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Piscicidal Constituents of *Calophyllum Inophyllum*

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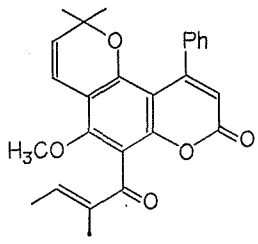
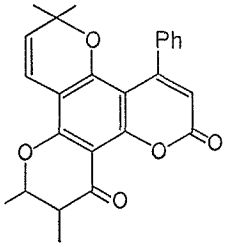
Five 4-phenylcoumarin derivatives have been isolated as piscicidal constituents of the leaves of *Calophyllum inophyllum*. Two of these have been shown to be (+)-inophyllolide and its *cis* isomer, and the other three the corresponding hydroxyl compounds of *trans*-(+)- and *cis*-(+)-inophyllolide.

Calophyllum inophyllum L. (*Guttiferae*) is a big tree commonly found in Southeast Asia. Its seed oil is reputed to be medicine in India, and its leaf has been used as fish poison.

Constituents of its nuts were investigated[1] and three 4-phenylcoumarin derivatives, calophyllolide (**1**), (±)-inophyllolide (**2**) and calophyllic acid (**3**) were isolated and their structures established.[2] From its bark inophylloidic acid (**4**) was isolated.[3] Four triterpenes of the friedelin group were isolated from its leaves[4] and xanthones



(1)



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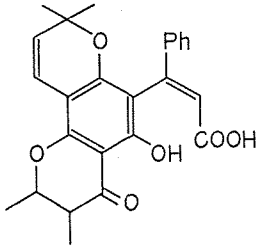

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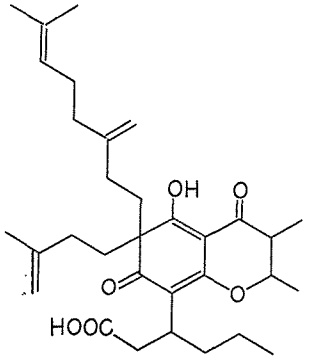

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from its heartwood.[5]

As part of an extensive study of active constituents of plants used as fish poison, we examined *Calophyllum inophyllum* and reported preliminarily the isolation and the characterization of three 4-phenylcoumarin derivatives as piscicidal constituents of its leaves.[6]

We wish to present here the details of the isolation and the structural elucidation of these three compounds as well as two additional piscicidal constituents.

The isolation procedure (Fig. 1) was guided by the killie-fish bioassay.[7] The leaves collected at Kepong, Malaysia were dried and ground. The ground leaves were continuously extracted with ether and the ether extract was chromatographed on silicic acid-Celite by stepwise elution with benzene containing an increasing ratio of ethyl acetate. The piscicidal activity was found in the fraction eluted with 11% ethyl acetate in benzene. This fraction showed five fluorescent spots under ultraviolet ray on silica gel G thin layer chromatogram (tlc) developed with isopropyl ether. Piscicidal test of each zone of the tlc exhibited that only these five fluorescent compounds have the activity. These five compounds were named inophyllum A, B, C, D and E in a decreasing order of Rf value on the tlc. Sprayed with 5% vanillin in sulfuric acid, inophyllum A, B and D gave reddish brown coloration, and inophyllum C and E yellow coloration on the tlc. This fraction was further chromatographed on Florisil and eluted stepwise with benzene-ethyl acetate. Piscicidal activity was found in fractions eluted with 15–20% and 50–70% ethyl acetate. Further separation by crystallization and preparative tlc of the former fractions gave inophyllum A, B and D. By similar procedures the latter fractions gave inophyllum C and E. The yields of inophyllum A, B, C, D and E were 631 mg, 113 mg, 112 mg, 50 mg and 19 mg, respectively from 1.4 kg of the dried leaves.

