

**Melanoxazal, New Melanin Biosynthesis Inhibitor Discovered by Using  
the Larval Haemolymph of the Silkworm, *Bombyx mori*  
Production, Isolation, Structural Elucidation, and Biological Properties**

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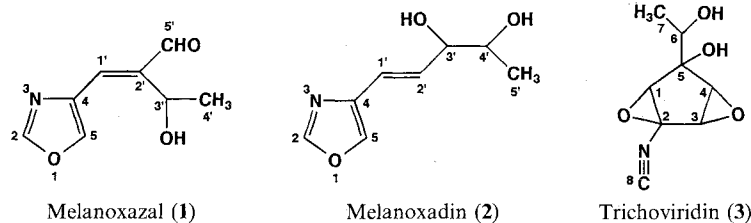
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A new melanin biosynthesis inhibitor, melanoxazal, was isolated from the fermentation broth of *Trichoderma* sp. ATF-451 by successive purification procedures of carbon adsorption, ethyl acetate extraction and silica gel column chromatography. The inhibitor possesses a novel oxazole-containing structure with molecular formula, C<sub>8</sub>H<sub>9</sub>NO<sub>3</sub>. The structure was determined by means of NMR analyses to be (*E*)-4-(2'-formyl-3'-hydroxybuten-1'-yl) oxazole, which is related to melanoxadin. Melanoxazal inhibited melanin formation in the larval haemolymph of the silkworm, *Bombyx mori*; IC<sub>50</sub> value = 30.1 μg/ml. Melanoxazal also showed a strong inhibitory activity against mushroom tyrosinase with IC<sub>50</sub> value = 4.2 μg/ml.

Tyrosinase mediated melanin biosynthesis in insects has been considered to involve three key reactions: the monophenoloxidase (tyrosinase) catalyzed oxidation of tyrosine to 3,4-dihydroxyphenylalanine (dopa) in the first step, the diphenoloxidase catalyzed conversion of dopa to dopachrome *via* dopaquinone in second step, and formation of melanin by spontaneous polymerization of dopachrome in third step.<sup>1)</sup> Several investigations on melanin biosynthesis inhibitors of microbial origin, feldamycin,<sup>2)</sup> melanostatin,<sup>3,4)</sup> albocycline, OH-3984 K1 and K2,<sup>5,6)</sup> MR304A<sup>7)</sup> have been reported with screening methods using mushroom tyrosinase, melanin formation in *Streptomyces bikiniensis* and B16 melanoma cells. We have previously reported our screening program for melanin biosynthesis inhibitors from microorganisms, using the larval haemolymph of the silkworm, *Bombyx*

*mori*, in which we have isolated melanoxadin<sup>8)</sup> (2) and trichoviridin<sup>9)</sup> (3) from the cultured broth of fungal strains ATF-606 and ATF-287, respectively. This method is advantageous in that it can screen many samples in a shorter time than other methods. Furthermore, this screening method provides a useful approach in that it can find novel enzymatic inhibitors, because it contains a wider variety of enzymatic systems in the melanin metabolic pathway than the other single enzymatic systems. During the course of searching for melanin biosynthesis inhibitors, a novel oxazole compound designated as melanoxazal (1) (Fig. 1) was isolated from fungi. Taxonomical study indicated that the producing organism was *Trichoderma* sp. ATF-451. Structural studies of 1 showed that it possesses an oxazole skeleton with a molecular formula of C<sub>8</sub>H<sub>9</sub>NO<sub>3</sub> which is struc-

Fig. 1. The structures of melanoxazal (1), melanoxadin (2) and trichoviridin (3).



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turally related to **2**. Melanoxazal (**1**) inhibited melanin formation in larval haemolymph of the silkworm, *Bombyx mori*, and against mushroom tyrosinase but did not show inhibitory activity of melanin formation in B16 melanoma cells.

In this paper we describe the production, isolation, structure elucidation and biological properties of melanoxazal.

