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Studies on Metabolites Produced by *Aspergillus terreus* var. *aureus*. I.[1] Chemical Structures and Antimicrobial Activities of Metabolites Isolated from Culture Broth

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A fungus which was identified as *Aspergillus terreus* var. *aureus* showed antifungal and antibacterial activities. Through the investigation of its metabolites, six compounds (**1**—**6**) were isolated; emodin (**1**), dihydrogeodin (**2**), questin (**3**), sulochrin (**5**), the new metabolite 2-(3-chloro-4-methyl-γ-resorcyloyl)-5-hydroxy-*m*-anisic acid methyl ester (**4**), which is a monochloro derivative of sulochrin (**5**), and compound (**6**), the chemical structure of which has not yet been clarified. Dihydrogeodin (**2**), sulochrin (**5**), **4** and **6** showed antifungal and antibacterial activities, and **6** had especially strong antifungal activity towards *Trichophyton mentagrophytes*.

Keywords——antifungal activity; antibacterial activity; *Aspergillus terreus* var. *aureus*; benzophenone; dihydrogeodin; sulochrin; 2-(3-chloro-4-methyl-γ-resorcyloyl)-5-hydroxy-*m*-anisic acid methyl ester; emodin; questin

Rather few potent antifungal substances have been discovered as yet, and we have therefore been engaged in screening studies for microorganisms which show antifungal activity.

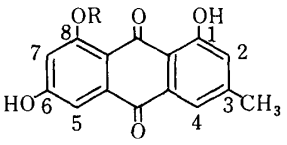
We successfully isolated a fungus which showed antifungal and antibacterial activities, and identified it as *Aspergillus terreus* var. *aureus*. From its culture broth, the metabolites (**1**—**6**) were isolated.

There have already been many reports about the metabolites of *Aspergillus terreus*, and many biologically active substances, such as asteriquinone[2] and quadrone[3] with antitumor activity, LL-S 88α[4] with antiviral activity, and geodin,[5] erdin,[5] geodoxin,[6] dihydrogeodin[7] and sulochrin[8] with antifungal activity, have been isolated. However, there is no previous report about the metabolite (**4**) isolated by the authors.

This paper deals with the chemical structures and antimicrobial activities of these metabolites. The culture broth of *Aspergillus terreus* var. *aureus* was extracted with ethyl acetate, and the metabolites (**1**—**6**) were isolated by silica gel chromatography using benzene, benzene–ethyl acetate and chloroform–methyl alcohol as eluents.

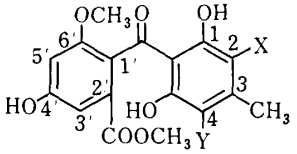
The metabolite (**1**) was obtained as yellow needles, mp 254—256 °C. The high resolution mass (MS) spectrum of **1** gave the molecular formula $C_{15}H_{10}O_5$. Absorption maxima in the ultraviolet (UV) spectrum were observed at 254, 292 and 450 nm. In the infrared (IR) spectrum, absorption bands were observed at 3380 (OH) and 1680 (C=O) cm^{-1}. The MS showed a peak due to M^+ at *m/z* 270. The proton magnetic resonance (^{1}H-NMR) data are listed in Table I together with those for other metabolites (**2**—**5**). All spectral data were very similar to those of emodin. Therefore, through direct comparison of its spectral data with

TABLE I. The Proton Magnetic Resonance Data for the Metabolites (**1**—**5**) (δ, ppm, DMSO-d_6)



1: R=H
3: R=CH_3

Metabolite	C_3–CH_3	C_8–CH_3	Aromatic protons
1	2.36 (3H, s)	—	6.50 (1H, d, J=3 Hz), 7.03 (1H, d, J=3 Hz), 7.06 (1H, d, J=2 Hz), 7.39 (1H, d, J=2 Hz)
3	2.33 (3H, s)	3.86 (3H, s)	6.76 (1H, d, J=3 Hz), 7.03 (1H, d, J=2 Hz), 7.14 (1H, d, J=3 Hz), 7.37 (1H, d, J=2 Hz)