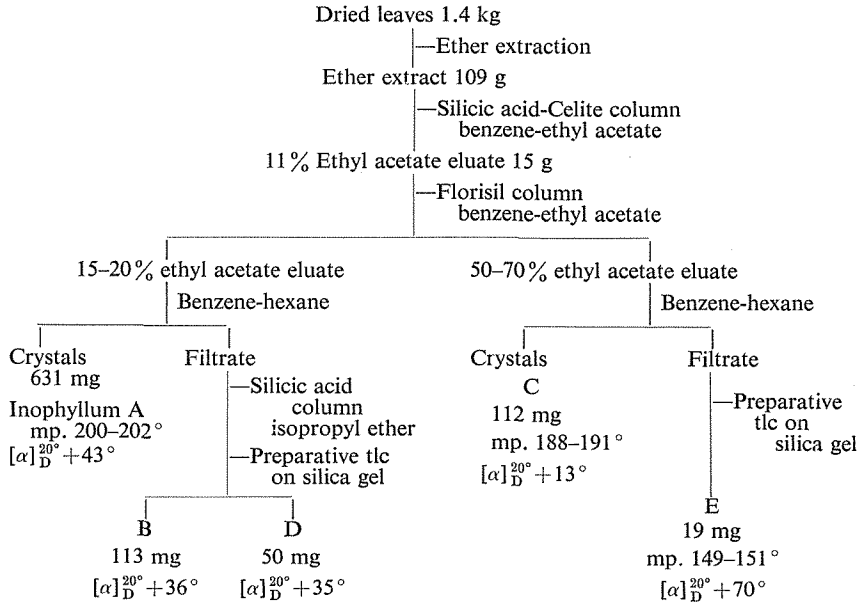


Fig. 1. Isolation procedure of the piscicidal constituents.

Inophyllum C (**5**), $C_{25}H_{22}O_5$, mp. 188–191°, $[\alpha]_D^{20°}+13°$ (*c* 1.1, chloroform) showed uv absorption maxima at 240 (shoulder) (ε 2.1×10^4), 257 (sh.) (2.9×10^4), 266 (3.1×10^4) and 302 nm (2.5×10^4) and ir absorption bands (Nujol mull) at 1727 (unsaturated δ-lactone), 1688 (aryl ketone), 767 and 703 cm^{-1} (monosubstituted benzene). Its mass spectrum showed the molecular ion peak at *m/e* 402 and prominent peaks at *m/e* 387 (M–15), 331 (M–15–56) and 303 (M–15–56–28). These spectral data which agree with those for (±)-inophyllolide, mp. 186–188°, reported by Polonsky,[2] indicate that inophyllum C is (+)-inophyllolide. This is supported by complete coincidence of the pmr spectra in carbon tetrachloride of both of them. It may be noteworthy that inophyllolide has been isolated in an optically active form from the leaves, while it was obtained in optically inactive form from the nuts of the same plant.

Inophyllum E (**6**), $C_{25}H_{22}O_5$, an isomer of inophyllum C, mp. 149–151°, $[\alpha]_D^{20°}+70°$ (*c* 1.2, chloroform) showed the same characteristic bands in the ir spectrum as inophyllum C. Its uv and mass spectra coincided with those of C. The pmr spectrum of E (Table 1) differs from that of C only in the chemical shifts of the methyl protons and the protons at C–10 and C–11 and the splitting pattern of the latter. These observations lead us to conclude that both compounds are the *cis* and *trans* isomers on C–10 and C–11. Comparison of the coupling constants, $J_{10,11}$ (11.5 Hz in C and 3.7 Hz in E) indicates that E is the *cis* isomer while C is the *trans*. It is of some biogenetic interest that both of the *cis* and *trans* isomers have been isolated from the same plant.

Table 1. Proton Magnetic Resonance Signals of Inophyllum C and E.

Inophyllum	C	E
	0.95, s	
C–6 $(CH_3)_2$	0.98, s	0.97, s
C–11 CH_3	1.24, d, J=7.2	1.18, d, J=7.2
C–10 CH_3	1.56, d, J=6.6	1.44, d, J=6.8
C–11 H	2.59, m, J=7.2, 11.5	2.67, m, J=7.2, 3.7
C–10 H	4.32, m, J=6.6, 11.5	4.73, m, J=6.8, 3.7
C–7 H	5.42, d, J=10	5.42, d, J=10
C–8 H	6.56, d, J=10	6.56, d, J=10
C–3 H	6.04, s	6.05, s
Phenyl H_5	7.3	7.3