### Materials and Methods

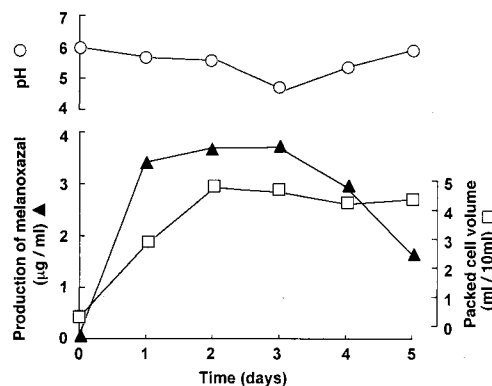
#### Microorganism

The fungal strain ATF-451 was isolated from a soil sample collected in Togane city, Chiba Prefecture, Japan. Colonies grown on 2% malt extract agar were more than 90 mm in diameter after incubation for 3 days at 25°C. This strain grew rapidly to form green to pale green colonies, with conidiophores borne from substrate hyphae. The colony surface was floccose. Conidia were produced in a slimy mass at the tip of the phialides, globe to subglobe, 3.5~5 × 2.5~3 μm. Phialides were generally borne in false verticils of two to five, flask-shaped, 4~6 × 3~4.5 μm. From the characteristics mentioned above, the fungal strain belongs to the genus *Trichoderma* and was named *Trichoderma* sp. ATF-451. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan, as *Trichoderma* sp. ATF-451 with an accession number of FERM P-14252.

#### Fermentation

A slant culture strain *Trichoderma* sp. ATF-451 grown on yeast-soluble starch (YpSs) agar [yeast extract 0.4% (Kyokuto Pharmaceutical Industrial Co.), soluble starch 1.5%, K<sub>2</sub>HPO<sub>4</sub> 0.1%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05% and agar 2.0%, pH 6.0] was transferred into 100-ml test tubes containing 10 ml of CzS-8 medium composed of glucose 1.5%, saccharose 1.5%, soybean powder 1.0% (Honen Co.), KH<sub>2</sub>PO<sub>4</sub> 0.1%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, KCl 0.05%, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.001%, CaCO<sub>3</sub> 0.5%. A CzS-8 medium was used for both seed and production cultures. The pH was adjusted to 6.0 before sterilization and incubated for 1 day at 27°C on rotary shaker at 230 rpm. Three ml of this seed culture was inoculated into 100 ml of the same medium in 500-ml Erlenmeyer flasks and incubated for 1 day at 27°C on rotary shaker at 230 rpm. Ninety ml of the seed culture were transferred into 3 liters of a production medium (CzS-8 medium), using a 5-liter mini-jar fermentor. The fermentation was carried out for 2 days at 27°C with an aeration at 3 liters per minute and an agitation speed of 250 rpm. The growth was monitored by packed cell volume measurement. The production of **1** was measured by analytical HPLC, using a Shimadzu LC-9A system (column, Senshu pack ODS-H-1251; 4.6 × 250 mm; mobile phase: acetonitrile - water, 15: 85; flow rate, 1 ml per minute; detection, absorbance

Fig. 2. Time course of melanoxazal (**1**) production by *Trichoderma* sp. ATF-451.



at 276 nm). The sample for HPLC analysis was prepared as follows: 5 ml of the culture broth was centrifuged at 3,000 × g for 10 minutes and adsorbed on a charcoal column (1 ml). After washing with water (5 ml), the column was eluted with 5 ml of 50% aqueous acetone. The eluate was concentrated *in vacuo* and dissolved in 5 ml of methanol. A 10 μl aliquot of the methanol solution was injected on the column. Melanoxazal (**1**) was eluted with a retention time at 11.3 minutes. A typical time course for the production in a 5-liter jar fermentor of **1** by *Trichoderma* sp. ATF-451 is shown in Fig. 2. The production of **1** reached a maximum on 2 to 3 days after inoculation.