2: X=Cl, Y=Cl
4: X=Cl
5: X=H, Y=H

Metabolite	C_4–CH_3	$C_{2'}$–$COOCH_3$	C_6–OCH_3	Aromatic protons
2	2.42 (3H, s)	3.67 (3H, s)	3.67 (3H, s)	6.69 (1H, d, J=2 Hz), 6.92 (1H, d, J=2 Hz)
4	2.20 (3H, s)	3.62 (3H, s)	3.62 (3H, s)	6.14 (1H, s), 6.66 (1H, d, J=2.2 Hz), 6.86 (1H, d, J=2.2 Hz)
5	2.15 (3H, s)	3.64 (3H, s)	3.64 (3H, s)	6.08 (2H, s), 6.65 (1H, d, J=2 Hz), 6.89 (1H, d, J=2 Hz)

those of an authentic sample, **1** was identified as emodin (Chart 1).

The metabolite (**2**), $C_{17}H_{14}Cl_2O_7$ (high resolution MS: m/z M^+ 400.0109), mp 223—227°C, was positive in the Beilstein reaction. All spectral data of **2** and its acetyl derivative (**2a**) were very similar to those of dihydrogeodin and dihydrogeodintriacetate, and through direct comparison of its spectral data with those of an authentic sample, **2** was identified as dihydrogeodin[7] (Chart 1). Compound (**2**) showed antifungal and antibacterial activities, as shown in Table II.

The metabolite (**3**) was obtained as brown needles, mp 255°C (dec.). The high resolution MS and the elemental analysis of **3** gave the molecular formula $C_{16}H_{12}O_5$. Absorption maxima in the UV spectrum were observed at 251, 286 and 441 nm, and in the IR spectrum absorption bands were observed at 3250 (OH) and 1625 (C=O) cm^{-1}. The MS showed a peak due to M^+ at m/z 284. These spectral data were similar to those of the methoxy derivative of emodin. Thus, **3** was demethylated with conc. sulfuric acid to yield **3a**. All spectral data of **3a** coincided with those of emodin. The present results suggest that **3** is a 1-, 6- or 8-methoxy derivative of emodin. We concluded that **3** was questin from the following results. Compound (**3**) was acetylated to yield a diacetate (**3b**). The ^{1}H-NMR spectrum of **3b** showed signals due to four protons (7.10, 7.22, 7.63 and 7.99 ppm, each J=2 Hz), each as a doublet, in the aromatic proton region. Upon irradiation at a broad methyl signal due to C-3 (2.38 ppm), two broad doublet signals due to C-2 and C-4 (1H each, 7.22 and 7.99 ppm) each changed into a sharp doublet. In addition, nuclear Overhauser effect (NOE) was observed between the C-8 methoxyl group and C-7 hydrogen in **3b**. These findings suggested that the methoxyl group should be at C-8. The spectral data of **3** were not identical with those of physion. Therefore, **3** was concluded to be questin[9] (Chart 1).

All spectral data of the metabolite (**5**), $C_{17}H_{16}O_7$ (high resolution MS: m/z M^+ 332.0893),