Inophyllum A (**7**), $C_{25}H_{24}O_5$, mp. 200–202°, $[\alpha]_D^{20°}+43°$ (*c* 1.8, acetone) showed uv absorption maxima at 235 (ε 1.5×10^4), 280 (sh.) (1.4×10^4), 286 (1.5×10^4) and 337 nm (8.9×10^3), and ir bands ascribed to hydroxyl (3430 cm^{-1}), α, β-unsaturated δ-lactone (1717 cm^{-1}) and monosubstituted benzene (767 and 703 cm^{-1}), but no band ascribed to aryl ketone. Its mass spectrum showed intense peaks due to the ions of $M-H_2O$ (*m/e* 386) and $M-CH_3-H_2O$ (*m/e* 371) as well as the characteristic peaks appearing in that of inophyllum C or E. Its pmr spectrum showed signals at 5.17 (1H, doublet, J=5.4 Hz, H–C(–OH)–) and at 3.32 ppm (1H, broad, hydroxyl proton) besides all signals observed in that of E. Inophyllum A was acetylated with acetic anhydride-pyridine to give a monoacetate. These data suggest that A is a dihydro derivative in which the C–12 ketone of E is replaced by a secondary alcohol group.

This was confirmed by the oxidation of inophyllum A with chromic anhydride-pyridine which gave a ketone, mp. 147–149°, $[\alpha]_D^{20°}+77°$ (*c* 1.1, chloroform) identical with inophyllum E in respects of mixed melting point, optical rotation and uv, ir and pmr spectra.

Inophyllum D (**8**), $[\alpha]_D^{20°}+35°$ (*c* 1.86, chloroform) and B (**9**), $[\alpha]_D^{20°}+36°$ (*c* 0.72, chloroform) showed uv spectra quite similar to that of A. Comparison of the pmr spectra (Table 2) of inophyllum A, B and D with those (Table 1) of C and E suggests that D as well as A is a hydroxy analog of inophyllum E, while B is that of C. This was confirmed by the oxidation of D and B which afforded E and C, respectively. The optical rotation of C was a little smaller than that of the oxidation product of B. This reminded us of the observation that inophyllolide isolated from the nuts was optically inactive. It may be assumed that inophyllolide in the leaves are also partially racemized.

Table 2. Proton Magnetic Resonance Signals of Inophyllum A, D and B.

Inophyllum	A	D	B
C–6 $(CH_3)_2$	0.94, s	0.95, s	0.91, s 0.97, s
C–11 CH_3	1.17, d, J=7.2	0.83, d, J=7.2	1.17, d, J=7.0
C–10 CH_3	1.43, d, J=7.0	1.45, d, J=6.7	1.47, d, J=6.8
C–11 H	2.27, m, J=7.2, 3.3, 5.4	1.99, m, J=7.2, 2.0, 2.0	2.03, m, J=7.0, 8.9, 7.4
C–10 H	4.43, m, J=7.0, 3.3	4.59, m, J=6.7, 2.0	3.97, m, J=6.8, 8.9
C–12 H	5.17, d, J=5.4	4.95, d, J=2.0	4.79, d, J=7.4
C–7 H	5.36, d, J=10	5.36, d, J=10.2	5.37, d, J=10
C–8 H	6.55, d, J=10	6.59, d, J=10.2	6.53, d, J=10
C–3 H	5.96, s	5.98, s	5.96, s
Phenyl H_5	7.3	7.3	7.3
OH	3.32, br.	2.67, br.	2.03, br.

It is therefore apparent that inophyllum D is the C–12 epimer of A and the alcohol derived from the *cis* ketone (inophyllum E), while B is that from the *trans* ketone (inophyllum C).

The dehydration of inophyllum A and B with p-toluenesulfonic acid in benzene afforded the same anhydro derivative whose pmr spectrum showed that the dehydration took place between C–11 and C–12. This indicates that inophyllum A, D and B, and hence inophyllum E and C have the same configuration about C–10.