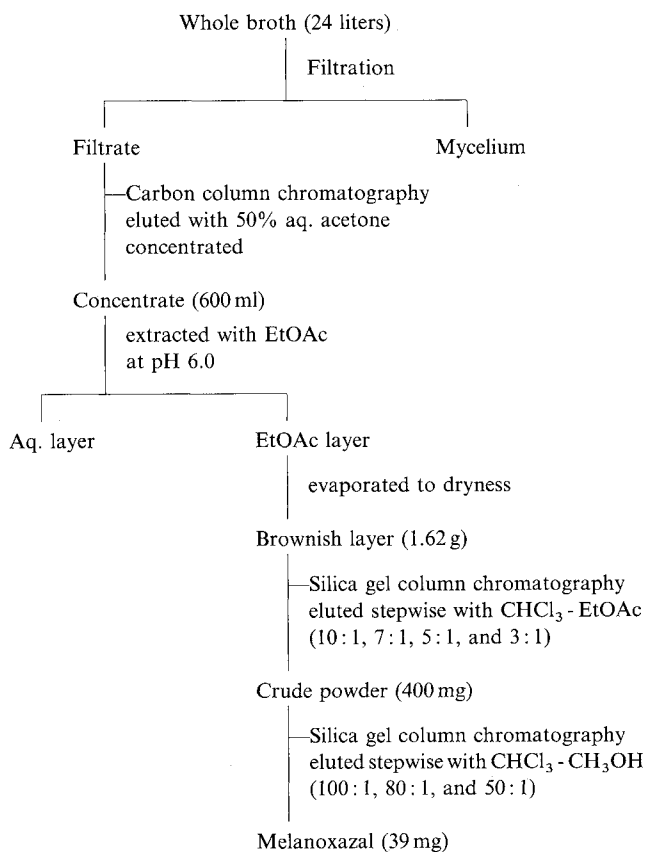
#### Isolation of Melanoxazal

The culture broth (24 liters) was filtered to remove the mycelium. The filtrate was adsorbed on an activated charcoal column (Wako Pure Chemical Industries, Ltd., 800 ml) and the components were sequentially eluted with water (500 ml), 50% aqueous acetone (500 ml) and 100% acetone (500 ml). The active substance was eluted with 50% aqueous acetone. The active eluate was concentrated under reduced pressure to aqueous solution (600 ml) and then extracted twice with 200 ml of ethyl acetate at pH 6.0. The organic layer was concentrated *in vacuo* to dryness to yield a brownish powder (1.62 g). The residue was subjected to silica gel column chromatography (Merck Kieselgel 60, 70~230 mesh) using a mixture of chloroform-ethyl acetate (10:1, 7:1, 5:1 and 3:1, stepwise, 500 ml each). The active fractions were combined and concentrated *in vacuo* to give 400 mg of crude powder. The powder was purified repeatedly by silica gel column chromatography, eluting with chloroform-methanol (100:1, 80:1 and 50:1, stepwise, 200 ml each). Evaporation of the active fractions afforded **1** (39 mg) as a white powder. (Fig. 3)

#### General Procedure

<sup>1</sup>H NMR (400 MHz) and <sup>13</sup>C NMR (100 MHz) spectra were recorded on a JEOL JNM EX-400 spectrometer in

Fig. 3. Isolation procedures of melanoxazal (1).



$\text{CDCl}_3$  solution with tetramethylsilane (TMS) as an internal standard and are expressed as ppm ( $\delta$ ). Mass spectra were recorded on a JEOL AX-505W mass spectrometer for FAB-MS and HRFAB-MS with a matrix of glycerol, 3-nitrobenzyl alcohol and polyethyleneglycol. Infrared spectra (KBr pellet) and Ultraviolet spectra were taken on a Shimadzu FTIR-4200 spectrophotometer and Shimadzu UV-3100 spectrophotometer, respectively. Optical rotation was recorded on a Horiba ESPA-200 polarimeter. Melting points were recorded on a Gallenkamp melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed using Merck Kieselgel 60  $\text{F}_{254}$  precoated glass plates (0.25 mm thick). Merck Kieselgel 60  $\text{F}_{254}$  precoated glass plates (0.5 mm thick) were used for preparative purpose. Analytical HPLC was carried out using a Shimadzu LC-9A system consisting of two LC-9A pumps, a SPD-6A variable UV/VIS-detector, a C-R4A CHROMATOPAC data processor and an ODS packed column (Senshu Scientific CO., LTD., ODS-H-1251,  $4.6 \times 250$  mm).

