Communications to the Editor

ISOLATION AND STRUCTURAL ELUCIDATION OF ANTIOXIDATIVE AGENTS, ANTIOSTATINS A_1 TO A_4 AND B_2 TO B_5

Sir:

Active oxygen species cause a variety of diseases such as ischemia-reperfusion, inflammation, autoimmune disease, diabetes, rheumatism, cardiovascular diseases and cancer-initiation[1,2]. Thus, it could be expected that antioxidative agents may prevent these diseases.

During the course of a screening program for novel compounds showing antioxidant activity, we obtained a new naphthoquinone derivative naphterpin[3]. Further screening resulted in the isolation of new antioxidative agents named antiostatins A_1 to A_4 and B_2 to B_5 from *Streptomyces cyaneus* 2007-SV_1. They showed strong inhibitory activity against lipid peroxidation induced by free radicals in rat liver microsome preparations free from vitamin E[4]. In this paper, we report the fermentation, isolation and structural studies of these new metabolites.

A stock culture of *S. cyaneus* 2007-SV_1 was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (starch 2.5%, soybean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4%, pH 6.2 before sterilization) and incubated at 27°C for 2 days on a rotary shaker. The seed culture (600 ml) thus obtained was transferred into a 50-liter jar fermenter containing 30 liters of the same medium, and cultivation was carried out at 27°C for 48 hours under agitation at 400 rpm and aeration at 30 liters per minute.

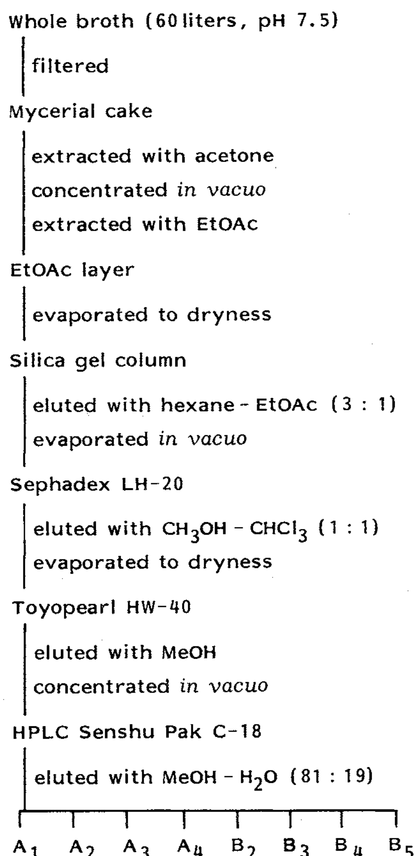
The active materials were isolated according to the procedures shown in Scheme 1. Each component of the antiostatins was separated at the final stage by reversed phase HPLC (Senshu Pak C-18) and was obtained as a pale yellow powder. The antiostatins were soluble in MeOH, $CHCl_3$, acetone and ethyl acetate, but insoluble in hexane and water. Based on the structural features of the substituent at C-4, they were divided into two groups, the A series and B series. Their structural studies were carried out using the main components A_1 (yield 2.5 mg) and B_4 (yield 2.0 mg) as explained in the following.

Antiostatin A_1: MP 180~183°C (dec); UV λ_{max}^{MeOH} nm (ε) 220 (28,500), 238 (26,200), 301 (16,100), 338 (4,250), 350 (4,250); IR ν_{max} (KBr) cm^{-1} 3350, 1620, 1585; HREI-MS (*m/z*) 324.1822 (M^+), calcd for $C_{20}H_{24}N_2O_2$, 324.1838.

Antiostatin B_4: MP 118~120°C†; UV λ_{max}^{MeOH} nm (ε) 218 (44,300), 238 (35,300), 301 (20,900), 338 (6,160), 352 (6,290); IR ν_{max} (KBr) cm^{-1} 3480, 3370, 3340, 1690, 1670, 1550; HRFAB-MS (*m/z*) 452.2763 (M^+), calcd for $C_{26}H_{36}N_4O_3$, 452.2787.

The UV spectral similarity between antiostatins A_1 and B_4, and carbazomycin B[5,6] suggested the presence of the carbazole nucleus in these compounds. Their structures were determined by ^1H and ^{13}C NMR spectral analyses and by comparison

Scheme 1. Isolation and purification of antiostatins.



† Antiostatins B_2 to B_5 gradually started to sublime at around 90°C.

Table 1. ^{13}C NMR data of antiostatins A_1 and B_4, and carazostatin[a].