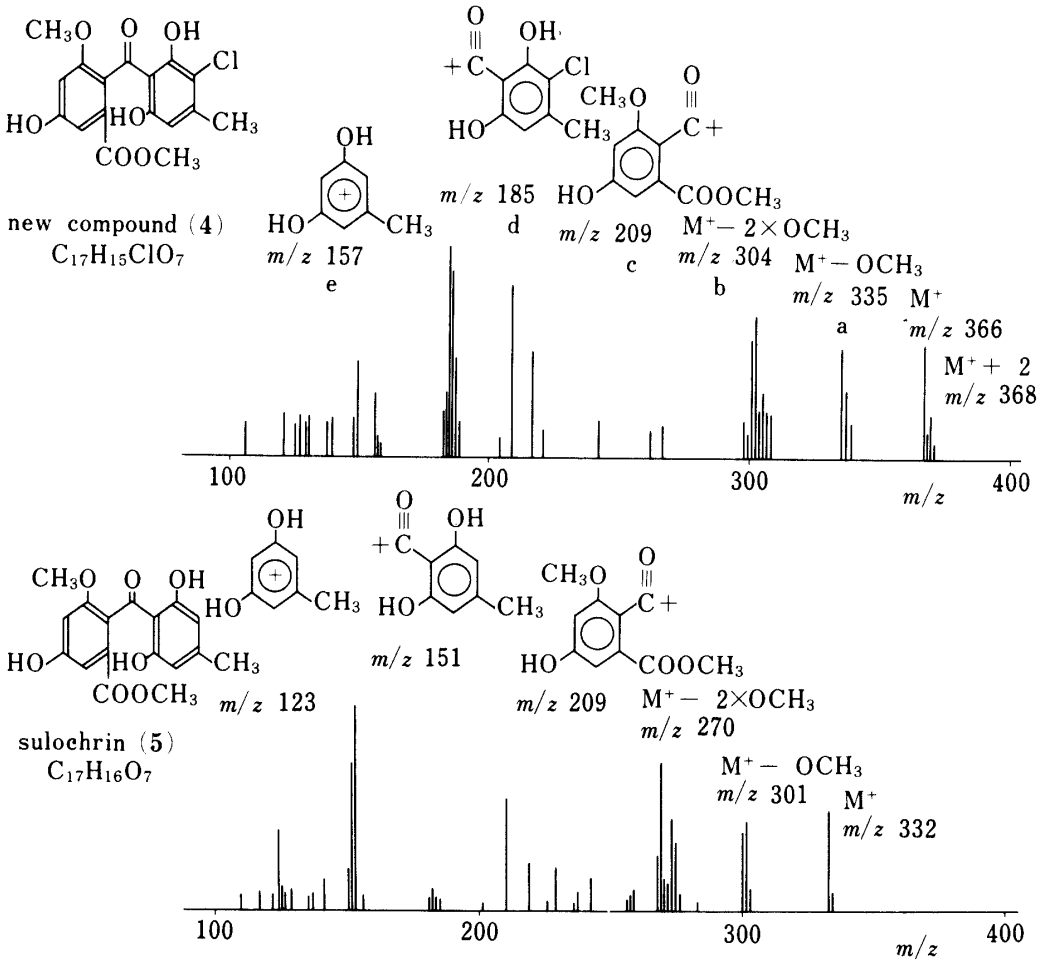


Fig. 1. Mass Spectra of the New Compound (**4**) and Sulochrin (**5**)

mp 240—245 °C, and its acetyl derivative (**5a**) were very similar to those of sulochrin and sulochrin triacetate. Through direct comparison of its spectral data with those of an authentic sample, **5** was identified as sulochrin[8] (Chart 1). Compound (**5**) showed antifungal and antibacterial activities (Table II).

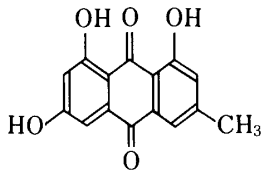
The metabolite (**4**) was obtained as pale yellow needles, mp 243—250 °C, exhibiting the Beilstein reaction. The high resolution MS of **4** gave the molecular formula $C_{17}H_{15}ClO_7$. An absorption maximum in the UV spectrum was observed at 282 nm, and appeared to be similar to those of sulochrin (**5**) and dihydrogeodin (**2**). This suggests that the skeleton of **4** is of benzophenone type. The IR spectrum of **4**, exhibiting absorption bands at 3400 (OH), 1700 (ester) and 1620 (C=O) cm^{-1}, was similar to that of sulochrin (**5**). In the ^1H-NMR spectrum (Table I) of **4** a signal corresponding to the 2H singlet of sulochrin (**5**) at 6.08 ppm appeared as a 1H singlet at 6.14 ppm, but the other signals were similar to those of sulochrin (**5**). The MS of **4** showed a peak due to M^+ at *m/z* 366 and fragment peaks a—e at *m/z* 335, 304, 209, 185 and 157, respectively, as shown in Fig. 1. These fragment peaks were 34 mass units higher than those of sulochrin (**5**). Thus, it was clear that **4** is a monochloro derivative of sulochrin (**5**), that is, 2-(3-chloro-4-methyl-γ-resorcyloyl)-5-hydroxy-*m*-anisic acid methyl ester (Chart 1). Compound (**4**) is a new substance which shows antifungal and antibacterial activities (Table II).