## Results and Discussion

### Physico-chemical Properties

The physico-chemical properties of melanoxazal (1) are summarized in Table 1. The UV spectrum of 1 showed

Table 1. Physico-chemical properties of melanoxazal (1).

Appearance	White powder
$[\alpha]_D^{24}$	+38.9° (c 0.105, MeOH)
Melting point	70.0 ~ 73.0 °C
Molecular formula	$\text{C}_8\text{H}_9\text{NO}_3$
FAB-MS ( $m/z$ )	168 (M+H) <sup>+</sup>
HRFAB-MS ( $m/z$ )	
Calcd.:	168.0661 (as $\text{C}_8\text{H}_{10}\text{NO}_3$ )
Found:	168.0644 (M+H) <sup>+</sup>
UV $\lambda_{\text{max}}$ ( $\epsilon$ ) in	
MeOH	274 (7,100)
IR (KBr) $\text{cm}^{-1}$	3330, 3135, 3123, 2982, 2971, 2932, 2838, 1682, 1642, 1526, 1458, 1435, 1360, 1144, 1111, 1100, 1074, 1038, 982, 916, 895, 820, 804, 689, 669, 619
Solubility	
Soluble:	MeOH, EtOH, Acetone, EtOAc, $\text{CHCl}_3$
Insoluble:	$\text{H}_2\text{O}$ , Hexane

a maximum absorption at 274 nm ( $\epsilon$  7100) in methanol. The IR spectrum of 1 showed the presence of a hydroxy group at  $3330 \text{ cm}^{-1}$  and an  $\alpha,\beta$ -unsaturated carbonyl group at  $1682 \text{ cm}^{-1}$ . The molecular weight of 1 was shown to be 167 from FAB-MS peaks at  $m/z$  168 (M+H)<sup>+</sup>. The high resolution measurement of the molecular ion peak in FAB-MS gave  $m/z$  168.0644.

### Structural Elucidation

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data of melanoxazal (1) in  $\text{CDCl}_3$  are summarized in Table 2. The  $^1\text{H}$  NMR spectrum (Fig. 4) indicated a total of nine protons: one methyl proton at 1.45 ppm, one oxymethine at 5.16 ppm, one olefinic proton at 6.99 ppm, two aromatic methines at 8.05 ppm and 8.06 ppm and one hydroxy proton at 5.65 ppm, respectively. The signal at 9.49 ppm apparently belong to the formyl proton. The  $^{13}\text{C}$  NMR spectrum (Fig. 5) showed eight resolved peaks, which were classified into one methyl carbon at 22.9 ppm, one oxymethine carbon at 63.7 ppm, three olefinic carbons at 134.5 ppm, 141.8 ppm and 151.7 ppm, one aldehyde at 193.9 ppm and two quaternary carbons at 135.8 ppm, 146.6 ppm. The connectivity of proton and carbon atoms was confirmed by  $^{13}\text{C}$ - $^1\text{H}$  COSY experiment. The  $^1\text{H}$ - $^1\text{H}$  COSY data revealed the coupling between 3'-H ( $\delta_{\text{H}}$  5.16) and 4'-H ( $\delta_{\text{H}}$  1.45), and between 3'-H and 3'-OH ( $\delta_{\text{H}}$  5.65). In addition, the heteronuclear multiple bond correlation (HMBC) spectrum indicated the following two- and three-bond connectivities between  $^1\text{H}$  and  $^{13}\text{C}$  from 1'-H ( $\delta_{\text{H}}$  6.99) to 2'-C ( $\delta_{\text{C}}$  146.6), 3'-C ( $\delta_{\text{C}}$  63.7) and 5'-C ( $\delta_{\text{C}}$  193.9), from 3'-H to 1'-C ( $\delta_{\text{C}}$  134.5) and 2'-C, from 4'-H to 2'-C, from 5'-H ( $\delta_{\text{H}}$  9.49) to 2'-C and 3'-C. These spectral analyses clarified the presence of a

Table 2. Chemical shift assignments of the  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  (100 MHz) NMR spectra of melanoxazal (1), (*E*)-acetylmelanoxazal (4) and (*Z*)-acetylmelanoxazal (5) in  $\text{CDCl}_3$ .

