COMMUNICATION TO THE EDITOR

A New Melanin Biosynthesis Inhibitor, Melanoxadin from Fungal Metabolite by Using the Larval Haemolymph of the Silkworm, *Bombyx mori*

Sir:

Melanin biosynthesis in insects involves the following three key steps: oxidation of tyrosine to dopa by monophenoloxidase (tyrosinase), conversion of dopa to dopachrome by diphenoloxidase and formation of melanin by polymerization of dopachrome.[1] When the larval haemolymph of *Bombyx mori* is exposed to air, it changes from pale yellow to black. This color change is based on melanin formation, by which tyrosine in the haemolymph is oxidized by tyrosinase. We have devised a screening system to search for melanin biosynthesis inhibitors from fungal metabolite using the larval haemolymph of the silkworm, *Bombyx mori* as an experimental animal model and demonstrated that this screening is an useful method to find the inhibitor.[2] We found that a strain of *Trichoderma* sp. ATF-606 isolated from a soil sample collected at Nikko city, Tochigi Prefecture, Japan produced a new melanin biosynthesis inhibitor, melanoxadin. In this communication we report the production, isolation, structure elucidation and biological properties of melanoxadin.

A slant culture of *Trichoderma* sp. ATF-606 which was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM P-14251, was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1.5%, saccharose 1.5%, soybean powder 1.0%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CaCO_3$ 0.5% (adjusted to pH 6.0 before sterilization). The fermentation was carried out at 27°C for 24 hours under aeration and agitation in a 5-liter jar fermentor containing 3 liter of the same medium.

The culture broth (42 liters) was centrifuged to separate the mycelial cake and supernatant. The supernatant was applied to a column of activated charcoal, which was washed with deionized water and then eluted with 50% of acetone. The active eluate was concentrated and lyophilized to obtain a crude powder (24.8 g), which was dissolved in methanol and further concentrated to dryness *in vacuo*. This residue (16.3 g) was chromatographed on a silica gel column using a mixture of chloroform - methanol (30 : 1 to 10 : 1). The active fraction was concentrated under reduced pressure to give a crude powder (386 mg). Further silica gel chromatography of this powder eluting with chloroform - ethyl acetate (3 : 1 to 2 : 1) afforded a white powder (56 mg). The powder was crystallized from a mixture of ethyl acetate and *n*-hexane to give colorless needles (43 mg) of melanoxadin.

The physico-chemical properties of melanoxadin were as follows: MP 73 ~ 74°C; $[\alpha]^{24}$ −20.0° (*c* 0.158, MeOH); UV λ_{max} nm (ε) 227 (19,800) in MeOH; IR (KBr) cm^{-1} 3353, 3285, 3102, 2982, 2932, 2889, 1516, 1468, 1437, 1375, 1327, 1258, 1121, 1094, 1061, 976, 910, 885, 781, 750. HRFAB-MS of melanoxadin revealed the molecular ion at *m/z* $(M+H)^+$ 170.0838 corresponding to the molecular formular $C_8H_{11}NO_3$ (calcd for $C_8H_{12}NO_3$, 170.0817). Melanoxadin is soluble in methanol, ethyl acetate, chloroform, and acetone and insoluble in *n*-hexane and water. The structure of melanoxadin was assigned successfully by ^1H and ^{13}C NMR spectral analyses and HMBC experiment. The 400 MHz ^1H NMR spectrum of melanoxadin taken in $CDCl_3$ is shown in Fig. 1. The ^1H NMR spectrum showed the presence of two aromatic protons (δ_H 7.60, s and 7.85, s), two

Fig. 1. ^1H NMR spectrum of melanoxadin (in $CDCl_3$).

