evaporated off under reduced pressure. Next, 10 ml of ethanol and an excess of 2,4-dinitrophenylhydrazine in 2N HCl were added to the residure. The amorphous crystalline product was recrystallized from ethanol–DMF. mp 242—243° *Anal.* Calcd for $C_{13}H_{10}N_4O_4$: C, 54.55; H, 3.52; N, 19.58. Found: C, 54.44; H, 3.38; N, 19.48. No mixed melting point depression was observed. The IR spectrum of the crystalline product was identical with that of an authentic sample.

2,4-Dinitrophenylhydrazones of crotonaldehyde, cinnamaldehyde and acrolein were identified by GLC using a column packed with silicone SF-96[9] and using a column packed with P.E.G. 20M, maintained at 250° and 160°, respectively.

c) Benzoic Acid: The IR spectrum of the product extracted from the electrolyzed solution with ether after addition of concentrated hydrochloric acid and 2,4-dinitrophenylhydrazine, and purified by sublimation, was identical with that of an authentic sample. The amount of the acid in the electrolyzed solution was estimated using i) the spectrophotometric method of Tamura[10] and ii) HPLC (Waters Associates Inc., model 6000, and Nihon Bunko UVIDEC-1). In HPLC, the solution (20 μl) was analyzed on a column packed with BONDAPAK C_{18} using 20% aqueous methanol as an eluent. The absorption maximum of benzoic acid at 223 nm was used for estimation.

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9) R.J. Soukur, R.J. Scarpellino and E. Danelzik, *Anal. Chem.*, **36**, 2255 (1964).
10) Y. Kasai, T. Tamura and Z. Tamura, *Anal. Chem.*, **47**, 34 (1975).

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Syntheses of Two Microbial Metabolites, 5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin and 8-Hydroxy-6-methoxy-3-methylisocoumarin[1]

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The racemate of 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (I), a fungal metabolite of *Periconia macrospinosa*, was synthesized from 6-chloro-3,5-dimethoxyhomophthalic acid (III) *via* compounds IV, VI, VII and VIII. Catalytic hydrogenation of V resulted in dechlorination, yielding 6-methoxy-8-hydroxy3-methylisocoumarin (II), another natural product from *Streptomyces mobaraensis*.

Keywords——fungal metabolite; *Periconia macrospinosa*; chloroisocoumarin; chloro-3,4-dihydroisocoumarin; 3,4-dihydro-8-hydroxyisocoumarin; *Streptomyces mobaraensis*

In 1969, Giles and Turner isolated a chlorine-containing metabolite from a fungus, *Periconia macrospinosa*, and identified it as 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin(I).[3] We have recently reported that oosponol(8-hydroxy-4-ω-hydroxyacetylisocoumarin) and oospolactone (8-hydroxy-3,4-dimethylisocoumarin), isolated by us from a wood-rotting basidiomycete, *Gloeophyllum sepiarium*, have antifungal activities[4,5] Later

1) This work was presented at the 99 th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August 1979, p. 308.
2) Location: *Ebara 2-4-41, Shinagawa-ku, Tokyo, Japan.*
3) D. Giles and W.B. Turner, *J. Chem. Soc.*, 2187 (1969).
4) S. Nakajima, K. Kawai. and S. Yamada, *Phytochemistry*, **15**, 327 (1976).
5) S. Nakajima, K. Kawai, S. Yamada, and Y. Sawai, *Agr. Biol. Chem.*, **40**, 811 (1976).

we discovered that the ingredients of *Hydrangea serrata* SERINGE var. *thunbergii* (Japanese name; Amacha), phyllodulcin(3,4-dihydro-8-hydroxy-3-(3'-hydroxy-4'-methoxyphenyl)-isocoumarin) and hydrangenol (3,4-dihydro-8-hydroxy-3-(4'-hydroxyphenyl)-isocoumarin), also possessed antifungal activity.[6] Thus, we came to consider that isocoumarin compounds with a hydroxyl group attached at the 8-position, *i.e.*, the position permitting chelation with the carbonyl moiety, are more or less antifungal. It was for this reason that we attempted to synthesize the above-mentioned fungal metabolite, 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (I), and related compounds in order to examine their antifungal properties. The synthetic scheme is shown in Chart 1.