Position	1		4		5	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$
2	151.7	8.06 (s)	151.5	8.14 (s)	151.8	7.96 (s)
4	135.8		135.7		136.2	
5	141.8	8.05 (s)	141.8	7.98 (s)	140.9	7.93 (s)
1'	134.5	6.99 (s)	135.7	7.11 (s)	128.8	7.20 (s)
2'	146.6		140.4		141.0	
3'	63.7	5.16 (dq, 11.7, 6.8)	66.6	6.53 (dq, 1.0, 6.8)	67.5	5.86 (dq, 1.0, 5.4)
4'	22.9	1.45 (d, 6.8)	18.2	1.57 (d, 6.8)	20.8	1.43 (d, 5.4)
5'	193.9	9.49 (s)	192.4	9.57 (d, 1.0)	192.5	10.86 (s)
3'-OH		5.65 (d, 11.7)				
3'-OCOCH <sub>3</sub>			21.0	2.04 (s)	21.2	2.10 (s)
3'-OCOCH <sub>3</sub>			170.3		169.7	

Chemical shifts in ppm from TMS as an internal standard.  
Multiplicity and *J* value (Hz) are in parentheses.

Fig. 4.  $^1\text{H}$  NMR spectrum of melanoxazal (1) in  $\text{CDCl}_3$  (400 MHz).

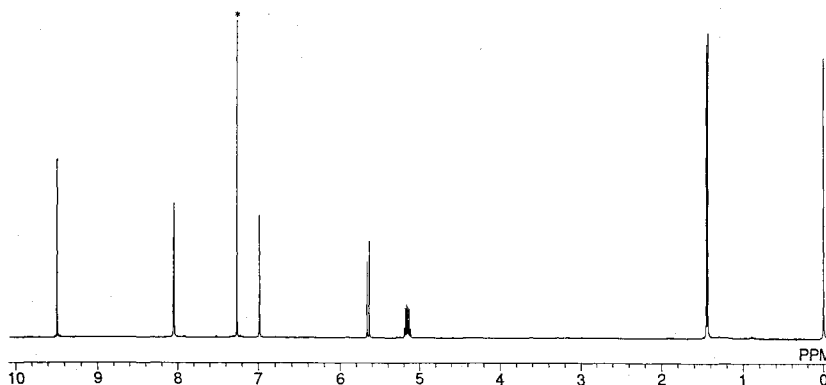
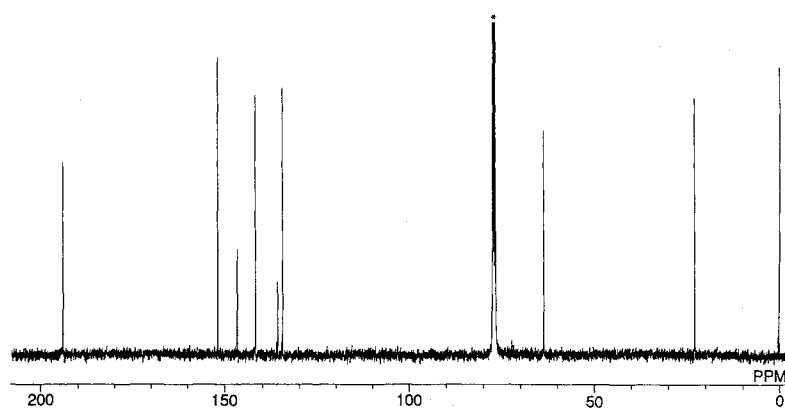
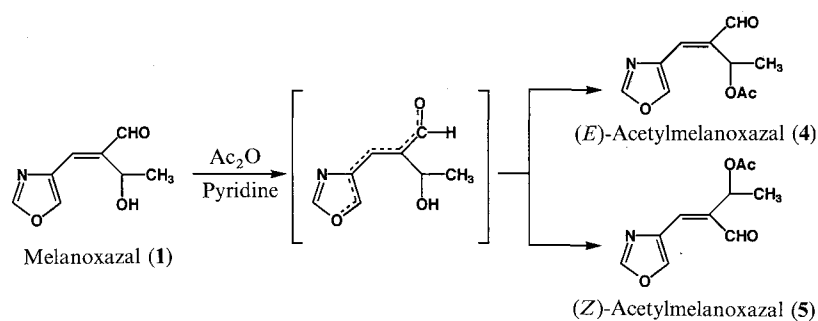


