NOTES

Potent Inhibitors of Cysteine Proteases from the Marine Fungus *Microascus longirostris*†

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Specific and selective protease inhibitors are potentially powerful tools in clinical therapy. These inhibitors could be used to inactivate the target proteases in the pathogenic processes of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancers, AIDS, and many others[1~3].

During our screening for biologically active compounds from marine sources, a strain of the fungus *Microascus longirostris* was found to produce secondary metabolites that strongly inhibited cysteine proteases. Bioassay-guided fractionation of the methanolic extract of the mycelia led to the isolation of three potent protease inhibitors. Based on spectroscopic data obtained from these three compounds, we have identified them as the epoxysuccinates **1**, **2** and **3**. While this work was being completed, another research group[4] reported the isolation of compounds **1** and **2**, or cathestatins B and A respectively, from a different fungal source (*Penicillium citrinum*). In this paper, we describe the production, isolation, structure determination, and inhibitory activities of cathestatins A and B (**2**, **1**), and of cathestatin C (**3**), a new and unreported member of this class of compounds.

The producing strain SF-73 was isolated from a marine sponge collected at Harrington Point, Otago Harbor, New Zealand, and was inoculated into ten 500-ml Erlenmeyer flasks each containing 100 ml of 2% molasses, 3% dextrin, 1.5% fish meal and 1.5% protein hydrolysate. The fermentation was carried out for 7 days at 25°C on a rotary shaker (210 rpm).

The active methanolic extract (4 g) of the mycelia was fractionated by reversed phase flash column chromatography, and the active fractions were then further purified by Sephadex LH-20 and reversed phase flash column chromatography. Finally, three extremely water soluble inhibitors, cathestatins A (3.6 mg), B (6.2 mg) and C (0.8 mg), were obtained by reversed phase high performance liquid chromatography on a Zorbax SB C-18 column eluting with 3.5% aqueous acetonitrile.

Compound **1**, an amorphous white solid, was determined to have the molecular formula $C_{17}H_{23}N_3O_6$ (*m/z* 366.1657 (MH^+), ΔM −0.8 mmu) on the basis of high resolution electrospray mass spectral data. The ^1H NMR (500 MHz) and ^{13}C NMR (125.7 MHz) data in D_2O are shown in Table 1 (the NMR data in the earlier work[4] were obtained in DMSO-d_6, and differed somewhat from those reported here). Together with the molecular formula, the NMR data suggested that this compound is a small peptide. The DEPT and HMQC experiments indicated the connectivities between protons and carbons, and the individual partial structures of epoxy-succinate, tyrosine and 1,4-diaminobutane were further assigned by interpretation of the ^1H-^1H COSY spectrum. Finally, the long-range ^1H-^{13}C correlation HMBC data in D_2O, as well as ^1H-^1H COSY and NOESY data in DMSO-d_6, allowed the assembly of partial structures into the final structure which is identical with the published cathestatin B[4] (Fig. 1). It is noteworthy that the latter NOESY experiment was the only way to distinguish the two epoxy carbons by the observation of the correlation between H-3 (δ 3.32) and the NH (δ 9.45) of the tyrosine moiety.

Compound **2** was also obtained as an amorphous white solid. The ^1H and ^{13}C NMR spectral data (Table 1) were similar to those of **1**. The molecular formula $C_{17}H_{23}N_3O_5$ (*m/z* 350.1716 (MH^+), ΔM 0.0 mmu), determined by high resolution electrospray mass spectrometry, indicated one oxygen atom less than in compound **1**. Comparison of the NMR data for **1** and **2** showed that the phenolic C-10 (δ 158.39) in **1** was replaced by an aromatic methine (δ_H 7.13, δ_C 130.05) in **2**. Therefore, the tyrosine residue in **1** is substituted by a phenylalanine in **2** and the full structure, confirmed by routine COSY, HMQC and HMBC experiments, was the same as that published for cathestatin A[4] (Fig. 1).

Fig. 1. Structures of cathestatins A, B and C.

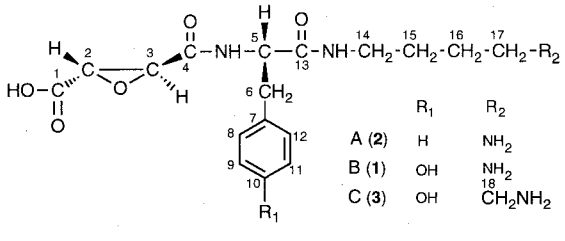

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Table 1. ^{1}H- and ^{13}C NMR data (D_2O) for Cathestatins A~C (**1**~**3**).