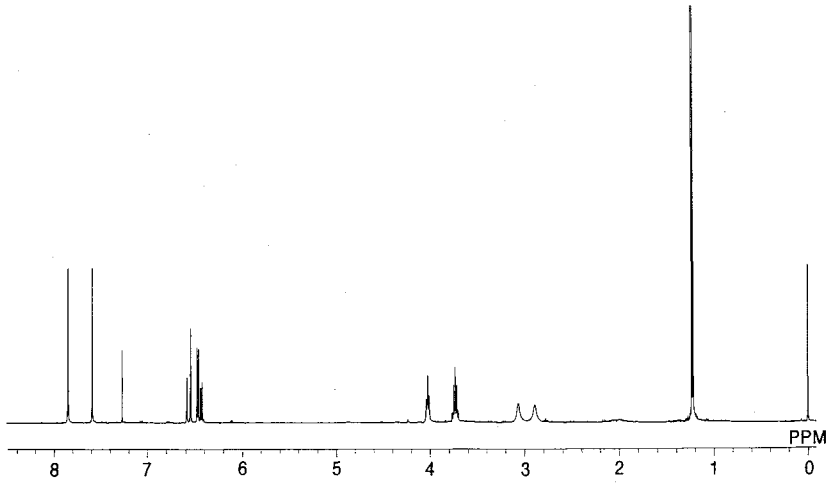


Table 1. ^{1}H and ^{13}C NMR assignments of melanoxadin and diacetylmelanoxadin.

	melanoxadin		diacetylmelanoxadin
	^{13}C chemical shift	^{1}H chemical shift	^{1}H chemical shift
2	151.3	7.85 s	7.83 s
4	137.8		
5	135.4	7.60 s	7.61 s
1'	119.9	6.57 d (15.6) [a]	6.54 d (15.6) [a]
2'	131.3	6.42 dd (6.5, 15.6)	6.36 dd (7.2, 15.6)
3'	76.9	4.03 dd (6.5, 6.3)	5.46 dd (6.4, 7.2)
4'	70.8	3.74 dq (6.3, 6.4)	5.10 dq (6.4, 6.4)
5'	19.0	1.23 d (6.4)	1.25 d (6.4)
4'-OH (OAc)		2.90 br s [b]	2.06 s [b]
3'-OH (OAc)		3.07 br s [b]	2.10 s [b]

[a] Coupling constants in J =Hz.

[b] These assignments are interchangeable.

Fig. 2. The structures and NMR chemical shift assignments of melanoxadin, phthoxazolin A, and oxazole.

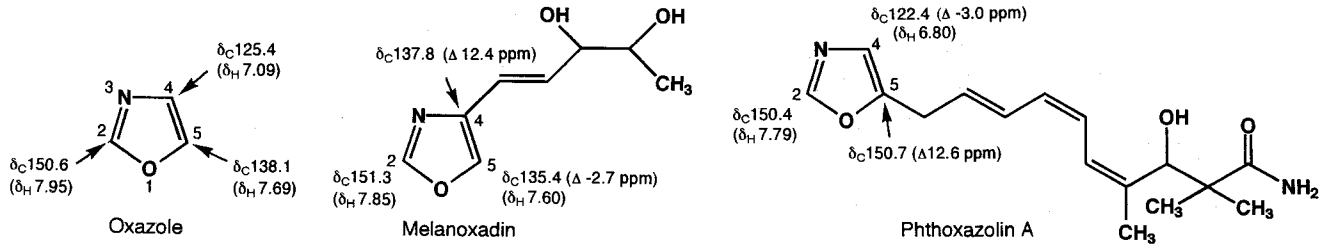
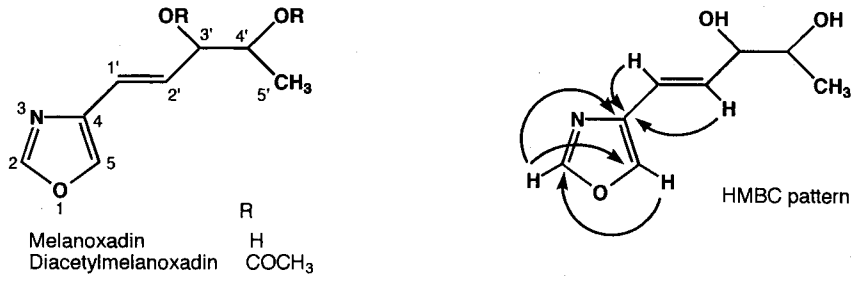


