J. Pesticide Sci. **10**, 101–106 (1985)

Original Article

Isolation and Structural Determination of Eriobofuran, A New Dibenzofuran Phytoalexin from Leaves of Loquat, *Eriobotrya japonica* L.

Masakazu MIYAKODO, Keisuke WATANABE, Nobuo OHNO, Fukuji NONAKA* and Akira MORITA**

Pesticides Research Laboratory, Takarazuka Research Center, Sumitomo Chemical Co., Ltd., Takatsukasa, Takarazuka 665, Japan
**Faculty of Agriculture, Saga University, Honjo, Saga 840, Japan*
***Nagasaki Fruit-Tree Experiment Station, Onihashi, Ohmura, 856–01, Japan*

(Received August 2, 1984)

An antimicrobial substance, named as eriobofuran (**Ia**) was isolated from loquat leaves [*Eriobotrya japonica* L. (Rosaceae)] inoculated with *Entomosporium eriobotryae*, a host-pathogenic fungus. The structure of eriobofuran was deduced to be 2,4-dimethoxy-3-hydroxydibenzofuran by spectroscopic, chemical evidence and biosynthetic considerations. Its structure was confirmed by direct comparison with a synthetic specimen of eriobofuran ethyl ether. Eriobofuran inhibited strongly the spore germination and germ tube growth of *Pestalotia funerea*, a host-pathogenic fungus of loquat tree.

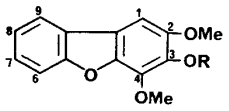
INTRODUCTION

The production of antimicrobial substances —phytoalexins— by injured plant cells has been recognized as part of a natural defence mechanism of plant against fungal attack.[1] All the known phytoalexins are secondary metabolites of low molecular weight that exhibited antimicrobial activities to one or several groups of microorganisms.

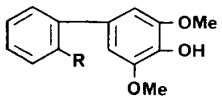
Previosuly, we reported the production of a biphenyl type phytoalexin, aucuparin (**IIa**), in the cortical layer of loquat tree (*Eriobotrya japonica* L., Japanese name "biwa") on inoculation with *Colletotrichum lindemuthianum*, a non-host-pathogenic fungus[2] (Fig. 1). As a minor antimicrobial metabolite, 2′-hydroxyaucuparin (**IIb**)[3] was also isolated from the inoculated layer. The production of aucuparin in loquat leaves was also observed when a definite amount of *Pseudomonas syringae* pv. *eriobotryae*, a host-pathogenic bacterium was inoculated.[4] From these observations, it was estimated that aucuparin (**IIa**) plays a possible role in disease resistance in the loquat.[5] When a host-pathogenic fungus, *Entomosporium eriobotryae* was inoculated on loquat leaves, the existence of aucuparin was also observed at the early stage. However, in course of time, the production of aucuparin ceased gradually, whereas the occurence of another kind of phytoalexin was observed.[6] We now report the isolation, structure determination of a new dibenzofuran phytoalexin, eriobofuran (**Ia**) from loquat leaves infected with *E. eriobotryae* and the synthesis of eriobofuran ethyl ether (**Ib**). Its antifungal activities against several kinds of phytopathogenic fungi are briefly described.

MATERIALS AND METHODS

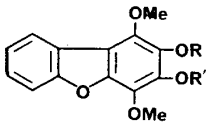
An EtOH extract of fungi-inoculated loquat leaves was purified by repeated preparative-layer chromatography with monitoring fractions for antimicrobial activity, to give a crystalline compound, mp 157–158°C, in 0.21% yield. Eriobofuran (**Ia**) which we named here, exhibited the following spectroscopic pro-



(Ia) R=H, Eriobofuran
(Ib) R=Et
(Ic) R=Ac



(IIa) R=H, Aucuparin
(IIb) R=OH



(IIIa) R=H, R'=Me, α-Pyrufuran
(IIIb) R=Me, R'=H, β-Pyrufuran

Fig. 1 The structure of phytoalexins from *Rosaceae* plants.