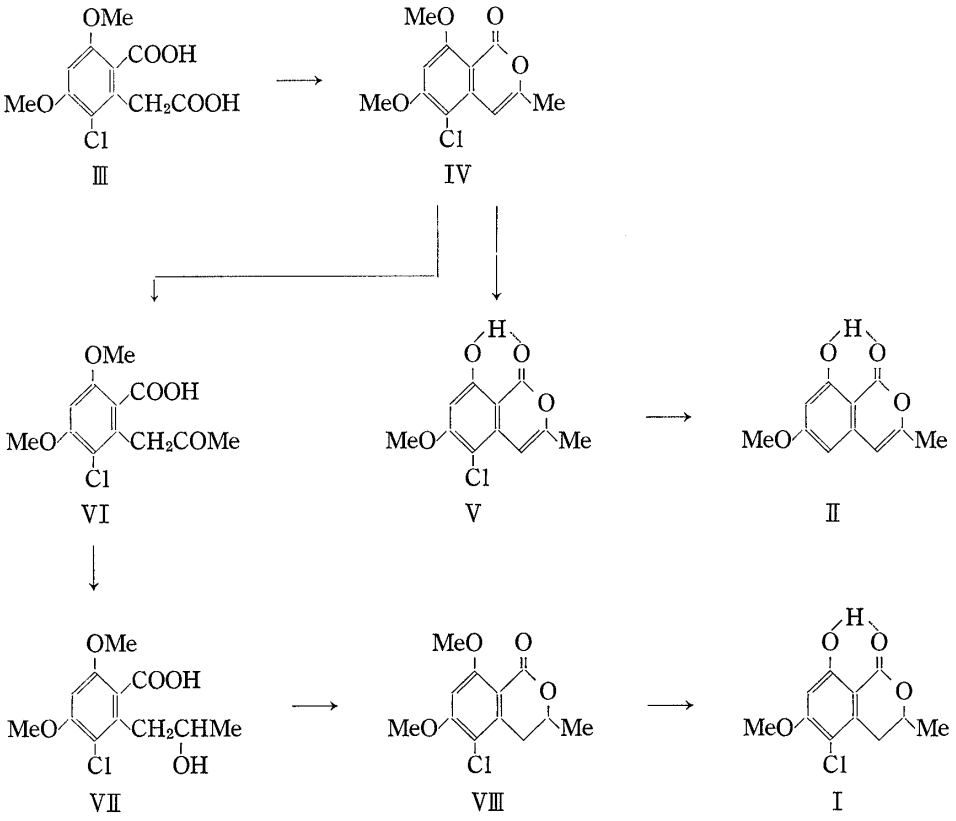

Chart 1

In the course of this synthesis, another microbial methabolite from *Streptomyces mobaraensis*,[7] 8-hydroxy-6-methoxy-3-methylisocoumarin (II), was synthesized. This compound was first synthesized by Hardegger *et al.* from 6,8-dimethoxy-3-methylisocoumarin.[8]

The starting material, 6-chloro-3,5-dimethoxyhomophthalic acid (III), was synthesized by the present authors *via* ten steps as described in the preceding paper.[9] Compound III was converted to 5-chloro-6,8-dimethoxy-3-methylisocoumarin (IV) by the action of acetic anhydride and pyridine. The formation of the isocoumarin structure in this reaction was confirmed by the proton nuclear magnetic resonance (PMR) spectrum of the product IV, which showod one methyl signal at 2.17 ppm slightly coupled (J=1 Hz) with the proton at 6.60 ppm on the double bond. The dimethoxy compound IV was partially demethylated with aluminum chloride in nitrobenzene to afford 5-chloro-8-hydroxy-6-methoxy-3-methylisocoumarin (V).

6) The 98 th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, Apr. 1978, p. 243.
7) M.A.W. Eaton and D.W. Hutchinson, *Tetrahedron Lett.*, 1337 (1971).
8) E. Hardegger, E. Widmer, K. Steiner and A, Pfiffner, *Helv. Chem. Acta*, **47**, 2013 (1964).
9) K. Nozawa, S. Nakajima, and K. Kawai, *Chem. Pharm. Bull.*, **28**, 1112 (1980).

The product V exhibited a blue color with ferric chloride and showed the IR absorption of chelated carbonyl at 1680 cm^{-1}, indicating that partial demethylation had occurred at the methoxyl not at the 6, but at the 8-position of the molecule. Expecting to obtain the target compound I, compound V was subjected to catalytic hydrogenation. Unexpectedly, however, the hydrogenation procedure gave not I but 8-hydroxy-6-methoxy-3-methylisocoumarin (II). The occurrence of dechlorination in this reaction is clear from the mass spectrum of II, which has no characteristic chlorine signal, and also from the PMR spectrum, in which the signals of two coupled aromatic protons are observed. We thus opened the ring of IV by alkali treatment, leading to 3-chloro-4,6-dimethoxy-2-(2'-oxopropyl)benzoic acid (VI), which was treated with sodium borohydride to give 3-chloro-4,6-dimethoxy-2-(2'-hydroxypropyl)benzoic acid (VII). Compound VII was again cyclized to 5-chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (VIII) by treatment with acetic anhydride, and demethylation provided the target compound I.

