NOTES

Phaffiaol, a New Antioxidant Isolated from a Yeast *Phaffia rhodozyma*

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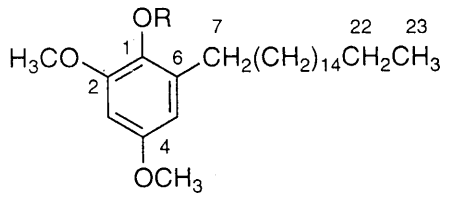
In recent years, it has been suggested that active oxygen species play an important part in the pathogenesis of various kinds of diseases, such as cerebral ischemia[1], PARKINSON's disease[2] and cancer[3]. Therefore, much efforts have been focused on exploring natural antioxidants[4~6].

In the course of our screening program for antioxidants, we have isolated phaffiaol (**1**)[7] from the culture of *Phaffia rhodozyma* ATCC 24201 which is a well-known red yeast[8] (Fig. 1). Antioxidant **1** was a new trisubstituted alkyl phenol. During the course of this work, structurally related compounds having a different side chain at the 6-position of phenols have been reported and some of them have biological activity[9~11]. However, their potency of antioxidative activity was not reported and **1** was the first trisubstituted alkyl phenol isolated from a fungus. In this paper, we describe the fermentation, isolation and structure determination of **1**, and examination of its antioxidative activity compared to that of α-tocopherol.

The producing stain *Phaffia rhodozyma* ATCC 24201 growing on potato dextrose agar was cultivated into twenty 300-ml Erlenmeyer flasks containing 50 ml of YM medium consisting of glucose 2%, meat extract 0.3%, peptone 0.5%, NaCl 0.2%, yeast extract 0.2%, pH 6.5 at 25°C for 2 days on a rotary shaker (150 rpm). The seed culture was inoculated into four hundred 2-liter Erlenmeyer flasks containing 400 ml of the same fresh medium and cultured at 21.5°C for 60 hours on a rotary shaker (150 rpm).

Dried culture (1.3 kg) was extracted with acetone (5 liters). After filtration, the extract was concentrated on a rotary evaporator. The residue (59.27 g) was partitioned between *n*-hexane (500 ml) and methanol - 10% aqueous NaOH solution (1 : 1) (500 ml) to exclude free fatty acids. The hexane layer was washed well with 50% methanol solution and brine until the aqueous layer was neutralized. The organic layer was concentrated under reduced pressure. The residue (28.17 g) was dissolved in hot ethanol (100 ml) and allowed to stand at 5°C overnight to precipitate triacylglycerols and sterols. Filtration and concentration of the ethanol solution gave an oil (7.50 g). The residue (5.13 g) was chromatographed on silica gel eluting with *n*-hexane - diethyl ether (95 : 5). The active fractions were collected, combined, and concentrated *in vacuo* and the residue (50.1 mg) was crystallized from hot methanol (8 ml). The resulting crystals (26.6 mg) were subjected to HPLC (Cosmosil $5C_{18}$, 10 mm i.d. × 250 mm, mobile phase EtOH - MeOH (1 : 3)) to furnish the pure antioxidant, phaffiaol (**1**)

Fig. 1. Chemical structures of phaffiaol (**1**) and its acetate (**2**).



1: R = H
2: R = $COCH_3$

Table 1. Physico-chemical properties of phaffiaol.

Appearance	Crystalline colorless powder
MP (°C)	74.8 ~ 77.3
Molecular formula	$C_{25}H_{44}O_3$
HREI-MS (*m/z*)	
Calcd for $C_{25}H_{44}O_3$:	392.3290
Found:	392.3294 (M^+)
EI-MS (*m/z*)	392 (M^+, 100%), 168 (99%), 167 (45%), 153 (16%), 139 (12%)
Elemental analysis	
Calcd for $C_{25}H_{44}O_3$:	C 76.48, H 11.30
Found:	C 76.45, H 11.58
UV (EtOH)	
λ_{max} nm (ε)	289 (3810)
IR (KBr) ν_{max} (cm^{-1})	3480, 2915, 2845, 1610, 1500

Table 2. ^{1}H and ^{13}C NMR spectral data of phaffiaol (**1**) and its acetate (**2**).

