Structure of Benthophoenin, a New Free Radical Scavenger Produced by Streptomyces prunicolor

Kazuo Shin-ya, Yoichi Hayakawa, and Haruo Seto

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STRUCTURE OF BENTHOPHOENIN, A NEW FREE RADICAL SCAVENGER PRODUCED BY *STREPTOMYCES PRUNICOLOR*[1]

KAZUO SHIN-YA, YOICHI HAYAKAWA, and HARUO SETO*

Institute of Molecular and Cellular Biosciences,[2] The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT.—A novel compound, benthophoenin [**1**], was isolated from the mycelium of *Streptomyces prunicolor*. The structure of **1** was determined mainly by nmr spectral analysis to be a phenazine carboxylic acid with a geranyl and two benzoyl residues.

Free radicals including active oxygen species cause a variety of diseases such as inflammation, autoimmune disease, diabetes, rheumatism, cardiovascular diseases, and cancer initiation (1–3). These diseases have been reported to be ameliorated by free radical scavengers (4).

In the course of our screening for free radical scavengers to ameliorate these diseases, we isolated naphterpin (5), antiostatins (6), pyridoxatin (7), benthocyanin A (8), and stealthins (9). Detailed investigation of minor components of benthocyanins (10) produced by *Streptomyces prunicolor* 1884-SVT2 has resulted in the isolation of a novel compound designated benthophoenin [**1**]. Compound **1** is a phenazine carboxylic acid with benzoyl moieties at the C-3 and C-7 positions and a geranyl substituent at a nitrogen and shows structural similarity to benthocyanin A (3). We report herein its isolation and structure determination.

RESULTS AND DISCUSSION

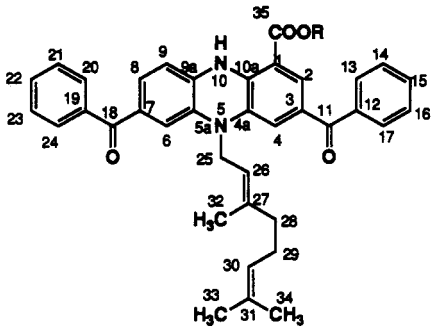
Compound **1** and its related compounds, benthocyanins, were extracted from the mycelium of *S. prunicolor* by Me_2CO, and **1** was purified by combined column chromatographies using Si gel, gel filtration through Sephadex LH-20, and preparative tlc to give a red powder.

The molecular formula of **1** was established by hrfabms to be $C_{37}H_{34}N_2O_4$ (*m/z* found 571.2597 $[M+H]^+$, calcd 571.2639). The ir absorption of **1** at 1680 cm^{-1} revealed the presence of an arylic carboxylic acid function as seen in benthocyanins (8,10). In common to benthocyanins, the ^{1}H-nmr spectrum of **1** showed signals assignable to a geranyl side chain and phenyl residues. Unlike benthocyanins, however, the aromatic protons were not observed as sharp signals. Therefore, **1** was treated with CH_2N_2 to give a monomethyl ester **2**, which gave well-resolved signals.

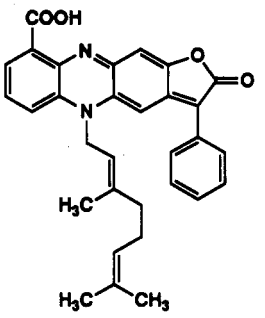
The ^{1}H-nmr spectral analysis of **2** revealed two phenyl residues from H-13 to H-17 and from H-20 to H-24. The other proton spin systems in the aromatic region were assigned to the 1,2,4-trisubstituted benzene ring and two *meta*-coupled protons. The geranyl side chain structure was established by comparison with benthocyanin A (8). The terminal methylene protons (H-25) of the geranyl residue were long-range-coupled to the carbons (C-4a and C-5a), which in turn showed long-range couplings to the amine proton H-10 in the phenazine skeleton. In addition, C-5a was long-range-coupled to H-9, which suggested a 1,2,4-trisubstituted benzene ring. Based on its ^{13}C chemical shift (46.8 ppm), C-25 was linked to the remaining nitrogen atom in the heterocyclic ring.

