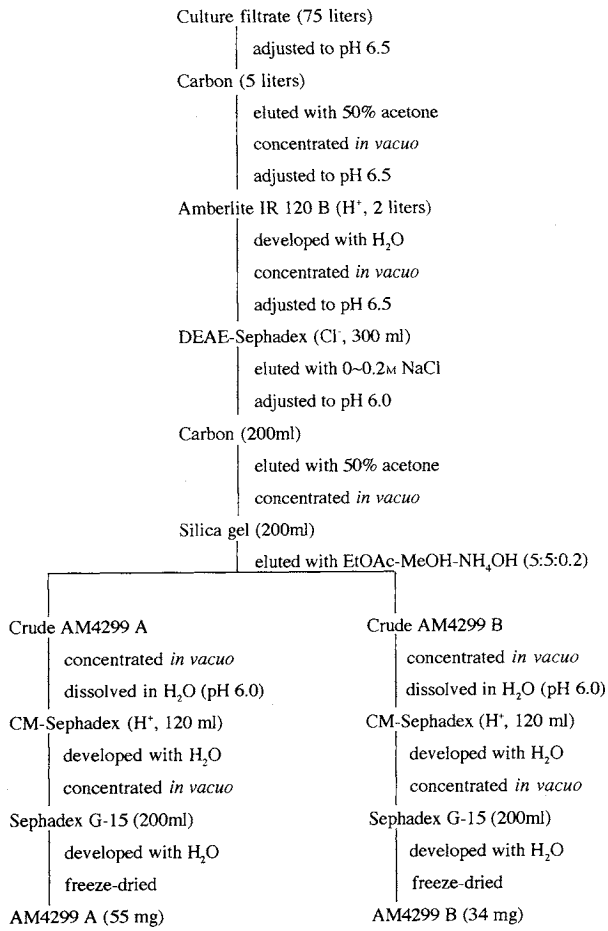

Table 1. Physico-chemical properties of AM4299 A and B.

	A	B
Elemental analysis	$C_{15}H_{26}O_6N_2 \cdot 2H_2O$	$C_{16}H_{27}O_7N_3 \cdot H_2O$
Calcd:	C 49.17, H 8.25, N 7.65	C 46.94, H 7.63, N 10.26
Found:	C 49.38, H 7.96, N 7.91	C 47.14, H 7.75, N 10.72
FAB-MS	*m/z* 331 (M+H)	*m/z* 375 (M+H)
$[\alpha]_D^{22}$	+14.6° (*c* 0.5, H_2O)	−7.2° (*c* 0.5, H_2O)
Amino acid composition		
	leucine	leucine (1 mol), lysine (1 mol)
Color reactions		
Positive:	Thiosulfate, Feigl	Thiosulfate, Feigl, Ninhydrin
Negative:	Ninhydrin, Molisch	Molisch
Rf value	0.24[a]	0.19[b]

[a] plate: Kieselgel 60 F_{254} (Merck), solvent: $CHCl_3$ - MeOH - acetic acid (5:2:0.1).
[b] plate: Kieselgel 60 F_{254} (Merck), solvent: $CHCl_3$ - MeOH - acetic acid (5:5:0.5).

Table 2. ^{1}H NMR data for AM4299 A and B.

A			B		
Position	Chemical shift	Coupling constant (J, Hz)	Position	Chemical shift	Coupling constant (J, Hz)
8	0.89 (3H, d)	6.4	8	0.90 (3H, d)	5.4
9	0.93 (3H, d)	6.4	9	0.94 (3H, d)	5.4
13	1.33 (2H, m)		13	1.40 (2H, m)	
12, 14	1.50~1.58 (4H, m)		6, 7, 12	1.53~1.67 (5H, m)	
6, 7	1.60~1.68 (3H, m)		14	1.88 (2H, m)	
11	3.21 (2H, m)		11	3.23 (2H, m)	
2	3.43 (1H, d)	2.4	2	3.45 (1H, d)	2.0
3	3.57 (1H, d)	2.4	3	3.59 (1H, d)	2.0
15	3.59 (2H, d)	6.8	15	3.74 (1H, t)	6.1
5	4.31 (1H, t)	5.6	5	4.32 (1H, t)	4.9

^{1}H NMR spectra were recorded at 400 MHz in D_2O at 27°C.

Fig. 2. Structures of AM4299 A and B.

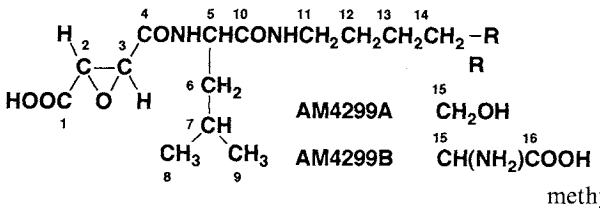

Table 3. ^{13}C NMR data for AM4299 A and B.

A		B	
Position	Chemical shift (δ, ppm)	Position	Chemical shift (δ, ppm)
		16	175.6 (s)
10	175.0 (s)	10	175.1 (s)
1	174.7 (s)	1	174.6 (s)
4	170.7 (s)	4	170.7 (s)
15	62.6 (t)	15	55.6 (d)
2	55.3 (d)	2	55.3 (d)
3	53.8 (d)	3	53.9 (d)
5	53.7 (d)	5	53.7 (d)
6	40.8 (t)	6	40.8 (t)
11	40.1 (t)	11	39.9 (t)
14	31.8 (t)	14	31.0 (t)
12	29.0 (t)	12	29.0 (t)
7	25.3 (d)	7	25.4 (d)
9	23.3 (q)	9	23.1 (q)
13	23.0 (t)	13	22.8 (t)
8	21.7 (q)	8	21.7 (q)

^{13}C NMR spectra were recorded at 100 MHz in D_2O at 27°C.

detected in AM4299 B.