The metabolite (**6**) was obtained as yellow needles, mp 265 °C and showed antifungal and antibacterial activities (Table II). In particular, it inhibited the growth of *Trichophyton mentagrophytes* IFO-5811 at 3.12 μg/ml and also the growth of *Bacillus subtilis* IFO-3513 at

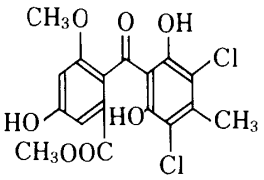
TABLE II. Antifungal and Antibacterial Activities of Metabolites (2), (4)—(6)

Microorganism	MIC (μg/ml)			
	2	4	5	6
Fungi				
Trichophyton mentagrophytes IFO-5811	50	6.25	>50	3.12
Candida albicans IAM-4966	>200	>200	>50	>200
Pyricularia oryzae IFO-5279	>200	>200	>50	>200
Aspergillus fumigatus IAM-2004	>200	>200	>50	>200
Helminthosporium sesamum IAM-5012	>200	>200	>50	>200
Bacteria				
Bacillus subtilis IFO-3513	6.25	>50	>50	6.25
Staphylococcus aureus IFO-12732	>100	>50	>50	12.5
Klebsiella pneumoniae IFO-3512	>100	>50	>50	>100
Serratia marcescens IAM-1136	>100	>50	>50	>100
Escherichia coli IFO-12734	>100	>50	>50	>100
Proteus vulgaris IFO-3851	>100	>50	>50	25
Pseudomonas aeruginosa IFO-12689	>100	>50	>50	>100
Mycobacterium smegmatis IFO-13167	>100	>50	>50	>100

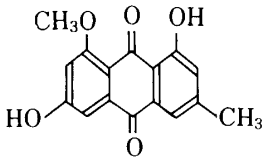
Culture conditions: fungi, 27 °C, 7 d (*Candida albicans*, 2 d); bacteria 37 °C, 18 h. Media: fungi, potato dextrose agar (BBL Co., Ltd.); bacteria, heart infusion agar (Eiken Co., Ltd.). Method: agar dilution method.



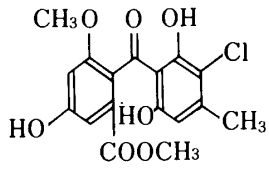
1 emodin $C_{15}H_{10}O_5$



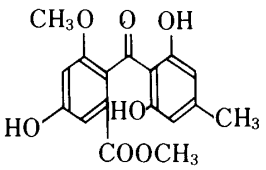
2 dihydrogeodin $C_{17}H_{14}Cl_2O_7$



3 questin $C_{16}H_{12}O_5$



4 new compound $C_{17}H_{15}ClO_7$



5 sulochrin $C_{17}H_{16}O_7$

Chart 1. Chemical Structures of Compounds Isolated from the Culture Broth of *Aspergillus terreus* var. *aureus*

6.25 μg/ml. However, the amount obtained was very small, and its chemical structure is still under investigation.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. The UV, IR, ^{1}H-NMR and mass spectra were taken on Shimadzu UV-200 A, Hitachi 285, JEOL FX-100 and Hitachi M-80 machines, respectively. ^{1}H-NMR chemical shifts are given on the δ(ppm) scale (s, singlet; d, doublet). Column chromatography was performed on silica gel (Wako, C-200), and thin-layer chromatography (TLC) was carried out on silica gel plates (Merck silica gel 60).

Identification of *Aspergillus terreus* var. *aureus* ——The results of morphological observation of the isolated

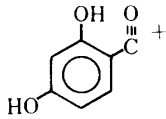
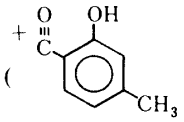
fungus through the slide culture process were as follows. Colonies: when cultivated on Czapek agar medium, they looked like cotton, and were yellow-brown. Conidial heads: on slide culture, they were completely cylindrical (180—450 × 35—50 μm) and yellow-brown in color. Conidiophore: smooth, slightly golden, 750—2000 × 5—8 μm. Vesicles: hemisphere of 8—20 μm in diameter. Sterigmata: these had two stages, the ones in the first stage being 5—6 × 2.0—2.5 μm and the others being 5.0 × 1.5—2.0 μm. Conidia: many were spherical but a few were elliptical and were 1.8—3.0 μm in diameter. Sclerotia: none could be recognized, but there were slightly enlarged cells. From these results the fungus was considered to be in the genus *Aspergillus terreus* (yellow-brown color, completely cylindrical heads, and two-staged sterigmata). It was identified as *Aspergillus terreus* THOM var. *aureus*, because it had no sclerotium but a long golden conidiophore of more than 500 μm.