Carbon No.	Phaffiaol		Phaffiaol acetate	
	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]
C-1		137.4 (s)		136.3 (s)
C-2		146.7 (s)		151.7 (s)
C-3	6.35 (d, J=2.5 Hz)	96.6 (d)	6.38 (d, J=3.0 Hz)	97.6 (d)
C-4		152.7 (s)		157.8 (s)
C-5	6.28 (d, J=2.5 Hz)	105.9 (d)	6.32 (d, J=3.0 Hz)	105.2 (d)
C-6		128.8 (s)		131.9 (s)
C-1-OH	5.24 (br s)			
C-2-OCH_3	3.85 (s)	56.0 (q)[c]	3.78 (s)	55.8 (q)[d]
C-4-OCH_3	3.75 (s)	55.8 (q)[c]	3.78 (s)	55.5 (q)[d]
C-7	2.60 (t, J=7.8 Hz)	31.9 (t)	2.45 (t, J=8.0 Hz)	31.9 (t)
C-8～C-22	1.5～1.6 (2H, m), 1.2～1.4 (28H, m)	30.0 (t), 29.9 (t), 29.7 (8C, each t), 29.6 (3C, each t), 29.4 (t), 22.7 (t)	1.5～1.6 (2H, m), 1.2～1.4 (28H, m)	30.5 (t), 30.0 (t), 29.7 (8C, each t), 29.6 (t), 29.5 (t), 29.4 (2C, each t), 22.7 (t)
C-23	0.87 (t, J=7.0 Hz)	14.1 (q)	0.88 (t, J=7.0 Hz)	14.1 (q)
$COCH_3$			2.30 (s)	169.2 (s), 20.5 (q)

[a] 500 MHz in $CDCl_3$ (ppm).
[b] 125 MHz in $CDCl_3$ (ppm).
[c,d] The assignments may be exchangeable.

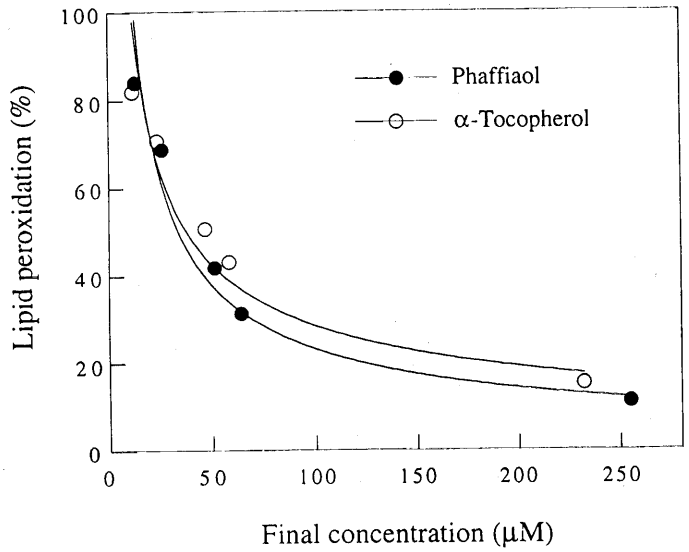
(17.0 mg) as a crystalline colorless powder.

The physico-chemical properties of phaffiaol (**1**) are summarized in Table 1. The molecular formula was established to be $C_{25}H_{44}O_3$ by the HR-EIMS and the elemental analysis data. The UV spectrum was indicative of the presence of a substituted benzene ring (289 nm). The IR spectrum of **1** contained a hydroxyl absorption band at 3480 cm^{-1} and aromatic absorption bands at 1610 and 1500 cm^{-1}.