The doublet protons due to one of the phenyl residues H-13 and H-17 (7.69 ppm), were coupled to a carbonyl carbon C-11 (194.8 ppm) which also showed long-range coupling with *meta*-coupled protons H-2 and H-4 (7.53 ppm and 6.67 ppm, respec-

[1]This manuscript is dedicated to the memory of the late Professor Edward Leete.
[2]Formerly, the Institute of Applied Microbiology.



1 R=H
2 R=Me



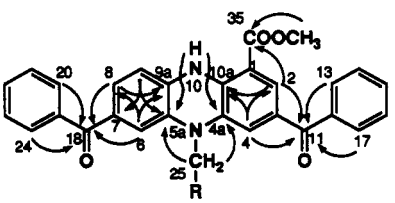
3

tively). Thus, one of the benzoyl moieties was located on C-3 lying between two aromatic methine carbons. In the same manner, the other benzoyl residue was placed at C-7 (132.7 ppm), since a carbonyl carbon C-18 (194.2 ppm) was long-range-coupled to H-6 (6.76 ppm), H-8 (6.98 ppm), and H-20 and H-24 (7.69 ppm).

The remaining carbonyl carbon (168.2 ppm), which proved to be a carboxylic acid by preparation of a monomethyl ester of **1** with CH_2N_2 ($C_{38}H_{36}N_2O_4$; hrfabms *m/z* found 585.2740 $[M+H]^+$; calcd for $C_{38}H_{37}N_2O_4$, 585.2753; -OMe, 3.81 ppm), was placed at C-1 (107.5 ppm) by the long-range coupling between the carbonyl carbon C-35 and H-2. All ^1H and ^{13}C assignments were established by analyzing the long-range couplings observed in heteronuclear multiple bond correlation (HMBC) (11) spectra as shown in Figure 1.

The free radical scavenging activity of **1**, in the assay system employing the modified Yagi method (12), was an order of magnitude more potent than that of vitamin E, which is a representative free radical scavenger. IC_{50} values of **1** and vitamin E were 0.15 and 10.8 μg/ml, respectively. Compound **1** also inhibited hemolysis of rat erythrocytes induced by radical initiators such as 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (13,14) at a concentration comparable to that of vitamin E, IC_{50} (0.8 μg/ml).

Benthophoenin is a member of the benthocyanin family, but it possesses two benzoyl residues at almost symmetrical positions instead of a phenyl-substituted δ-lactone. Since the benthocyanins lack phenolic OH groups, which are believed to be essential for antioxidative activity (15), the mechanism of action of benthophoenin and benthocyanins to prevent lipid peroxidation remains unclear. Biological and chemical investigations of their mechanism of action may open a way for developing powerful free radical scavengers. The reason of the signal broadening of the aromatic proton region of **1**, which was not observed in benthocyanins, is unclear.



2 R=C_9H_{15}

FIGURE 1. Proton and carbon connectivities of **2** as revealed by HMBC experiments. Arrows indicate long-range ^{13}C-^1H couplings.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra were measured on a JASCO A-102 spectrophotometer. ^{1}H-(500 MHz) and ^{13}C-(125 MHz) nmr spectra were recorded on a JEOL JNM GSX-500 spectrometer using TMS as the internal standard. Chemical shifts are reported in δ (ppm) values . Fabms were obtained with a JEOL HX-110. Analytical and preparative tlc was carried out on Si gel 60 F_{254} plate (Merck).

EXTRACTION AND ISOLATION.—The mycelial cake of *S. prunicolor* 1884-SVT2 (FERN BP-2728), collected by centrifugation from whole fermentation broth (1600 liters), was stirred with 800 liters of Me_2CO. The solvent extract was concentrated in vacuo, and active materials were extracted with EtOAc, dried over Na_2SO_4, and concentrated in vacuo. The residue was subjected to Si gel cc eluting with a gradient of $CHCl_3$/MeOH. Active fractions eluted with $CHCl_3$-MeOH (10:1) were concentrated and applied to Sephadex LH-20 [$CHCl_3$-MeOH (1:1)]. The eluate was further purified by preparative tlc [Si gel, 0.5 mm, $CHCl_3$-MeOH-concentrated NH_4OH (50:10:1)] to give 25 mg of **1**.

PREPARATION OF METHYL ESTER OF **1**.—To a $CHCl_3$ solution of **1** (10 mg in 5 ml) was added an aliquot of CH_2N_2 followed by stirring at room temperature and monitoring by tlc [$CHCl_3$-MeOH (10:1)]. After 30 min, the reaction mixture was evaporated in vacuo, and the residue was subjected to Si gel cc packed with $CHCl_3$ to give 8 mg of pure **2**.