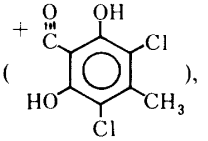
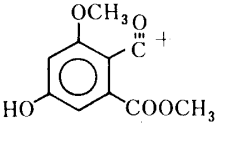
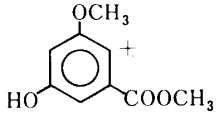
Medium and Cultivation——Medium: 4% soluble starch, 0.5% peptone, 0.7% beef extract and 0.5% NaCl (pH 6.0). Cultivation: a loopful of spores of *Aspergillus terreus* var. *aureus* was transferred to a 500 ml flask containing 100 ml of medium and this flask was incubated with reciprocal shaking (120 rpm, amplitude 7 cm) at 27 °C for 7 d.

Determination of Antibiotic Potency——Agar dilution method. Antifungal Activity Test: Fungi; *Trichophyton mentagrophytes* IFO-5811, *Candida albicans* IAM-4966, *Pyricularia oryzae* IFO-5279, *Aspergillus fumigatus* IAM-2004 and *Helminthosporium sesamum* IAM-5012. Minimal inhibitory concentration (MIC) were determined by the following method. That is, the test microorganism was applied to potato dextrose agar (BBL Co., Ltd.) containing various concentrations of the metabolites. Then the plates were incubated at 27 °C for 7 d (*Candida albicans*: 2 d) and growth was observed with the naked eye.

Antibacterial Activity Test: Bacteria; *Bacillus subtilis* IFO-3513, *Staphylococcus aureus* IFO-12732, *Klebsiella pneumoniae* IFO-3512, *Serratia marcescens* IAM-1136, *Escherichia coli* IFO-12734, *Proteus vulgaris* IFO-3851, *Pseudomonas aeruginosa* IFO-12689 and *Mycobacterium smegmatis* IFO-13167. MIC values were determined by the following method. That is, the test microorganism was applied to heart infusion agar (Eiken Co., Ltd.) containing various concentrations of the metabolites. Then the plates were incubated at 37 °C for 18 h and growth was observed with the naked eye.

Extraction and Separation of the Metabolites——The culture broth (17 l) were extracted three times with AcOEt. The AcOEt layer, after being dried with Na_2SO_4 overnight, was evaporated to dryness under reduced pressure. The AcOEt extract (*ca.* 30 g) was chromatographed on silica gel with benzene, benzene–AcOEt and $CHCl_3$–MeOH as eluents to give **1** (emodin, 150 mg), **2** (dihydrogeodin, 1200 mg), **3** (questin, 1000 mg), **4** (unknown, 45 mg), **5** (sulochrin, 800 mg) and **6** (under investigation, 10 mg).

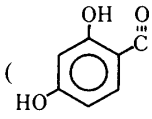
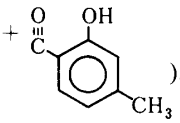
Emodin (1)——**1** was recrystallized from AcOEt as yellow needles, mp 254—256 °C. TLC *Rf*: 0.59 (benzene : AcOEt = 1 : 1), 0.43 (benzene : AcOEt = 3 : 1), 0.32 (benzene : AcOEt = 5 : 1), 0.53 ($CHCl_3$: MeOH = 10 : 1). MS *m/z*: 270.0534 (M^+, Calcd for $C_{15}H_{10}O_5$: 270.057), base peak, 255 ($M^+ - CH_3$), 253 ($M^+ - OH$), 242 ($M^+ - CO$), 136 (), 134 (). UV λ_{max}^{EtOH} nm (log ε): 254 (4.19), 292 (4.14), 450 (3.84). IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1615 (C=O). The ^{1}H-NMR data are summarized in Table I.

