Benthocyanins B and C, New Free Radical Scavengers from *Streptomyces prunicolor*

Kazuo Shin-ya, Kazuo Furihata,† Yoshihiro Teshima, Yoichi Hayakawa, and Haruo Seto*

Institute of Applied Microbiology, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, and Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Recently, a variety of diseases such as atherosclerosis, inflammation, Parkinson's disease, and ischemia-reperfusion injury including cerebral trauma and stroke have been proven to be caused by oxygen-derived free radicals.[1-3] These diseases were reported to be ameliorated or overcome by substances which exhibited free radical scavenging activities.[4]

In the course of our screening for free radical scavengers of microbial origin,[5-7] we isolated benthocyanin A (3)[8] from the mycelium of *Streptomyces prunicolor* and determined its structure by NMR and X-ray crystallographic experiments. As shown in Figure 1, 3 is a unique phenazine carboxylic acid fused with a phenyl-substituted γ-lactone and a geranyl substituent at a nitrogen. Further investigation has resulted in the isolation of minor congeners of 3, designated benthocyanins B (1) and C (2) (Figure 1). We report herein on structural studies of these metabolites.

Isolation of 1–3 from the mycelium of *Streptomyces prunicolor* was carried out by combined column chromatography (see Experimental Section). Benthocyanin B (1) and benthocyanin C (2) were obtained as dark blue plates and violet powder, respectively. The physicochemical properties and ^1H and ^{13}C NMR chemical shifts of 1–3 are compared in Tables I and II.

The UV and visible spectra of 1 were very similar to those of 3 suggesting the presence of the same chromophore in these two compounds. The IR absorption of 1 at 1740 and 1710 cm^{-1} revealed the presence of ester and carboxylic acid functions as seen in 3. The molecular formula of 1, $C_{31}H_{28}N_2O_4$, was determined to be identical with that of 3 by HRFAB-MS (*m/z* found 493.2106 $[M + H]^+$, calcd for $C_{31}H_{29}N_2O_4$ 493.2127). In common to 3, there were observed ^1H and ^{13}C NMR signals assignable to a geranyl side chain and a phenyl residue together with an aromatic proton (4-H) in 1 (see Table II). However, there existed clear differences between them; 1-H in 3 was absent in 1 and the three consecutive aromatic proton system in 3 was replaced by a four-proton system consisting of 6-H to 9-H in 1.

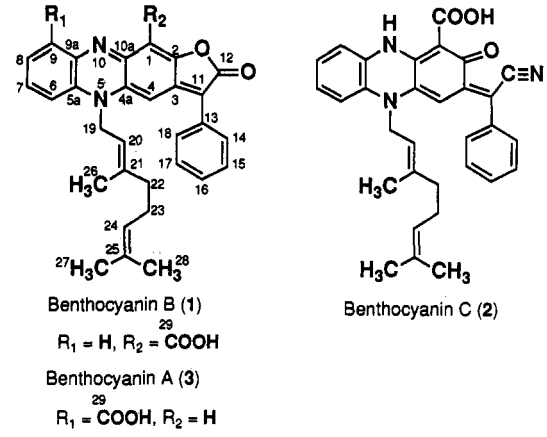

Figure 1. Structure of benthocyanins.

The sequence from 6-H to 9-H was established by ^1H NMR spectral analysis and ascribed to an *ortho*-disubstituted benzene ring (Figure 2). The terminal methylene protons (19-H) of the geranyl side chain were coupled to the carbon (C-5a), which in turn was long range coupled to 7-H. Based on its ^{13}C chemical shift (46.8 ppm), C-19 was linked to a nitrogen atom. Thus, the geranyl side chain was connected to the *ortho*-disubstituted benzene ring through a nitrogen atom.