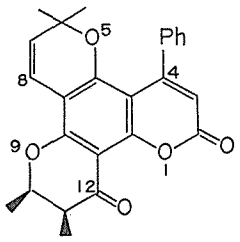
Observation of the coupling constants between protons at C–10 and C–11 (J=8.9 Hz) and between protons at C–11 and C–12 (J=7.4 Hz) of inophyllum B enables us to conclude that the protons on C–10, C–11 and C–12 are situated vicinally *trans*.

The relative configuration of the hydroxyl group of inophyllum A and D, a pair of epimers, are not suggested from the coupling constants of protons on C–11 and C–12,

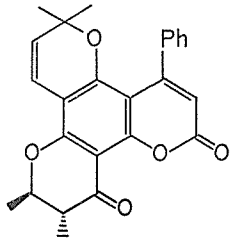
because the coupling constants of both A (J=5.4 Hz) and D (J=2.0 Hz) are rather small compared with that of B.

On treatment with acidic methanol both A and D gave the same methyl ether as a sole product which had the same configuration as D. This indicates that the etherification has occurred with inversion at C–12 in A, but with retention of configuration in D. This reaction is assumed to be solvolytic and of first order. Methanol attacks a resulting carbonium ion from the less hindered side, the side opposite to methyl groups on C–11 and C–12, to afford a methyl ether in which the methoxyl group is situated *trans* to C–11 methyl. It is obvious that the hydroxyl group of A is *cis* and that of D is *trans* to C–11 methyl group.

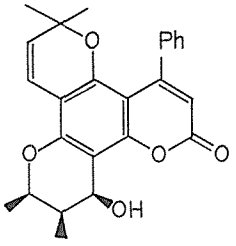
On the basis of the above evidence the stereostructures (**5**–**9**) except for the absolute configuration of C–10 were given to the five piscicidal constituents from *Calophyllum inophyllum* leaves.



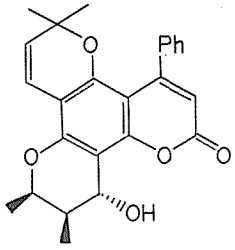
Inophyllum E (**6**)



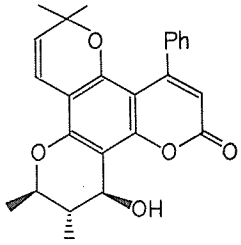
Inophyllum C (**5**)



Inophyllum A (**7**)



Inophyllum D (**8**)



Inophyllum B (**9**)

Inophyllum A, B and D are the first naturally occurring 4-phenylcoumarin derivatives possessing a 2,3-dimethylchromanol ring though costatolide, a 4-n-propylcoumarin derivative with a 2,3-dimethylchromanol ring was isolated from the bark resin of *Calophyllum costatum*.[8]

It is of some biogenetical interest that in contrast to the nuts, the leaves contain as major constituents the corresponding hydroxy compounds of the ketone, the main component of the nuts, and contain both of the *cis* and *trans* isomers.

The piscicidal activity of these constituents are given in Table 3. The ketones are more active than the alcohols, among the alcohols inophyllum B having all *trans* configuration is the most active.

Table 3. Toxicity of the Piscicidal Constituents to *Oryzias Latipes*

Compounds	TL*m* (24 hr) ppm
Inophyllum C	5.0—3.6
Inophyllum E	6.4—4.9
Inophyllum B	13.7—8.3
Inophyllum A	25.0—18.8
Inophyllum D	33.3—25.0

EXPERIMENTAL

Unless otherwise stated, the uv spectra were taken in ethanol, and the pmr spectra were taken in deuteriochloroform at 60 MHz. Chemical shifts are expressed in ppm from tetramethylsilane as internal standard and coupling constants in Hz. Singlet, doublet, triplet, quartet and multiplet are abbreviated to s, d, t, q and m, respectively.