The compound thus synthesized gave a blue color with ferric chloride. The UV spectrum was characteristic of this type of isocoumarin, coinciding with that of the natural compound I,[3] with peaks at 313, 267 and 220 nm. It is a recemate, and melted at 119°, whereas the natural product melted at 123 to 124°.[3] The PMR spectra indicated complete identity. The positions of the methoxy and chlorine moieties on the benzene ring were determined by Giles *et al.* merely on the basis of biosynthetic and PMR-spectral speculations.[3] However, now that the racemate has been synthesized, their structure has been definitely proved correct.

Compound I thus synthesized inhibited the growth of *Alternaria maritima* and *Fusarium splendens* with a minimun inhibition concentration (MIC) of 200 μg per ml. It was also found that compounds II and IV showed antifungal activity against *Fusarium splendens*, both with MIC values of 200 μg per ml. The MIC of compound IV against *Alternaria maritima* was 50 μg per ml.

Experimental

Melting points are uncorrected. The IR spectra were taken in KBr in the case of solid samples and by the film method for liquid samples, using a Hitachi 215 grating spectrophotometer. The PMR spectra were measured on a JEOL JNM-FX 100 FT NMR spectrometer at 100MHz, using $(CH_3)_4$ Si as an internal standard. Low resolution and high resolution MS spectra were obtained on a JEOL JMS-D 300 spectrometer. UV spectra were obtained on a Hitachi 124 spectrophotometer in ethanol.

5-Chloro-6,8-dimethoxy-3-methylisocoumarin (IV)——A mixture of compound III (1.0 g), benzene (20 ml), Ac_2O (3 ml) and 6 drops of pyridine was refluxed for 4 hr. The reaction mixture was washed consecutively with 5% HCl (20 ml) and water (40 ml), then dried over Na_2SO_4 and concentrated. The residue was washed with ether (40 ml), and dried over P_2O_5 to give crude IV (450 mg). The washings yielded further crude IV (140 mg). Combined crude IV was purified by passage through a column of silica gel, followed by recrystallization from benzene–ether, to give pure IV as thin yellow needles, mp 204°. *Anal.* Calcd for $C_{12}H_{11}ClO_4$: C, 56.58; H, 4.25. Found: C, 56.60; H, 4.34. IR ν_{max}^{KBr} cm^{-1}: 1720 (lactone C=O), 1662 (double bond). PMR (in $CDCl_3$) δ: 2.17 (3H, d, $J=1$ Hz, Me), 4.02 (6H, s, OMe), 6.49 (1H, s, arom. –H), 6.60 (1H, d, $J=1$ Hz, –CH=C(Me)–O–). MS *m/e*: 256 (M^+ for ^{37}Cl), 254 (M^+ for ^{35}Cl).

5-Chloro-8-hydroxy-6-methoxy-3-methylisocoumarin (V)——$AlCl_3$ (0.95 g) was added to a solution of compound IV (360 mg) in freshly distilled nitrobenzene (16 ml). The mixture was heated at 50° for 4.5 hr, then poured into ice-water (20 ml). After the addition of conc. HCl (4 ml), the mixture was extracted with ether (50 ml), and the extract was washed consecutively with 10% HCl (20 ml) and water (20 ml), then dried over Na_2SO_4 and concentrated. The residue was partitioned between 10% NaOH (60 ml) and ether (20 ml), and the aqueous layer was acidified with conc. HCl to give crude V (85 mg) as a precipitate. Additional crude V (69 mg) was obtained by extraction of the mother liquor with ether. Combined crude crystals of V were purified by passage through a column of silica gel using CH_2Cl_2 as an eluent, followed by recrystallization from benzene, to give pure V as colorless microcrystals, mp 146—147°. *Anal.* Calcd for $C_{11}H_9ClO_4$: C, 54.90; H, 3.69. Found: C, 54.90: H, 3.77. IR ν_{max}^{KBr} cm^{-1}: 1680 (chelated C=O). PMR (in $CDCl_3$) δ: 2.31 (3H, d, $J=1$ Hz, Me), 3.96 (3H, s, OMe), 6.52 (1H, s, arom. –H), 6.63 (1H, d, $J=1$ Hz, –CH=C(Me)–O–), 11.25 (1H, s, OH). MS *m/e*: 242 (M^+ for ^{37}Cl), 240 (M^+ for ^{35}Cl).

8-Hydroxy-6-methoxy-3-methylisocoumarin (II)——Compound V (100 mg) in acetone (30 ml) was hydrogenated over 10% palladized charcoal (100 mg). After removal of the catalyst by filtration, the solvent was evaporated off *in vacuo*, and the residue was crystallized from hexane to give II as colorless needles (15 mg), mp 127° (lit.[7] 129°). IR ν_{max}^{KBr} cm^{-1}: 1680 (chelated C=O). PMR (in $CDCl_3$) δ: 2.18 (3H, s, Me), 3.93 (3H, s, OMe), 6.19 (1H, s, $-C\underline{H}=\overset{Me}{\overset{|}{C}}-O-$), 6.29 (1H, d, $J=4$ Hz, arom. –H), 6.45 (1H, d, $J=4$ Hz, arom.–H). High resolution MS *m/e*: Calcd for $C_{11}H_{10}O_4$ (M^+) 206.0579. Observed: 206.0523.