The isolated aromatic proton (4-H) was coupled to C-2, C-4a, C-10a, and C-11. In addition, C-11 was coupled to aromatic protons on the phenyl ring (14-H and 18-H) (Figure 2). Additionally, C-4a was coupled to 19-H. The ^{13}C NMR spectrum of 1 also showed that the methine carbon C-1 (102.9 ppm) in 3 was replaced by a quaternary carbon in 1 (101.4 ppm). The very similar ^{13}C chemical shift values of these two carbons in 1 and 3 suggested that C-1 was substituted by an electron-withdrawing substituent, *i.e.*, a carboxylic acid, the presence of which was confirmed by preparation of a monomethyl ester of 1 with diazomethane [$C_{32}H_{30}N_2O_4$; HRFAB-MS (*m/z* found, 507.2290 $[M + H]^+$; calcd for $C_{32}H_{31}N_2O_4$, 507.2284; OCH_3, 4.06 ppm)]. The relationships between 4-H, 6-H, 14/18-H, and 19-H were established by NOE experiments as shown in Figure 2.

These data showed that 1 is a regioisomer of 3 with the carboxylic acid at C-1 in place of C-9 in 3. The other remaining carbons were reasonably assigned as shown in Table II. Further analyses of the HMBC and NOE spectra and ^{13}C chemical shifts (see Table II and Figure 2) supported the structure of 1.

The violet color of 2 different from 1 and 3 indicated that 2 contained a different chromophore. The IR absorption at 2180 and 1667 cm^{-1} revealed the presence of a nitrile and a carboxylic acid residue, respectively. Its molecular formula established as $C_{31}H_{29}N_3O_3$ from HR-FAB-MS (*m/z* found 492.2279 $[M + H]^+$, calcd 492.2287) showed that one oxygen atom in 3 was replaced by NH in 2.

In the ^1H and ^{13}C NMR spectra of 2, a geranyl side chain, a phenyl ring, an *ortho*-disubstituted benzene group, and an isolated singlet proton (4-H) were observed as in 1. In addition, two hydrogen-bonded exchangeable protons were observed at an extremely low field (14.54 and 15.70 ppm). An NOE experiment irradiating at 9-H enabled us to assign one of the exchangeable protons to an amine proton 10-H (14.54 ppm). In the HMBC

† Department of Agricultural Chemistry.

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Table I. Physicochemical Properties of Benthocyanins B, C, and A

	benthocyanin B (1)	benthocyanin C (2)	benthocyanin A (3)
appearance	dark blue plate	violet powder	dark blue plate
mp	235–236 °C	157–158 °C	196–197 °C
molecular formula	$C_{31}H_{28}N_2O_4$	$C_{31}H_{29}N_3O_3$	$C_{31}H_{28}N_2O_4$
HRFAB-MS (m/z) $(M + H)^+$			
found	493.2106	492.2279	493.2071
calcd	493.2127	492.2287	493.2127
UV λ_{max}^{MeOH} nm (ϵ)	249 (24400)	246 (18100)	247 (29000)
	344 (15900)	283 (11300)	342 (18600)
	415 (6100)	445 (5700)	424 (7400)
	430 (6100)	470 (5300)	622 (17200)
	615 (15700)	570 (11700)	
$\lambda_{max}^{MeOH + HCl}$ nm (ϵ)	245 (26300)	247 (21100)	247 (30600)
	335 (15200)	280 (7800)	344 (18800)
	632 (16400)	325 (12100)	638 (16200)
		410 (5300)	
		430 (6400)	
		665 (13100)	
$\lambda_{max}^{MeOH + NaOH}$ nm (ϵ)	250 (26400)	251 (16300)	247 (31400)
	343 (17400)	335 (8000)	340 (17400)
	415 (6500)	410 (3600)	407 (8000)
	430 (6500)	440 (4400)	428 (8000)
	615 (17300)	535 (11600)	616 (18400)
IR ν_{max} (KBr) cm^{-1}	3430, 1760, 1745	3430, 2180, 1735, 1670	3450, 1740, 1720