Benthophoenin [**1**].—Hrfabms *m/z* 571.2597 (calcd for $C_{37}H_{35}N_2O_4$, 571.2639); mp 189–190; ir ν max (KBr) cm^{-1} 1640, 1680; uv λ max (ε) (MeOH) 234 (27500), 248 (28000), 309 (36500), 388 (7100), 520 (12400); ^{1}H nmr (CD_3OD) δ 7.73 (1H, bs, H-2), 7.668 (4H, dd, 8.0, 1.0 Hz, H-13, -17, -20, -24), 7.59 (2H, m, 15, H-22), 7.50 (4H, dt, 8.0, 1.0 Hz, H-14, -16, -21, -23), 6.99 (1H, dd, 8.0, 2.0 Hz, H-8), 6.69 (2H, bs, 4, H-6), 6.32 (1H, bs, H-9), 5.06 (2H, bs, H-26, -30), 2.08 (4H, m, H-28, -29), 1.68 (3H, s, H-32), 1.61 (3H, s, H-33), 1.59 (3H, s, H-34); ^{13}C nmr (CD_3OD) δ 197.1 (C-11, -18), 173.8 (C-35), 143.8, (C-10a), 141.7 (C-27), 139.9 (C-9a, -12, -19), 136.8 (C-3), 135.9 (C-7), 132.8 (C-4a, -5a), 132.6 (C-15, -22), 132.5 (C-31), 131.8 (C-2), 130.3 (C-13, -17, -20, -24), 129.3 (C-14, -16, -21, -23), 128.3 (C-8), 124.9 (C-1, -30), 119.5 (C-26), 113.0 (C-6), 112.9 (C-9), 112.7 (C-4), 44.9 (C-25), 40.5 (C-28), 27.2 (C-29), 25.8 (C-33), 17.8 (C-34), 16.5 (C-32).

Benthophoenin methyl ester [**2**].—Hrfabms *m/z* 585.2740 (calcd for $C_{38}H_{37}N_2O_4$, 585.2753); ir ν max (KBr) cm^{-1} 1640, 1680; uv λ max (ε) (MeOH) 225 (33900), 251 (31100), 302 (43900), 390 (7000), 508 (12400); ^{1}H nmr ($CDCl_3$) δ 9.80 (1H, H-10, NH), 7.69 (4H, dt, 8.0, 1.0 Hz, H-13, -17, -20, -24), 7.55 (1H, dt, 8.0, 1.0 Hz, H-15), 7.53 (1H, bs, H-2), 7.53 (1H, dt, 8.0, 1.0 Hz, H-22), 7.45 (2H, dt, 8.0, 1.0 Hz, H-14, -16), 7.43 (2H, dt, 8.0, 1.0 Hz, H-21, -23), 6.98 (dd, 8.2, 2.0 Hz, H-8), 6.76 (1H, bd, 2.0 Hz, H-6), 6.67 (1H, bd, 2.0 Hz, H-4), 6.25 (1H, d, 8.0 Hz, H-9), 5.02 (2H, m, H-26, -30), 3.81 (3H, s, -OMe), 2.01 (4H, m, 28, H-29), 1.62 (3H, s, H-32), 1.61 (3H, s, H-34), 1.56 (3H, s, H-33); ^{13}C nmr ($CDCl_3$) δ 194.8 (C-18), 194.2 (C-11), 168.2 (C-35), 143.4 (C-10a), 141.4(C-27), 138.4 (C-19), 138.1 (C-12), 136.4 (C-9a), 136.1 (C-4a), 134.8 (C-5a), 132.7 (C-7), 131.7 (C-15, -22, -31), 129.5 (C-3), 129.4 (C-13, -17, -20, -24), 128.1 (C-14, -16, -21, -23), 127.5 (C-2), 126.6 (C-8), 123.7 (C-30), 117.1 (C-26), 113.2 (C-4), 112.4 (C-6, -9), 107.5 (C-1), 52.1 (-OMe), 44.2 (C-25), 39.5 (C-28), 26.4 (C-29), 25.6 (C-33), 17.7 (C-34), 16.3 (C-32).

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