Carbon	A_1	B_4	Carazostatin
1	123.3 (s)	123.7 (s)	124.1 (s)[b]
2	125.8 (s)	126.0 (s)	121.4 (s)
3	144.3 (s)	143.9 (s)	148.2 (s)
4	118.3 (s)	117.6 (s)	103.0 (s)
4a	115.5 (s)	115.7 (s)	120.9 (s)
4b	123.8 (s)	123.7 (s)	123.7 (s)
5	122.8 (d)	122.6 (d)	120.0 (d)
6	119.2 (d)	119.7 (d)	118.9 (d)
7	125.8 (d)	126.0 (d)	125.2 (d)
8	111.8 (d)	112.1 (d)	110.6 (d)
8a	141.4 (s)	141.8 (s)	139.8 (s)
9a	135.1 (s)	135.3 (s)	134.0 (s)
10	14.6 (q)	13.3 (q)	12.0 (q)
1′	30.0 (t)	29.7 (t)	28.8 (t)
2′	30.1 (t)	31.1 (t)	29.5 (t)
3′	33.1 (t)	31.0 (t)	30.0 (t)
4′	23.5 (t)	30.6 (t)	29.3 (t)
5′	13.0 (q)	33.2 (t)	31.7 (t)
6′	—	23.8 (t)	22.7 (t)
7′	—	14.9 (q)	14.1 (q)
1″	172.1 (s)	155.9 (s)	
2″	23.7 (q)	156.8 (s)	
3″		48.3 (t)	
4″		30.1 (d)	
5″		20.7 (q)	
6″		20.7 (q)	

[a] Solvent: A_1 and B_4 in $(CD_3)_2CO$, carazostatin in $CDCl_3$.
[b] $q=CH_3$, $t=CH_2$, $d=CH$, $s=-C$.

Fig. 1. Partial structure of antiostatin A_1 as revealed by HMBC analysis.

Arrows indicate ^{13}C-^1H long range couplings.

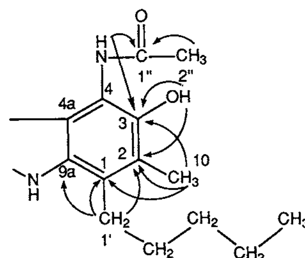
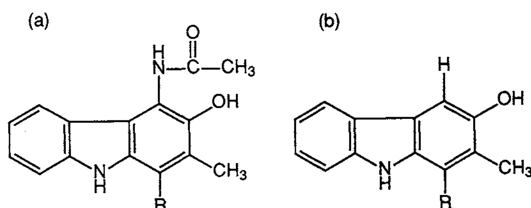


Fig. 2. Structures of antiostatins A series and carazostatin.

(a) Antiostatin series: A_1, $R=(CH_2)_4CH_3$; A_2, $R=(CH_2)_2CH(CH_3)CH_2CH_3$; A_3, $R=(CH_2)_4CH(CH_3)_2$; A_4, $R=(CH_2)_6CH_3$. (b) Carazostatin, $R=(CH_2)_6CH_3$.



with a carbazomycin related compound carazostatin[7] (Fig. 2).

Detailed analysis of the ^1H NMR spectral data (500 MHz, acetone-d_6) of antiostatin A_1 revealed the presence of an *n*-pentyl side chain (CH_3, t, 0.89 ppm; $3\times CH_2$, each m, 1.37, 1.45 and 1.65 ppm; CH_2, t, 2.97 ppm), an *N*-acetyl group (CH_3, s, 2.47 ppm, NH, br s, 9.68 ppm), an aromatic methyl group (CH_3, s, 2.40 ppm), a 1,2-disubstituted benzene ring (8.13, 7.10, 7.30 and 7.45 ppm), a phenolic hydroxy group (s, 8.05 ppm) and an imino proton of a carbazole nucleus (br s, 10.16 ppm). Based on heteronuclear multiple-bond correlation (HMBC)[8] spectral analysis (*vide infra*) and comparison of the ^{13}C NMR spectral data (125 MHz, acetone-d_6) of antiostatin A_1 and carazostatin (Table 1), these groups were arranged on the carbazole nucleus as shown in Fig. 1.

Thus, the methylene proton (1′-H, 2.97 ppm) of the *n*-pentyl side chain was coupled to C-9a (135.1 ppm), C-1 (123.3 ppm) and C-2 (125.8 ppm). The aromatic methyl proton was coupled to the last two carbons and C-3 (144.3 ppm), which in turn was coupled to the hydroxy proton and amide proton. Additional ^{13}C-^1H couplings observed between the amide carbonyl carbon (C-1″) and acetyl methyl and amide protons together with the data just described corroborated the relationship among C-1 to C-4 of the carbazole nucleus as well as the substituents on these carbons as shown in Fig. 1. Thus, the structure of antiostatin A_1 has been determined to be 1-*n*-pentyl-2-methyl-3-hydroxy-4-acetylaminocarbazole (Fig. 2).