5-Chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (VIII)——A suspension of compound IV (310 mg) in 10% NaOH (3 ml) was refluxed for 30 min, during which time all the compound went into solution. After adding water (27 ml) and raising the temperature of the solution to 70°, $NaBH_4$ (70 mg) was added with vigorous stirring. Additional $NaBH_4$ (50 mg) was added after 30 min, and the mixture was stirred for a further 10 min. The mixture was then filtered. The filtrate was acidified with conc. HCl, then extracted with AcOEt (100 ml), and the extract was washed with water (100 ml), dried over Na_2SO_4, and concentrated. The crude VII thus obtained as the residue was heated for 1.5 hr with Ac_2O (1 ml). After decomposing excess Ac_2O by the addition of water (20 ml), crude VIII precipitated was collected by filtration, washed consecutively with water (50 ml) and hot MeOH (5 ml), and purified by recrystallization from AcOEt–hexane to give pure VIII as colorless needles, 134° (180 mg). *Anal.* Calcd for $C_{12}H_{13}ClO_4$: 56.15; H, 5.11. Found: C, 55.97; H, 5.17. PMR (in $CDCl_3$) δ: 1.50 (3H, d, $J=6$ Hz, Me), 2.71 (1H, dd, $J=11$ Hz and 17 Hz, $-C\underline{H}_2-\overset{Me}{\overset{|}{C}}H-O-$), 3.24 (1H, dd, $J=3$ Hz and 17,Hz, $-C\underline{H}_2-\overset{Me}{\overset{|}{C}}H-O-$), 3.99 (6H, s, $2\times$OMe), 4.48 (1H, ddq, J= 11 Hz, 3 Hz and 6 Hz, $-CH_2-\overset{Me}{\overset{|}{C}}\underline{H}-O-$), 6.48 (1H, s, arom. –H). MS *m/e*: 258 (M^+ for ^{37}Cl), 256 (M^+ for ^{35}Cl).

5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (I)——$AlCl_3$ (0.47 g) was added to a solution of compound VIII (140 mg) in freshly distilled nitrobenzene (10 ml), and the mixture was stirred at 50° for 6 hr, then poured into ice-water (20 ml). After acidification with HCl, the mixture was extracted with ether. The ethereal extract was mixed with 10% NaOH and shaken well. The aqueous layer was separated and acidified with HCl, and extracted again with ether. The extract was washed with water, dried over Na_2SO_4, and concentrated. The residue was purified by chromatography on a column of silica gel with benzene–$CHCl_3$ (1 : 1) as an eluent, to afford I as colorless crystals. It was further purified by recrystallization from AcOEt–hexane to give pure I as colorless needles (72 mg), mp 119°. *Anal.* Calcd for $C_{11}H_{11}ClO_4$: C, 54.45; H, 4.57. Found: C, 54.24; H, 4.55. IR ν_{max}^{KBr} cm^{-1}: 2500—3200 (chelated OH), 1655 (chelated C=O), 1610, 1580 and 1500 (phenyl), 1370 (CH_3), 1240 and 1055 (OMe). UV λ_{max}^{EtOH} nm (log ε): 313 (3.76), 267 (4.67), 220 (4.37). PMR (in $CDCl_3$) δ: 1.55 (3H, d, $J=6$ Hz, Me), 2.75 (1H, dd, $J=11$ Hz and 17 Hz, $-C\underline{H}_2-\overset{Me}{\overset{|}{C}}H-O-$), 3.26 (1H, dd, $J=3$ Hz and 17 Hz, $-C\underline{H}_2-\overset{Me}{\overset{|}{C}}H-O-$), 4.68 (1H, ddq, $J=3$ Hz, 11 Hz and 6 Hz, $-CH_2-\overset{Me}{\overset{|}{C}}\underline{H}-O-$), 6.45 (1H, s, arom. –H). MS *m/e*: 244 (M^+ for ^{37}Cl), 242 (M^+ for ^{35}Cl).

Antimicrobial Activity Test——The antifungal activity was determined by the cylinder-agar plate assay method with *Alternaria maritima* (IFO 8618) and *Fusarium splendens* (IFO 7711) as test organisms. The methodology was the same as that reported in the preceding paper.[10]

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10) S. Nakajima and K. Nozawa, *J. Nat. Prod.*, **42**, 423 (1979).