Table 2 lists the ^{1}H and ^{13}C NMR spectral data of **1** and its acetate (**2**). The 500 MHz ^{1}H NMR spectrum of **1** indicated the presence of meta-coupled aromatic protons at δ 6.35 (1H, d, J=2.5 Hz) and 6.28 (1H, d, J=2.5 Hz), two methoxy groups at δ 3.85 and 3.75, and protons of a long alkyl chain at δ 2.60 (2H, t, J=7.8 Hz), 1.5～1.6 (2H, m), 1.2～1.4 (28H, m), and 0.87 (3H, t, J=7.0 Hz). The 125 MHz ^{13}C NMR spectrum showed signals for the tetrasubstituted benzene ring at δ 152.7 (s, C-4), 146.7 (s, C-2), 137.4 (s, C-1), 128.8 (s, C-6), 105.9 (d, C-5), and 96.6 (d, C-3), signals for the methoxy group at δ 56.0 and 55.8 (each q), and signals for the alkyl side chain at δ 31.9 to 14.1 accounted for 17 carbons. These data indicated that phaffiaol was an asymmetric phenol that had two methoxy groups and a long side chain consisting of a saturated alkyl group on its ring. It was supported by the observation of the fragment ion peak m/z 168 in the EI-mass spectrum produced by the McLafferty rearrangement. The positions of the substituents on the benzene ring were determined by the differences of the ^{13}C NMR signals for the benzene ring between phaffiaol and its acetate. In the ^{13}C NMR spectrum of the acetate, the signals of non-substituted carbons that came at δ 105.2 (d, C-5) and 97.6 (d, C-3) were almost unchanged, whereas the signals of substituted carbons that came at δ 157.8 (s, C-4), 151.7 (s, C-2), and 131.9 (s, C-6) were shifted to 3～5 ppm downfield. These data suggested that non-substituted carbons (C-3 and C-5) were meta-position for the carbon (C-1) linked to the hydroxyl group. To confirm the positions of substituents, the different NOE correlations were obtained. Irradiation of the methoxy signal at δ 3.75 gave NOEs on two aromatic protons (δ 6.35 and 6.28), whereas irradiation of the methoxy signal at δ 3.85 gave NOE only on the aromatic proton at δ 6.35. These results provided unambiguous evidence that phaffiaol is 2,4-dimethoxy-6-heptadecylphenol.

The antioxidative activity of phaffiaol was compared with that of authentic α-tocopherol by using the t-butylhydroperoxide-initiated lipid peroxidation of rabbit erythrocyte ghost membrane[12]. Phaffiaol was considerably effective and its activity was almost equivalent to that of α-tocopherol, as shown in Fig. 2.

Fig. 2. Antioxidative assay of phaffiaol and tocopherol by erythrocyte membrane ghost system.



In conclusion, we have found a novel naturally occurring antioxidant, phaffiaol (**1**), which is the first trisubstituted alkyl phenol isolated from a fungus, *Phaffia rhodozyma* ATCC 24201. The antioxidative activity was demonstrated to be equivalent to α-tocopherol.

Experimental

Detection of Antioxidants

Antioxidants in the *Phaffia rhodozyma* were detected on a TLC plate (E. Merck, silica gel plates 60 F_{254}, 0.25 mm) according to the method of SEINO *et al.*[13] with some modification. The test sample was charged on a TLC plate and developed with *n*-hexane-diethyl ether-AcOH (70:30:1). On this plate was sprayed a 5% ethyl icosapentaenoate (EPA) in 2,2,4-trimethylpentane solution. The plate was successively heated at 40°C for 15 minutes to generate peroxides of EPA. Then *N,N*-dimethyl-1,4-phenylendiamine-AcOH solution was used as a coloring reagent for the peroxides. In this method, 0.2 μg of α-tocopherol was detected on a TLC plate.