Piscicidal test. The piscicidal test was carried out using the killie-fish (*Oryzias latipes, Himedaka* in Japanese) according to the procedure in the preceding paper.[7]

Isolation of the piscicidal constituents. The leaves of *Calophyllum inophyllum* L. were harvested and dried at Kepong, Malaysia. The dried leaves (1.4 kg) were ground and continuously extracted with ether. The ether extract was concentrated at 40–45° *in vacuo* to give a residue (109 g). The residue (3.3 g) adsorbed on silicic acid (Mallinckrodt) (16 g) was placed on the top of a column (6×80 cm) packed with a mixture of silicic acid (150 g) and Celite (300 g), and eluted with 1 liter fractions of benzene containing increasing amounts (0, 5, 10, 11, 12 and 15%) of ethyl acetate. The piscicidal activity was found in the fraction eluted with 11% ethyl acetate. The column chromatography of this scale was performed 33 times and the 11% ethyl acetate eluates were combined and concentrated to give the active fraction (17.2 g).

Further chromatography of the active fraction on Florisil was carried out. Florisil (Floridin Co.) 150 times as much as the material to be chromatographed was used. Eluant was a mixture of benzene and ethyl acrtate in which amount of ethyl acetate was increased in 5% step.

The 15–20% ethyl acetate eluates were concentrated and the residue was crystallized from benzene-hexane to give inophyllum A; yield 631 mg. From the filtrate inophyllum B (113 mg) and D (50 mg) were obtained by silicic acid column chromatography in isopropyl ether and preparative tlc on silica gel G (Merck). Inophyllum C (112 mg) was crystallized from a benzene-hexane solution of the fractions eluted with 50–70% ethyl acetate. The filtrate was separated by preparative tlc on silica gel G to afford inophyllum E (19 mg).

Inophyllum A, mp. 200–202° (from benzene-hexane or from ethyl acetate), $[\alpha]_D^{20°}+43°$ (*c* 1.82, acetone). Found: C, 74.35; H, 6.20. Calcd. for $C_{25}H_{24}O_5$; C, 74.24; H, 5.98%. MS, *m/e* (relative intensity), 404 (25.3%), 389 (100), 386 (15.7), 371 (59.6), 333 (66.4), 305 (7.0); uv, 235 (ε 1.5×10^4), 280 (sh.) (1.4×10^4), 286 (1.5×10^4), 337 nm (8.9×10^3); ir (Nujol) 3430, 1717, 767, 703 cm^{-1}; pmr, see Table 2.

Inophyllum B, $[\alpha]_D^{20°}+36°$ (*c* 0.72, chloroform); uv, 233 (ε 1.8×10^4), 277 (sh.) (1.5×10^4), 287 (1.7×10^4), 335 nm (1.0×10^4); pmr, see Table 2.

Inophyllum C, mp. 188–191° (from benzene-hexane or from ethyl acetate), $[\alpha]_D^{20°}$+13° (*c* 1.1, chloroform). MS, 402 (31.7%), 387 (100), 331 (93.7), 303 (8.5); uv, 240 (sh.) (ε 2.1×10^4), 257 (sh.) (2.9×10^4), 266 (3.1×10^4) and 302 nm (2.5×10^4); ir (Nujol) 1727, 1688, 767, 703 cm^{-1}; pmr, see Table 1.

Inophyllum D, $[\alpha]_D^{20°}$+35° (*c* 1.86, chloroform); uv, 232 (ε 1.7×10^4), 277 (sh.) (1.4×10^4), 286 (1.6×10^4), 334 nm (9.6×10^3); pmr, see Table 2.

Inophyllum E, mp. 149–151° (from benzene-hexane), $[\alpha]_D^{20°}$+70° (*c* 1.2, chloroform). MS, 402 (33.3%), 388 (27.7), 387 (100), 331 (75.7), 303 (7.7); uv, 240 (sh.) (ε 2.0×10^4), 257 (sh.) (2.7×10^4), 266 (2.9×10^4), 302 nm (2.4×10^4); ir (Nujol) 1727, 1688, 767, 703 cm^{-1}; pmr, see Table 1.