Dihydrogeodin (2)——**2** was recrystallized from AcOEt as pale yellow needles, mp 223—227 °C, Beilstein reaction (+). TLC *Rf*: 0.50 (benzene : AcOEt = 1 : 1), 0.30 (benzene : AcOEt = 3 : 1), 0.18 (benzene : AcOEt = 5 : 1), 0.42 ($CHCl_3$: MeOH = 10 : 1). MS *m/z*: 400.0109 (M^+, Calcd for $C_{17}H_{14}Cl_2O_7$: 400.0116), base peak, 369 ($M^+ - OCH_3$), 214 (), 209 (), 182 (). UV λ_{max}^{EtOH} nm (log ε): 283 (3.82). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1715 (ester), 1590 (C=O). The ^{1}H-NMR data are summarized in Table I.

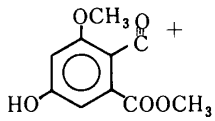
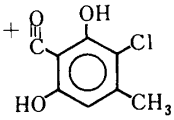
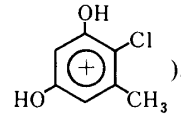
Acetylation of Dihydrogeodin (2)——A mixture of **2** (30 mg), AcONa (60 mg) and Ac_2O (3 ml) was refluxed for 4 h on an oil bath. The product was recrystallized from MeOH to give the triacetate (**2a** 28 mg) as colorless plates, mp 163—165 °C. MS *m/z*: 526.0449 (M^+, Calcd for $C_{23}H_{20}Cl_2O_{10}$: 526.0433), base peak, 484 ($M^+ - CH_2C=O$), 442 ($M^+ - 2 \times CH_2C=O$). UV λ_{max}^{EtOH} nm (log ε): 250 (3.73), 309 (3.40). IR ν_{max}^{KBr} cm^{-1}: 1765 (acetyl C=O), 1710 (ester C=O), 1675 (C=O). ^{1}H-NMR ($CDCl_3$): 2.07 (6H, s), 2.33 (3H, s), 2.58 (3H, s), 3.68 (3H, s), 3.75 (3H, s), 6.85 (1H, d, *J* = 3 Hz), 7.26 (1H, d, *J* = 3 Hz).

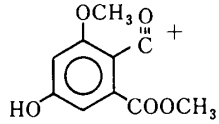
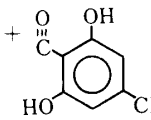
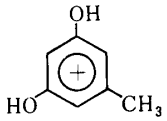
Questin (3)——**3** was recrystallized from AcOEt as brown needles, mp 255 °C (dec.). TLC *Rf*: 0.37 (benzene : AcOEt = 1 : 1), 0.18 (benzene : AcOEt = 3 : 1), 0.11 (benzene : AcOEt = 5 : 1), 0.47 ($CHCl_3$: MeOH = 10 : 1). *Anal.* Calcd for $C_{16}H_{12}O_5$: C, 67.60, H, 4.26. Found: C, 67.44, H, 4.21. MS *m/z*: 284.067 (M^+, Calcd for $C_{16}H_{12}O_5$: 284.0686), base peak, 269 ($M^+ - CH_3$), 267 ($M^+ - OH$). UV λ_{max}^{EtOH} nm (log ε): 251 (4.20), 286 (4.24), 441 (2.88). IR ν_{max}^{KBr} cm^{-1}: 3250 (OH), 1625 (C=O). The ^{1}H-NMR data are summarized in Table I.

Demethylation of Questin (3)——A mixture of **3** (50 mg) and conc. H_2SO_4 (5 ml) was heated for 30 min on an oil bath (150 °C). The product was recrystallized from MeOH to give **3a** (30 mg), as yellow needles, mp 258.5—262 °C. MS *m/z*: 270.0534 (M^+, Calcd for $C_{15}H_{10}O_5$: 270.0570), base peak, 255 ($M^+ - CH_3$), 253 ($M^+ - OH$), 242 ($M^+ - CO$),

136 (), 134 (). UV λ_{max}^{EtOH} nm (log ε): 254 (4.06), 291 (4.08), 448 (3.82). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1625 (C=O). ^{1}H-NMR (DMSO-d_6) : 2.36 (3H, s), 6.50 (1H, d, J=3 Hz), 7.02 (1H, d, J=3 Hz), 7.06 (1H, d, J=2 Hz) 7.39 (1H, d, J=2 Hz).