The ^1H and ^{13}C NMR spectral comparison of antiostatin B_4 and antiostatin A_1 showed that the acetyl group in A_1 was substituted by an isobutylamino group ($-NH-CH_2$, 3.19 ppm, CH, 1.88 ppm, $2\times CH_3$, 0.98 ppm) in B_4 in addition to the replacement of the alkyl side chain in A_1 by an *n*-heptyl residue (CH_3, t, 0.89 ppm, $5\times CH_2$, m, 1.28, 1.30, 1.36, 1.47 and 1.66 ppm, CH_2, t, 2.98 ppm) in B_4 (Fig. 3).

In the HMBC spectrum of antiostatin B_4, the methylene proton (1′-H, 2.98 ppm) of the *n*-heptyl chain was coupled to C-1 (123.7 ppm), C-2 (126.0 ppm) and C-9a (135.3 ppm). The aminomethylene proton of the isobutylamino group (3″-H, 3.19 ppm) was coupled to a carbonyl carbon

Fig. 3. Partial structure of antiostatin B_4 as revealed by HMBC analysis.

Arrows indicate ^{13}C-^1H long range couplings.

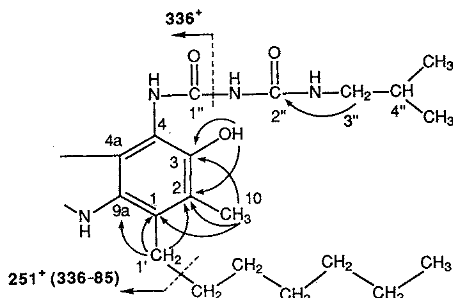
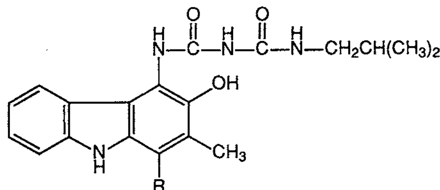


Fig. 4. Structures of antiostatin B series.

B_2, $R=(CH_2)_5CH_3$; B_3, $R=(CH_2)_4CH(CH_3)_2$; B_4, $R=(CH_2)_6CH_3$; B_5, $R=(CH_2)_5CH(CH_3)_2$.



(C-2″, 156.8 ppm) indicating the presence of a unit $(CH_3)_2CH-CH_2-NH-C=O$. Thus, there remained one carbonyl or amidino carbon (155.9 ppm), two exchangeable protons (6.92 and 10.90 ppm), one oxygen and two nitrogens to be explained. Since the carbon chemical shifts of C-4 in B_4 and A_1 were almost identical (117.6 vs. 118.3 ppm), C-4 was substituted by a nitrogen atom. Therefore, the functional group at C-4 in B_4 was either $C_4-NH-C(=O)-NH-C(=O)-NH-C_4H_9$ or $C_4-NH-C(=NH)-O-C(=O)-NH-C_4H_9$. This final problem was solved by the fragmentation ions observed in the HRFAB-MS at 336.1846 ($C_{21}H_{24}N_2O_2$, calcd 336.1838) and 251.0826 ($C_{21}H_{24}N_2O_2-C_6H_{13}$, calcd 251.0821) as shown in Fig. 3. Based on these results, the structure of antiostatin B_4 has been determined as shown in Fig. 4.

Because of the paucity of the samples available, the structures of the remaining components, antiostatins A_2, A_3, A_4 (Fig. 2), B_2, B_3 and B_5 (Fig. 4) were established by comparison of ^1H NMR and MS spectral data. Their molecular formulae and mp's are as follows; A_2 (yield 0.8 mg) $C_{21}H_{26}N_2O_2$, 195~197°C; A_3 (0.75 mg) $C_{22}H_{28}N_2O_2$, 190~192°C; A_4 (1.5 mg) $C_{22}H_{28}N_2O_2$, 191~193°C; B_2 (0.5 mg) $C_{25}H_{34}N_4O_3$, 119~120°C; B_3 (1.3 mg) $C_{26}H_{36}N_4O_3$, 117~118°C; B_5 (1.2 mg) $C_{27}H_{38}N_4O_3$, 92~94°C.

Antiostatins are the first carbazole derivatives that possess an acetamide group or a substituted urea chain in addition to a long alkyl chain. The IC_{50} values of antiostatin A_1 and antiostatin B_4 for the assay system mentioned above were 0.207 and 0.211 μg/ml, respectively, while that of vitamin E was 10.8 μg/ml. The remaining members of the antiostatins showed similar IC_{50} values to those of A_1 and B_4.

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