Acetylation of inophyllum A. Inophyllum A (9.5 mg) dissolved in pyridine (1 ml) was treated with acetic anhydride (2 ml). The reaction mixture was kept at room temperature overnight. Water was added to the mixture, and the monoacetate was crystallized. Recrystallization from benzene-methanol gave crystals (7.6 mg), mp. 205–208°, $[\alpha]_D^{19°}$+8.8° (*c* 1.4, chloroform); ir (Nujol), 1742, 1722, 1632, 703 cm^{-1}; pmr, 0.94, s, C–6 $(CH_3)_2$; 1.06, d, *J*=7.2, C–11 CH_3; 1.40, d, *J*=6.9, C–10 CH_3; 2.17, s, AcO; 2.45, m, C–11 H; 4.40, m, C–10 H, 5.37, d, *J*=10.2, C–7 H; 5.96, s, C–3 H; 6.41, d, *J*=5, C–8 H; 6.53, d, *J*=10.2, C–12 H; 7.3, phenyl H_5.

Oxidation of inophyllum A and D with chromic anhydride-pyridine. Chromic anhydride (500 mg) dissolved in water (0.3 ml) was mixed with pyridine (5 ml) in an ice-bath. To this solution (3.5 ml) was added inophyllum A (197 mg) in pyridine (1.2 ml). After standing overnight the mixture was treated with water (15 ml), and extracted with ethyl acetate. Removal of ethyl acetate gave crystals (158 mg). Recrystallized from benzene-hexane, the crystals gave colorless needles (80 mg), mp. 147–149°, $[\alpha]_D^{20°}$+77.7° (*c* 1.08, chloroform), which was identified with inophyllum E in respects of mixed melting point, optical rotation, and uv, ir and pmr spectra.

In a similar procedure, D (17 mg) gave a ketone (14 mg), mp. 147–149°, $[\alpha]_D^{20°}$+70° (*c* 0.57, chloroform) which was identical with E in all respects.

Oxidation of inophyllum B with chromic anhydride-pyridine. In a similar manner to the oxidation of A and D, B (22 mg) was oxidized to a ketone (21 mg), mp. 180–183°, $[\alpha]_D^{20°}$+54° (*c* 0.85, chloroform), which was identical with C in all respects except optical rotation.

Dehydration of inophyllum A and B. Inophyllum A (30 mg) with a small amount of p-toluenesulfonic acid in dry benzene (2.8 ml) was refluxed for 20 minutes. Water was added and the benzene layer was washed thoroughly with water. Removal of the benzene afforded yellow crystalls, which was recrystallized from benzene-hexane; yellow needles, 16.5 mg, mp. 198–200°, $[\alpha]_D^{20°}$−40° (*c* 1.0, chloroform), pmr, 0.94, s, C–6 $(CH_3)_2$; 1.41, d, *J*=6.8, C–10 CH_3; 1.88, m, C–11 CH_3; 4.93, q, *J*=6.8, C–10 H; 5.39, d, *J*=10, C–7 H; 5.95, s, C–3 H; 6.54, d, *J*=10, C–8 H; 6.70, m, C–12 H; 7.35, phenyl H_5.

In a similar procedure inophyllum B (29 mg) gave an anhydro derivative (8.7 mg), mp. 192–195°, $[\alpha]_D^{20°}$ −42° (*c* 4.1, chloroform), which was identified with the dehydration product from inophyllum A in respects of the optical rotation and the pmr spectra.

Methylation of inophyllum A and D. A solution of inophyllum A (31.6 mg) in

methanol (5 ml) containing a small amount of 2N-hydrochloric acid was boiled under reflux for 5 minutes, and concentrated. Water being added, the reaction mixture was extracted with ether. Removal of the ether gave a sole product. Crystallization from *n*-hexane afforded colorless prisms (14.7 mg), mp. 164–167°, $[\alpha]_D^{20°}$ +58.3° (*c* 0.6, chloroform); pmr. 0.80, d, *J*=7.2, C–11 CH_3; 0.94 s, C–6 $(CH_3)_2$; 1.43, d, *J*=6.5, C–12 CH_3; 2.06, m, *J*=7.2, 1.9, 2.2, C–11 H; 3.60, s, OCH_3; 4.47, d, *J*=2.2, C–12 H; 4.55, m, *J*=6.5, 1.9, C–10 H; 5.34, d, *J*=10, C–7 H; 5.97, s, C–3 H; 6.55, d, *J*=10, C–8 H; 7.3, phenyl H_5.

In a similar procedure, inophyllum D gave the same methylated product.

ACKNOWLEDGMENTS

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