Table II. The ^{13}C and ^1H NMR Chemical Shifts of Benthocyanins B, C, and A Taken in $CDCl_3$

	benthocyanin B (1)		benthocynin C (2)		benthocynin A (3)	
no.	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	101.4		95.2		102.9	6.96
2	161.1		180.3		158.6	
3	140.2		139.7		140.2	
4	92.4	6.71	101.3	6.25	92.5	6.76
4a	132.4		130.3		133.3	
5a	131.7		130.4		132.2	
6	113.7	7.43	113.5	7.04	117.8	7.53
7	132.7	7.67	128.4	7.31	131.0	7.66
8	125.1	7.42	123.6	7.15	128.4	8.30
9	127.9	7.80	119.1	7.30	125.3	
9a	128.0		123.9		134.6	
10a	148.3		148.8		150.2	
11	110.9		110.8		112.0	
12	167.4		120.9		168.3	
13	130.8		135.4		131.4	
14,18	128.7	7.77	129.7	7.50	128.1	7.81
15,17	128.9	7.46	129.5	7.44	129.1	7.48
16	127.9	7.37	129.1	7.43	128.9	7.38
19	46.8	4.92	45.9	4.39	47.1	4.87
20	115.7	5.15	116.1	4.95	115.9	5.16
21	143.2		142.4		143.6	
22	39.4	2.12	39.2	1.97	39.7	2.13
23	26.1	2.12	26.2	1.97	26.5	2.13
24	122.9	4.98	123.1	4.97	123.1	5.00
25	132.7		132.2		132.8	
26	17.1	1.94	16.6	1.39	17.4	1.94
27	25.6	1.55	25.7	1.64	25.8	1.57
28	17.7	1.51	17.7	1.56	17.9	1.52
29	162.1		172.1		166.3	
10-NH				14.54		
29-OH				15.70		

spectrum of 2, both the terminal methylene protons of the geranyl side chain (19-H) and amine protons (10-H) were coupled to the same carbons C-4a and C-5a (Figure 3). In addition, C-4a displayed a long-range coupling to the isolated proton 4-H. Additional important long-range correlations were observed from 4-H to C-2, C-10a, and C-11. The other hydrogen-bonded exchangeable proton was ascribed to the hydroxy proton of a carboxylic acid residue due to the coupling to the carboxylic acid carbon C-29 and its adjacent carbon C-1. In addition to this correlation, the HMBC spectral analyses with the *ortho*-disubstituted benzene unit and the phenyl ring revealed

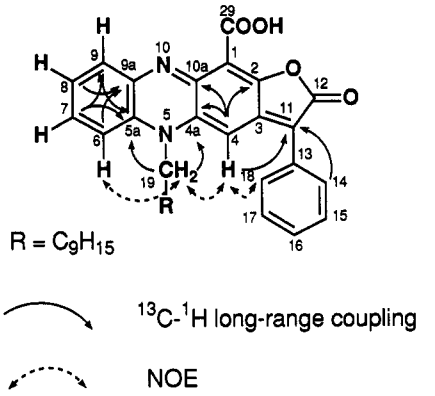

Figure 2. NMR analyses of benthocyanin B (1).

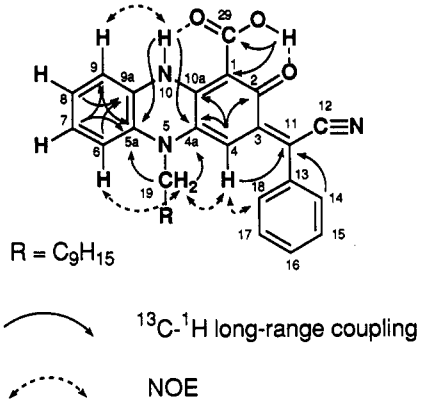

Figure 3. NMR analysis of benthocyanin C (2).

the phenazine skeleton in 2 (Figure 3). The remaining isolated unit was shown to be a nitrile residue at C-11 by the IR absorption (2180 cm^{-1}) and its ^{13}C chemical shift (120.9 ppm). Thus, the structure of 2 was determined as shown in Figure 1.

Both 1 and 2 inhibited lipid peroxidation in rat microsomes[9] at low concentrations. IC_{50} values of 1 and 2 were 0.16 and 0.29 μg/mL, respectively, which were 30–70 times as strong as that of vitamin E. They also showed

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inhibitory effects on rat erythrocyte hemolysis[10] with IC_{50} values, 0.56 and 1.30 μg/mL, respectively. Further studies on other biological activities are now under way.