Acetylation of Questin (3)——A mixture of **3** (30 mg), AcONa (60 mg) and Ac_2O (3 ml) was refluxed for 4 h on an oil bath. The product was recrystallized from MeOH to give the diacetate (**3b**, 25 mg), as colorless plates, mp 166—167 °C. MS *m/z*: 368.0871 (M^+, Calcd for $C_{20}H_{16}O_7$: 368.0898), base peak, 326 ($M^+ - CH_2C=O$), 284 ($M^+ - 2\times CH_2C=O$). UV λ_{max}^{EtOH} nm (log ε): 258 (4.53), 375 (3.78). IR ν_{max}^{KBr} cm^{-1}: 1760 (acetyl C=O), 1660 (C=O), 1590 (aromatic ring). ^{1}H-NMR ($CDCl_3$): 2.38 (3H, s), 2.50 (6H, s), 4.01 (3H, s), 7.10 (1H, d, J=2 Hz), 7.22 (1H, d, J=2 Hz), 7.63 (1H, d, J=2 Hz), 7.99 (1H, d, J=2 Hz).

Metabolite (4)——**4** was recrystallized from AcOEt as pale yellow needles, mp 243—250 °C, Beilstein reaction (+). TLC *Rf*: 0.40 (benzene : AcOEt=1 : 1), 0.19 (benzene : AcOEt=3 : 1), 0.11 (benzene : AcOEt=5 : 1), 0.35 ($CHCl_3$: MeOH=10 : 1). MS *m/z*: 366.0491 (M^+, Calcd for $C_{17}H_{15}ClO_7$: 366.0506). base paek, 368 (M^++2), 335 ($M^+ - OCH_3$), 304 ($M^+ - 2\times OCH_3$), 209 (), 185 (), 157 (). UV λ_{max}^{EtOH} nm (log ε) : 282 (3.58). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1700 (ester), 1620 (C=O). The ^{1}H-NMR data are summarized in Table I.

Sulochrin (5)——**5** was recrystallized from AcOEt as pale yellow needles, mp 240—245 °C. TLC *Rf*: 0.42 (benzene : AcOEt=1 : 1), 0.18 (benzene : AcOEt=3 : 1), 0.09 (benzene : AcOEt=5 : 1), 0.31 ($CHCl_3$: MeOH=10 : 1). MS *m/z*: 332.0893 (M^+, Calcd for $C_{17}H_{16}O_7$: 332.0896), base peak, 301 ($M^+ - OCH_3$), 270 ($M^+ - 2\times OCH_3$), 209 (), 151 (), 123 (). UV λ_{max}^{EtOH} nm (log ε) : 283 (4.13). IR ν_{max}^{KBr} cm^{-1} : 3320 (OH), 1685 (ester), 1580 (C=O). The ^{1}H-NMR data are summarized in Table I.

Acetylation of Sulochrin (5)——A mixture of **5** (20 mg), AcONa (40 mg) and Ac_2O (2 ml) was refluxed for 6 h on an oil bath. The product was recrystallized from MeOH to give the triacetate (**5a**, 16 mg), as colorless needles, mp 167—168.5 °C. MS *m/z*: 458.1216 (M^+, Calcd for $C_{23}H_{22}O_{10}$: 458.1213), base peak, 427 ($M^+ - OCH_3$), 416 ($M^+ - CH_2C=O$), 374 ($M^+ - 2\times CH_2C=O$), 332 ($M^+ - 3\times CH_2C=O$). UV λ_{max}^{EtOH} nm (log ε) : 251 (4.13), 303 (3.72). IR ν_{max}^{KBr} cm^{-1}: 1765 (acetyl C=O), 1720 (ester), 1670 (C=O). ^{1}H-NMR ($CDCl_3$): 1.99 (6H, s), 2.30 (3H, s), 2.37 (3H, s), 3.64 (3H, s), 3.72 (3H, s), 6.78 (2H, s), 6.80 (1H, d, J=2.5 Hz), 7.24 (1H, d, J=2.5 Hz).

Metabolite (6)——**6** was recrystallized from AcOEt as yellow needles mp 265 °C.

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References and Notes

1) This work was presented at the 32nd Kinki Regional General Meeting of the Japanese Society of Pharmacy, Osaka, November 1982. The isolation number for *Aspergillus terreus* THOM var. *aureus* is IFO-31217.
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