Fig. 3. The structures of melanoxadin and diacetylmelanoxadin.



olefinic protons (δ_H 6.42, dd and 6.57, d), two oxy methines (δ_H 3.74, dq and 4.03, dd), two hydroxyls (δ_H 2.90, br s and 3.07, br s, disappeared with D_2O) and one methyl (δ_H 1.23, d). The presence of a vicinal diol group was confirmed from appearance of two acetoxyl signals (δ_H 2.06, s and 2.10, s) and two oxy methines (δ_H 5.10, dq, 4'-H and 5.46, dd, 3'-H) in the ^{1}H NMR spectrum of diacetylmelanoxadin (FAB-MS m/z $(M+H)^+$ 254) which was obtained by acetylation of melanoxadin with Ac_2O/Py. ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY spectral analyses of melanoxadin deduced the presence of 3',4'-dihydroxypentenyl moiety. According to the molecular formular, seven atoms remain undefined; three carbons, two hydrogens, one nitrogen, and one oxygen. These carbons were reasoned to construct a monosubstituted oxazole ring, based on the chemical shifts of NMR assignable to two aromatic methines (δ_C 151.3/δ_H 7.85 and δ_C 135.4/δ_H 7.60) and one aromatic quaternary carbon (δ_C 137.8), because of the unsaturation index 3 for this moiety. ^{1}H and ^{13}C NMR assignments of melanoxadin and its acetate are shown in the Table 1. The substitution pattern of 3',4'-dihydroxypentenyl moiety to an oxazole ring was determined by comparison of ^{1}H and ^{13}C chemical shift values of melanoxadin with

those of oxazole[3] and phthoxazolin A[4]. A low field shift (*ca.* 12.5 ppm) of each substituted quaternary carbon at C-4 in melanoxadin and at C-5 on oxazole ring in phthoxazolin A, compaired with the ^{13}C chemical shift values at C-4 and C-5 on oxazole implies that 3',4'-dihydroxypentenyl moiety bonds to C-4 on an oxazole ring, but not to C-5, as shown in Fig. 2. The connectivity was also evidenced from HMBC experiment. In the HMBC experiment of melanoxadin, two olefinic protons at δ_H 6.57 (1'-H) and δ_H 6.42 (2'-H) were coupled to a carbon at δ_C 137.8 (C-4). A proton at δ_H 7.85 (2-H) was coupled to a sp^2 quaternary carbon at δ_C 137.8 (C-4) and a sp^2 carbon at δ_C 135.4 (C-5). From the above mentioned results, the structure of melanoxadin was determined to be (*E*)-4-(3',4'-dihydroxypentenyl) oxazole, as shown in Fig. 3.

The inhibition of melanin formation in larval haemolymph of *Bombyx mori* was examined by observing the change in color after incubation of a solution of melanoxadin and the haemolymph using a 96-well plate format at 25°C for one hour. The inhibitory activity toward melanin formation was indicated by the 50% inhibition (IC_{50}) values of the blackish change of the larval haemolymph, which was measured by a photospectrometer (450 nm wavelength). The IC_{50} value of the inhibitor was 22.3 μg/ml, and may be compared to that of trichoviridin[2] (IC_{50}: 13.1 μg/ml) which has also been found in this screening program using the larval haemolymph of the silkworm, *Bombyx mori*. Melanoxadin also exhibited inhibitory activity (IC_{50}: 98 μg/ml) toward the enzymatic reaction of mushroom tyrosinase. The inhibitor showed no effect on melanogenesis of B16 melanoma cell and no antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi or yeast at a concentration of 500 μg/ml.

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RYUJU HASHIMOTO
SENJI TAKAHASHI
KUNIKATSU HAMANO†
AKIRA NAKAGAWA*

Department of Biosciences, Teikyo University,
1-1 Toyosatodai, Utsunomiya, Tochigi 320, Japan
† Faculty of Agriculture,
Tokyo University of Agriculture and Technology,
3-5-8 Saiwaicho, Fuchu, Tokyo 183, Japan

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