Position	Cathestatin B (**1**)		Cathestatin A (**2**)		Cathestatin C (**3**)	
	δ ^{13}C (ppm)	δ ^{1}H (ppm)	δ ^{13}C (ppm)	δ ^{1}H (ppm)	δ ^{13}C (ppm)	δ ^{1}H (ppm)
1	176.46	—	176.42	—	176.47	—
2	57.11	3.08 (d)	57.11	3.02 (d)	57.16	3.08 (d)
3	55.56	3.32 (d)	55.54	3.30 (d)	55.60	3.32 (d)
4	172.28	—	172.34	—	172.25	—
5	58.42	4.24 (t)	58.13	4.33 (t)	58.38	4.25 (t)
6	38.89	2.78 (d)	39.67	2.84 (dd)	38.95	2.79 (d)
				2.93 (dd)		
7	130.14	—	139.03	—	130.82	—
8	133.36	6.91 (d)	131.99	7.07 (d)	133.40	6.94 (d)
9	118.68	6.62 (d)	131.61	7.19 (t)	118.35	6.66 (d)
10	158.39	—	130.05	7.13 (t)	157.37	—
11	118.68	6.62 (d)	131.61	7.19 (t)	118.35	6.66 (d)
12	133.36	6.91 (d)	131.99	7.07 (d)	133.40	6.94 (d)
13	175.40	—	175.36	—	175.23	—
14	41.26	2.87 (m)	41.29	2.94 (m)	41.65	2.83 (m)
		2.97 (m)				2.95 (m)
15	28.09	1.21 (m)	28.08	1.25 (m)	30.47	1.15 (m)
16	26.94	1.22 (m)	26.88	1.31 (m)	25.60	0.94 (m)
17	41.91	2.71 (t)	41.87	2.74 (t)	29.16	1.37 (m)
18	—	—	—	—	42.16	2.73 (t)

Table 2. Inhibitory effects of cathestatins A, B, C and E-64 on cysteine proteases.

Cysteine protease	IC_{50} (nM)			
	A	B	C	E-64
Papain	11.2	4.6	20.0	2.5
Cathepsin B	177.6	8.8	114.3	5.0
Cathepsin L	1.4	3.0	11.1	15.0

The minor compound **3** of the active mixture showed a protonated molecular ion by the high resolution liquid secondary ion mass spectrometry at *m/z* 380.1817, corresponding to a molecular formula of $C_{18}H_{25}N_3O_6$ (ΔM -0.5 mmu). The molecular formula of **3** differs from that of **1** by the addition of one methylene group. Inspection of the ^{1}H and ^{13}C NMR data (Table 1) revealed that a structural variation is at the diamine moiety. The ^{1}H NMR spectra of **3** displayed three multiple signals at δ 1.15, 0.94 and 1.37 attributed to three methylene groups, which were accordingly assigned to H_2-15, H_2-16 and H_2-17 of the 1,5-diaminopentane moiety based on DEPT, HMQC, COSY and HMBC experiments. In contrast, the proton signal for the two methylene groups, H_2-15 and H_2-16 of the 1,4-diaminobutane moiety in **1** appeared as an overlapped multiplet. Therefore, the structure of this new compound cathestatin C is represented as **3** (Fig. 1).

The *trans* configuration of the epoxysuccinate moiety in these compounds was assigned based on the small coupling value ($J_{H,H}=2.0$ Hz) between H-2 and H-3. The absolute stereochemistry of the epoxysuccinate moiety has also been assigned (Fig. 1) by virtue of comparison with that of its analog E-64, an important member of this class of inhibitors, which has been studied by X-ray structure analysis[5]. In all cases the amino acid moieties were determined to possess the L-configuration by HPLC analyses of 1-fluoro-2,4-dinitro-phenyl-5-L-alanine amide (FDAA) derivatives of their acid hydrolysate[6]. Therefore, the structures of cathestatins A, B and C are concluded to be (2*S*,3*S*,5*S*)-epoxysuccinyl-phenylalanyl-putrescine (**2**), (2*S*,3*S*,5*S*)-epoxysuccinyl-tyrosyl-putrescine (**1**) and (2*S*,3*S*,5*S*)-epoxysuccinyl-tyrosyl-cadaverine (**3**), respectively.

Cathestatin C, like cathestatins A, B and E-64, exhibited potent and irreversible *in vitro* inhibition against the cysteine proteases papain, and both cathepsin B and L respectively, with IC_{50} values in the low nanomolar range shown in Table 2. Although this class of protease inhibitors possessing an epoxysuccinate functional group is reported to induce hepatic injury[7], thereby limiting their potential as new pharmaceutical agents, they may become useful reagents for biological research.

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