The ^{1}H NMR (400 MHz, D_2O) and ^{13}C NMR (100 MHz, D_2O) data for AM4299 A and B are shown in Tables 2 and 3. In the ^{1}H-^{1}H COSY spectrum of AM4299 A, the connectivity between the epoxide methine protons 2-H and 3-H was observed. Spin proton systems were also observed from the methine proton (5-H) to methyl protons (8-H and 9-H) through methylene protons (6-H) and the methine proton (7-H), and from methylene protons (11-H) to methylene protons (15-H) through methylene protons (12-H, 13-H and 14-H). From these data, partial structures of epoxysuccinate, leucine and aminopentyl alcohol moieties were assigned. Similarly, partial structures of AM4299 B were assigned. C-H connectivity was assigned by ^{13}C-^{1}H COSY. In the heteronuclear multiple-bond connectivity (HMBC) spectra of AM4299 A and B, the carbonyl carbons of leucine (C-10) showed long-range CH couplings with methylene protons (11-H), and the carbonyl carbons of epoxysuccinates (C-4) showed long-range CH couplings with methine protons (5-H). Based on these data and DEPT spectrum, the connectivities of amino acids and epoxysuccinates were assigned. We propose the structures for AM4299 A and B as shown in Fig. 2. The structures were similar to E-64[5,6] and estatins[1], which were isolated from fungal strains as thiol protease-specific inhibitors. All of these compounds have amino acids and epoxysuccinate moieties but AM4299 A and B differ from others in lacking agmatine moieties in their structures.

AM4299 A and B had no antimicrobial activity against bacteria and fungi at least 100 μg/ml, and had a low toxicity; no deaths occurred after its intravenous injection of 200 mg/kg to mice. AM4299 A and B exihibited strong inhibitory activities against cathepsin B, cathepsin L and papain but showed no inhibition against α-chymotrypsin and trypsin (Table 4). Accordingly, AM4299 A and B

Table 4. Inhibitory effect of AM4299 A and B on various proteases.

Enzyme	Quantity of enzyme (μg)	IC_{50} (M)	
		AM4299 A	AM4299 B
Cathepsin B (bovine spleen)[a]	0.7	7.3×10^{-8}	1.3×10^{-7}
Cathepsin L (human kidney)[b]	0.5	3.9×10^{-7}	1.0×10^{-6}
Papain[c]	20	8.8×10^{-8}	2.8×10^{-7}
Trypsin[a]	40	$>8.0\times10^{-5}$	$>7.0\times10^{-6}$
α-Chymotrypsin[d]	12	$>8.0\times10^{-5}$	$>7.0\times10^{-6}$

[a] Sigma.
[b] Nova Biochem.
[c] Boehringer.
[d] P-L Biochemicals.

were specific inhibitors of thiol proteases. Thiol proteases have been implicated to involve in resorption of the organic matrix of bone and some inhibitors of thiol proteases have been found to reduce this process[7,8]. Thiol protease activity is also essential in providing nutrients for the growth and survival of schistosomula in its mammalian host. ZARDA *et al.*[9] showed that inhibitors of the enzymes repress the growth and survival of the parasite. Thus, thiol protease specific inhibitors like AM4299 A and B may be useful for treatment of osteoporosis and as anti-parasitic agents.

Acknowledgments

We are indebed to Mrs. Y. YATA and Miss N. UMEHARA for their technical assistance.

References

1) YAGINUMA, S.; A. ASAHI, A. MORISHITA, M. HAYASHI, M. TSUZINO & M.TAKADA: Isolation and characterization of new thiol protease inhibitors estatins A and B. J. Antibiotics 42: 1362~1369, 1989
2) HENNEBERT, G. L.: *Botrytis*-like genera. Persoonia 7: 183~204, 1973
3) HENNEBERT, G. L. & R. P. KORF: The peat mold, *Chromelosporium ollare*, conditional state of *Peziza ostracoderma*, and its misapplied names, *Botrytis crystallina*, *Botrytis spectabilis*, *Ostracoderma epigaeum* and *Peziza atrovinosa*. Mycologia 67: 214~240, 1975
4) BAJKOWSKI, A. S. & A. FRANKETER: Specific spectrophotometric assays for cathepsin B_1. Anal. Biochem. 68: 119~127, 1975
5) HAMADA, K.; M. TAMAI, M. YAMAGISHI, S. OHMURA, J. SAWADA & I. TANAKA: Isolation and characterization of E-64, a new thiol protease inhibitor. Agric. Biol. Chem. 42: 523~528, 1978
6) HAMADA, K.; M. TAMAI, S. OHMURA, J. SAWADA, T. SEKI & I. TANAKA: Structure and synthesis of E-64, a new thiol protease inhibitor. Agric. Biol. Chem. 42: 529~536, 1978
7) DELAISSE, J. M.; Y. EECKHOUT & G. AAES: Inhibition of bone resorption in culture by inhibitiors of thiol proteinases. Biochem. J. 192: 365~368, 1980
8) DELAISSE, J. M.; A. BOYDE, E. MACONNACHIE, N. N. ACI, C. H. J. SEAR, Y. EECKHOUT, G. VAES & S. J. JONES: The effects of inhibitors of cysteine-proteinases and collagenase on the resorptive activity of isolated osteoclasts. Bone 8: 305~313, 1987
9) ZERDA, K. S.; M. H. DRESDEN & C. L. CHAPPELL: *Schistosoma mansoni*: Expression and role of cysteine proteinases in developing schistosomula. Experimental Parasitology 67: 283~246, 1988