Experimental Section

General Experimental Procedure. Spectral data were collected on the following instruments: IR, JASCO A-102; UV/visible, Shimazu UV-300; FAB-MS, JEOL HX-110, *m*-nitrobenzyl alcohol matrix; NMR, JEOL JNM GSX-500. ^1H NMR chemical shifts are reported in ppm relative to TMS and ^{13}C NMR chemical shifts are reported in ppm relative to $CDCl_3$ (77.0 ppm). One bond ^1H–^{13}C connectivities were determined *via* ^{13}C–^1H COSY experiments, and multiple-bond ^1H–^{13}C connectivities were revealed through an HMBC experiment. Silica gel 60 F_{254} plates (Merck) were used for analytical and preparative TLC.

Purification of 1 and 2. The producing microorganism of benthocyanins, *Streptomyces prunicolor* 1884-SVT2, was cultivated in a producing medium (starch 2.5%, soybean meal 1.5%, dry yeast 0.2%, $CaCO_3$ 0.4%, pH 7.2). Cultures were incubated in jar fermenters at 27 °C for 2 days. The mycelial cake collected by centrifugation from the whole fermentation broth (1600 L) was stirred in acetone. The solvent extract was concentrated *in vacuo* and the aqueous residue was extracted with EtOAc, dried over Na_2SO_4, and concentrated *in vacuo*. After removal of the solvent, the residue was applied to a silica gel column packed with $CHCl_3$. Following the elution of **3**, a mixture of **1** and **2** was eluted with $CHCl_3$/MeOH (10:1). After removal of the organic solvent, the residue was rechromatographed on a silica gel column with $CHCl_3$/MeOH (20:1) to give crude **1** and **2**. Gel filtration on Toyopearl HW-40F (MeOH) and Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) gave pure **1** as dark blue plates. Crude powder of **2**, which was obtained by silica gel column chromatography, was further purified by preparative TLC ($CHCl_3$/MeOH, 20:1). Finally, gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) gave pure **2** as a violet powder.

Preparation of the Methyl Ester of 1. To a $CHCl_3$ solution (5 mL) of **1** (10 mg) was added an aliquot of diazomethane, and the mixture was stirred for 1 h at room temperature with monitoring by silica gel TLC ($CHCl_3$/MeOH 10:1). After evaporation of the solvent, the residue was passed through a silica gel column (4.5 × 25 cm) with $CHCl_3$ to afford a monomethyl ester of **1** (8 mg) as dark blue plates: HRFAB-MS *m/z* 507.2290 $(M + H)^+$ (calcd for $C_{32}H_{31}N_2O_4$ 507.2284); IR ν_{max} (KBr) cm^{-1} 3440, 1730, 1720 (sh), 1235; UV λ_{max} (ε) in MeOH 244 (22300), 345 (11200), 365 (sh, 9600), 420 (4000), 620 (10500); ^1H NMR ($CDCl_3$) δ 7.93 (1H, dd, 8.0, 1.5 Hz, 9-H), 7.80 (2H, dd, 8.0, 1.0 Hz, 14,18-H), 7.58 (1H, dd, 8.0, 1.5 Hz, 7-H), 7.45 (2H, dt, 8.0, 1.0 Hz, 15,17-H), 7.38 (2H, m, 6,8-H), 7.33 (1H, dt, 8.0, 1.0 Hz, 16-H), 6.63 (1H, s, 4-H), 5.13 (1H, m, 24-H), 4.98 (1H, m 20-H), 4.89 (2H, bd, 5.0 Hz,, 19-H), 4.06 (3H, s, OCH_3), 2.11 (4H, m 22,23-H), 1.93 (3H, s, 26-H), 1.56 (3H, s, 27-H), 1.51 (3H, s, 28-H).

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Supplementary Material Available: Copies of spectra of benthocyanins B and C (5 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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