STUDIES ON FREE RADICAL SCAVENGING SUBSTANCES FROM MICROORGANISMS

III. ISOLATION AND STRUCTURAL ELUCIDATION OF A NOVEL FREE RADICAL SCAVENGER, RESORSTATIN

SHINICHIRO KATO, KAZUTOSHI SHINDO, HIROYUKI KAWAI, MICHIKO MATSUOKA and JUNICHIRO MOCHIZUKI

Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd., 3 Miyahara-cho, Takasaki-shi, Gunma 370-12, Japan

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In the course of our screening program for free radical scavenging substances from microorganisms, which are expected to be useful as therapeutic agents for myocardial and cerebral ischemia[1,2], inflammation[3] and other diseases, we have isolated carazostatin[4] and neocarazostatin[5], as reported previously. Further screening has resulted in the isolation of an alkylresorcinol-type compound named resorstatin (**I**) from the culture of *Pseudomonas* sp. DC165 (Fig. 1). Resorstatin is structurally related to DB-2073 previously reported by KANDA *et al.*[6,7]. Resorstatin has shown a strong inhibitory activity against lipid peroxidation induced by free radicals in rat brain homogenate. In this paper, we wish to report the isolation, structural elucidation and biological activity of this compound.

The organism was isolated from a soil sample collected in Taketomi-cho, Okinawa Prefecture, Japan and taxonomic studies indicated that it belonged to the genus *Pseudomonas*. The strain was cultivated on a rotary shaker at 27°C for 5 days in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of glycerol 3.0%, fish meal 2.0% and calcium carbonate 0.2%. The fermentation broth (1 liter) was filtered and the cell mass was extracted with acetone. After removal of acetone, the pH of this extract was adjusted to 7 and extracted with ethyl acetate. After drying over anhydrous Na_2SO_4, the extract was concentrated *in vacuo* to dryness. The residue was dissolved in 10 ml of chloroform, and 60 ml of hexane was added to it. The formed precipitate was removed by centrifugation, and the supernatant was concentrated *in vacuo* to give a crude oil (200 mg), which was chromatographed on a silica gel column (3.5 × 30 cm) with hexane-ethyl acetate (50:1). The active fraction was further purified by a Sephadex LH-20 column (2 × 40 cm) with methanol. After concentration, we obtained 49 mg of **I**. DB-2073 could be also isolated and purified from the same culture.

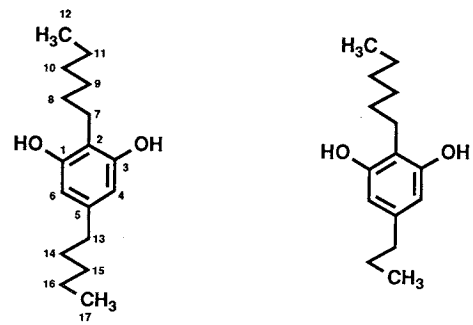
I is a pale yellowish powder: molecular formula $C_{17}H_{28}O_2$; HREI-MS (M^+ m/z 264.2110, calcd: 264.2089; mp 88~91°C (dec.); UV λ_{max} nm (ε) 206 (29,500), 274 (1,210), 280 (1,140); IR (KBr) ν cm^{-1} 3400, 3260, 2910, 2840, 1630, 1580, 1460, 1440, 1320, 1260, 1160, 1110, 1000, 830. The UV and IR spectra of **I** were characteristic for dihydroxybenzene moiety[6,8~10].

I is soluble in methanol, ethanol, chloroform, benzene, acetone, ethyl acetate and *n*-hexane, but not soluble in water. **I** showed a positive reaction for phenolic OH with $FeCl_3$.