Acetylation of Phaffiaol

To a solution of phaffiaol (7 mg, 0.018 mmol) in pyridine (0.8 ml) was added acetic anhydride (0.8 ml), and the mixture was stirred at room temperature for 1 hour. The solution was concentrated under reduced pressure and the residue was purified by preparative TLC (E. Merck, silica gel plates 60 F_{254}, 0.5 mm) with *n*-hexane-diethyl ether (10:1) to afford 7 mg of phaffiaol monoacetate (**2**): EI-MS *m/z* 434 (M^+, 6%), 392 (M^+-42, 100%), 168 (20%), 167 (20%), 153 (5%), 139 (4%). UV (EtOH) λ_{max} nm (ε): 279 (2530). IR (KBr) ν_{max} (cm^{-1}): 2923, 2850, 1756, 1589, 1489. *Anal.* Calcd for $C_{27}H_{46}O_4$: C 74.61; H 10.67. Found: C 74.25; H 10.88.

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References

1) TRYSTMAN, R. J.; J. R. KIRSCH & R. C. KOEHLER: Oxygen radical mechanisms of brain injury following ischemia and reperfusion. J. Appl. Physiol. 71: 1185~1195, 1991
2) SOTOMATSU, A.: Brain damage and free radicals. The Saishin-igaku 45: 1748~1753, 1990
3) KENSLER, T. W. & B. G. TAFFE: Free radicals in tumor promotion. Adv. in Free Radical Biology & Medicine 2: 347~387, 1986
4) SHIOMI, K.; H. YANG, Q. XU, N. ARAI, M. NAMIKI, M. HAYASHI, J. INOKOSHI, H. TAKESHITA & R. MASUYA: Phenopyrrozin, a new radical scavenger produced by *Penicillium* sp. FO-2047. J. Antibiotics 48: 1413~1418, 1995
5) KIM, W.-G.; J.-P. KIM, C.-J. KIM, K.-H. LEE & I.-D. YOO: Benzastatins A, B, C, and D: New free radical scavengers from *Streptomyces nitrosporeus* 30643. J. Antibiotics 49: 20~25, 1996
6) MURAKAMI, Y.; S. KATO, M. NAKAJIMA, M. MATSUOKA, H. KAWAI, K. SHIN-YA & H. SETO: Formobactin, a novel free radical scavenging and neuronal cell protecting substance from *Nocardia* sp. J. Antibiotics 49: 839~845, 1996
7) HATA, K. (Nippon Suisan Kaisha, Ltd.): Manufacture of

antioxidant phenols with *Phaffia rhodozyma*. Jpn. Kokai Tokkyo Koho JP 04 145 036 A2, May 19, 1992

8) MILLER, M. W.; M. YONEYAMA & M. SONEDA: *Phaffia*, a new yeast genus in the *Deuteromycotina* (*Blastomycetes*). Int. J. Syst. Bacteriol. 26: 286~291, 1976

9) FUKUYAMA, Y.; J. OKINO & M. KODAMA: Structures of belamcandol A and B isolated from the seed of *Belamcanda chinesis*. Chem. Pharm. Bull. 39: 1877~1879, 1991

10) SEKI, K.; K. HAGA & R. KANEKO: Phenols and a dioxotetrahydrodibenzofuran from seeds of *Iris pallasii*. Phytochemistry 38: 965~973, 1995

11) METZGER, P. & E. CASADEVALL: Aldehydes, very long chain alkenylphenols, epoxides and other lipids from an alkadiene-producing strain of *Botryococcus braunii*. Phytochemistry 28: 2097~2104, 1989

12) SU, J.-D.; T. OSAWA, S. KAWAKISHI & M. NAMIKI: Tannin antioxidants from *Osbeckia chinensis*. Phytochemistry 27: 1315~1319, 1988

13) SEINO, H.; S. WATANABE & Y. ABE: Studies on the antioxidative compounds in the deodorizer sludge of soybean oil. Yukagaku 20: 218~223, 1971