Fig. 5.  $^{13}\text{C}$  NMR spectrum of melanoxazal (1) in  $\text{CDCl}_3$  (100 MHz).



Scheme 1. Proposed mechanism for the acetylation of melanoxazol (1).



2-formyl-3-hydroxybuten-1-yl moiety in **1**. According to the molecular formula, seven atoms remain undefined; three carbons, two hydrogens, one nitrogen, and one oxygen. These remaining atoms should construct a monosubstituted oxazole ring, based on the chemical shifts of <sup>13</sup>C NMR assignment. The oxazole moiety was also elucidated by the following connectivities in the HMBC spectrum, from 1'-H to 4-C ( $\delta_c$  135.8) and 5-C ( $\delta_c$  141.8), from 2-H ( $\delta_H$  8.06) to 4-C and 5-C, from 5-H ( $\delta_H$  8.05) to 2-C ( $\delta_c$  151.7) and 4-C. Furthermore, the <sup>13</sup>C NMR shift differences from the corresponding free oxazole<sup>10</sup>;  $\Delta\delta$  (ppm) = [ $\delta$ (melanoxazol) -  $\delta$ (oxazole)] are exhibited as follows (2-C:  $\Delta\delta_c$  1.1 ppm, 4-C:  $\Delta\delta_c$  10.4 ppm, 5-C:  $\Delta\delta_c$  3.7 ppm). The compound **1** showed larger  $\Delta\delta$  for quarternary carbon at 4-C than for tertiary carbons at 2-C and 5-C which resulting from the electronic effects of the attached alkyl group. The geometry of the double bond (1'-C to 2'-C) was determined to be *E* by NOESY spectrum and NOE data between 1'-H and 5'-H. The stereochemistry at 3'-C has not been established. Thus, the planer structure of **1** was determined to be a (*E*)-4-(2'-formyl-3'-hydroxybuten-1'-yl) oxazole.

#### Acetylation of Melanoxazol

Melanoxazol (**1**) (5.7 mg) was acetylated in a mixture of acetic anhydride (0.2 ml) and pyridine (0.4 ml). The solution was allowed to stand for 18 hours at room temperature. Water was added to the solution and the resulting mixture was extracted with ethyl acetate. After washing the organic layer with saturated aqueous NaHCO<sub>3</sub>, the solvent was dried on anhydrous Na<sub>2</sub>SO<sub>4</sub> and then evaporated to give a residue. The products were purified by preparative TLC using chloroform-ethyl acetate (2:1) to give two monoacetates (**4**: 3.0 mg and **5**: 2.1 mg), which are detectable by UV irradiation at 254 nm. The R<sub>f</sub> values of **4** and **5** on Merck silica gel

plates with chloroform-ethyl acetate (2:1) as development solvents were 0.42 and 0.53, respectively. Both monoacetates possess the same molecular weight, 209 from FAB-MS peaks at  $m/z$  210 (M+H)<sup>+</sup>. The formation of two monoacetates was speculated from the presence of a conjugated skeleton between the 2-formyl-3-hydroxybuten-1-yl moiety and oxazole ring of the melanoxazol under basic conditions as shown in Scheme 1. Both <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra (CDCl<sub>3</sub>) showed eleven proton signals and ten carbon signals, respectively. The connectivity of proton and carbon atoms of two monoacetates (**4** and **5**) and the structures was assigned by <sup>1</sup>H and <sup>13</sup>C NMR spectral analyses, as shown in Table 2. Additionally, the NOE experiment was observed between 1'-H ( $\delta_H$  7.11) and 5'-H ( $\delta_H$  9.57), indicating *E* configuration for **4**. On the other hand, no observation of NOE between 1'-H ( $\delta_H$  7.20) and 5'-H ( $\delta_H$  10.86) indicated the *Z* configuration for **5**.