In the ^1H NMR spectrum of **I** in $CDCl_3$, the signals were classified into the three groups as follows: the two aromatic protons δ_H 6.22 (2H, s, 4-H and 6-H); the protons of the normal-type alkyl side chains δ_H 2.57 (2H, t, J=7.9 Hz, 7-H_2), 2.45 (2H, t, J=7.9 Hz, 13-H_2), 1.55 (4H, m, 8-H_2 and 14-H_2), 1.38~1.30 (10H, m, 9-H_2~11-H_2, 15-H_2 and 16-H_2), 0.89 (3H, t, J=6.7 Hz, 17-H_2) and 0.88 (3H, t, J=6.7 Hz, 12-H_3); and the two exchangeable protons δ_H 4.65 (2H, br s) ascribed to phenolic OH. The chemical shifts of these proton signals were closely related to those of DB-2073 and indicated that **I** was a di-alkyl-substituted resorcinol.

In order to determine the length of two alkylated groups and assign ^{13}C NMR signals, we performed the analysis of phase sensitive C-H HOHAHA. Couplings were observed from 8-H to C-7 and C-9, from 12-H to C-10 and C-11, from 14-H to C-13 and C-15, and from 17-H to C-15 and C-16, respectively. These results indicate the existence of *normal*-type hexyl and pentyl side chain. Since the

Fig. 1. Structures of resorstatin and DB-2073.



Resorstatin DB-2073

Table 1. ^{13}C chemical shifts of resorstatin in $CDCl_3$.

Carbon	δ_C	Carbon	δ_C
1	154.3 (s)	10	31.8 (t)
2	112.8 (s)	11	22.6 (t)
3	154.3 (s)	12	14.0 (q)
4	107.8 (d)	13	35.4 (t)
5	142.0 (s)	14	30.7 (t)
6	107.8 (d)	15	31.5 (t)
7	23.1 (t)	16	22.5 (t)
8	29.2 (t)	17	13.9 (q)
9	29.4 (t)		

Fig. 2. The NOE in the structure of resorstatin (arrows).

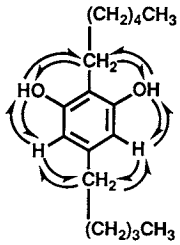


Table 2. Inhibitory effects of resorstatin and DB-2073 on lipid peroxidation in rat brain homogenate.

Drug	Concentration (μg/ml)	Inhibition (%)
Resorstatin	3.0	100.0
	1.0	99.8
	0.3	2.2
DB-2073	3.0	100.0
	1.0	80.8
	0.3	0.0
Flunarizine 2HCl	47.8 (100 μM)	62.7
	14.3 (30 μM)	38.1
	4.8 (10 μM)	8.8
Butylated hydroxytoluene	3.0	100.0
	1.0	99.8
	0.3	5.3

The inhibitory activity was measured according to the method of KUBO *et al.*[11], in the presence of Fe^{2+} (10 μM) and ascorbic acid (100 μM).

nuclear Overhauser effects (NOE) were remarkably observed as shown in Fig. 2, the structure of **I** has been determined to 2-*n*-hexyl-5-*n*-pentyl-1,3-benzenediol (Fig. 1).

Inhibitory effects of **I** and DB-2073 on lipid peroxidation induced by free radicals generated in the presence of Fe^{2+} (10 μM) and ascorbic acid (100 μM) in rat brain homogenate are shown in Table 2. IC_{50} values of **I** and DB-2073 were 2.06 μM and 2.74 μM, respectively. They were much more active than flunarizine (IC_{50}; 55.0 μM) which is a brain protective drug with free radical scavenging activity[11], and were almost as active as butylated hydroxytoluene (BHT, IC_{50}; 2.44 μM) which is a well known antioxidant.

I had a weak antibacterial activity as DB-2073, which inhibit growth of *Bacillus subtilis* at the concentration of 1 mg/ml. **I** had low toxicity; there was no death after intraperitoneal injection to mice with 100 mg/kg.

Our results suggest that resorstatin may be useful for the alleviation of tissue damage due to generation of free radicals such as superoxide radical and subsequent peroxidative disintegration of cell membranes.

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