#### Biological Properties

##### Biological Activity of Melanoxazol using the Larval Haemolymph of the Silkworm, *Bombyx mori*

Fifth instar larvae at day three of *Bombyx mori* (Chinese No. 601 × Japanese No. 601), which had been reared on an artificial diet (chlorella assorted diet for silkworm, Yakult Honsha Co.) were used as the experimental insects for biological assay. The abdominal leg of each larva was pricked with an injection needle, and 1.5 ml haemolymph samples were collected into microtubes. These were stored at -20°C, and then quickly thawed with warm water for use. A sample was dissolved in small amount of methanol, and then it was diluted with distilled water to make a 1000 μg/ml sample solution. Fifty μl of the sample solution was diluted serial twofold with distilled water from 1000 μg/ml to 7.8 μg/ml on a 96-well plate. Fifty μl of the thawed larval haemolymph was added to each of the dilutions. After they were

incubated at 25°C for one hour, the changes in color of the haemolymph was observed by the naked eye. Phenylthiourea, which disturbs the blackish change of the larval haemolymph of *Bombyx mori*, was used as a positive control. The inhibitory activity is indicated by the 50% inhibition (IC<sub>50</sub>) value of the blackish change, which was measured by a spectrophotometer (450 nm wavelength: MPR-4Ai, TOSOH). It has been reported<sup>8,9)</sup> that kojic acid, phenylthiourea, melanoxadin (**2**) and trichoviridin (**3**) showed inhibitory activity in this screening program. The IC<sub>50</sub> value of melanoxazol (**1**) in larval haemolymph of the silkworm, *Bombyx mori* was 30.1 µg/ml which was weaker inhibitory active than **2** (IC<sub>50</sub> 22.3 µg/ml) and **3** (IC<sub>50</sub> 13.1 µg/ml).

#### Biological Activity of Melanoxazol using the Mushroom Tyrosinase

The reaction mixture consisted of serial two-fold dilutions of 50 µl of the sample solution and 160 U/ml mushroom tyrosinase (Sigma) in 25 µl of a 0.06 M phosphate buffer (pH 6.8). Twenty-five µl of a solution of L-3,4-dihydroxyphenylalanine (dopa) (1500 µg/ml) was added to the reaction mixture in a 96-well plate and incubated at 25°C for 10 minutes. The optical density at 450 nm was measured by a spectrophotometer. The inhibitory activity of the sample was expressed as the concentration which inhibits 50% of the enzyme activity (IC<sub>50</sub> value). Melanoxazol (**1**) showed a strong inhibition against mushroom tyrosinase with an IC<sub>50</sub> value of 4.2 µg/ml, which was 23-fold more active than that of structurally similar melanoxadin (**2**). The IC<sub>50</sub> value of trichoviridin (**3**) was 6.6 µg/ml, which is almost the same as **1**. Under the same conditions, the IC<sub>50</sub> values of monoacetates, **4** and **5** were 85.9 µg/ml and 6.1 µg/ml, respectively.

#### Other Biological Activities

The antimicrobial activity of **1** was compared at the concentration of 1000 µg/ml by the agar plate diffusion assay (8 mm diameter, ADVANTEC). Melanoxazol (**1**) showed no antimicrobial activity against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Bacteroides fragilis*, *Acholeplasma laidlawii*, *Candida albicans*, *Saccharomyces sake*, *Aspergillus niger*, *Mucor racemosus* and *Pyricularia oryzae*.

#### Acknowledgments

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