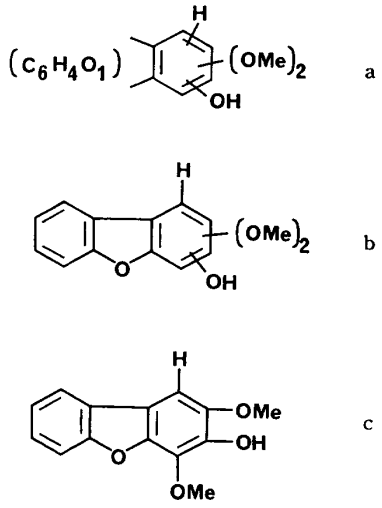


Fig. 2 Partial and total structure of eriobofuran.

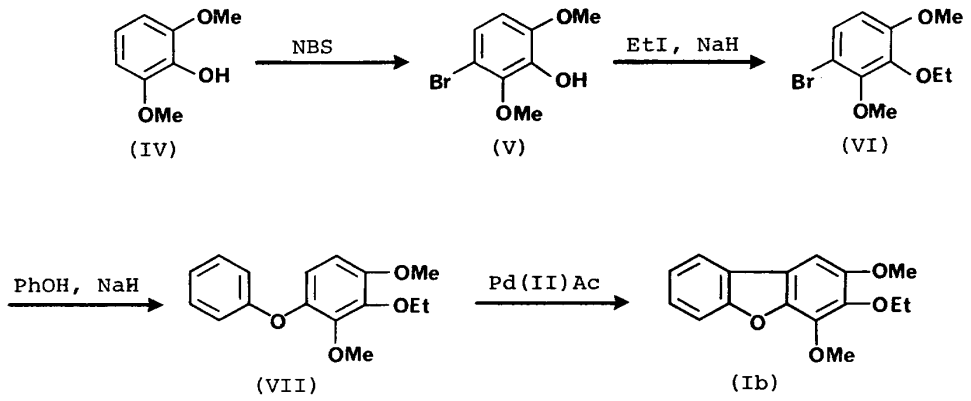
perties: the mass spectrum of (**Ia**) gave a molecular ion peak at m/z 244 (100%, $C_{14}H_{12}O_4$) and two fragment peaks at 229 (M^+–CH_3, 75%) and 201 (M^+–CH_3–CO, 11%). Its ^{1}H NMR spectrum showed two methoxy signals at δ 3.99 and 4.21, a replaceable broad singlet, probably a hydroxy proton at δ 5.48 and five aromatic protons (δ7.00, 1 H, s; 7.10–7.37, 2 H, m; 7.46, 1 H, dd, J=8.0, 1.5 Hz; 7.71, 1 H, dd, J=8.0, 1.5 Hz).

On acetylation with Ac_2O and pyridine, eriobofuran (**Ia**) gave monoacetyleriobofuran (**Ic**) (δ 2.40, 3 H, s, for acetyl-CH_3) in nearly quantitative yield indicating the presence of a phenolic hydroxy group in the molecule. A sharp siglet at δ 7.17 assigned to an aromatic proton comprises that this hydrogen as well as two methoxy and a hydroxy group were attached to the same benzene ring. All the twelve protons of eriobofuran could be thus accounted for, indicating two methoxy and one hydroxy groups attached to a polycylic aromatic ring of ($C_{12}H_5O$) as shown in Fig. 2 a.

^{13}C NMR spectrum of eriobofuran showed twelve resonances in the region of δ 96–157 ppm of which five signals appeared as doublets on off-resonance measurement, indicating the presence of five aromatic carbons with hydrogens. The other seven singlet signals were assigned to substituted aromatic carbon atoms. Two signals at δ 56.9 and 61.1 ppm corresponded to two methoxy carbons. In its UV spectrum, the absorption at 300 nm was characteristic for a dibenzofuran with bathochromic substituents[8] (two OCH_3, one OH) on a benzene ring. From these considerations, eriobofuran was estimated to be a dibenzofuran with a hydroxy and two methoxy groups on one benzene ring as shown in Fig. 2 b. However, it was difficult to determine location of the three substituents on benzene ring only by spectroscopic consideration. On the basis of biosynthetic relevancy and structural similarities of eriobofuran (**Ia**) with that of aucuparin (**IIa**) and 2′-hydroxyaucuparin (**IIb**), it was estimated that the three substituents of eriobofuran would be located at C_2, C_4 (methoxy groups) and C_3 (hydroxy group) as shown in Fig. 2 c. Although the structure for eriobofuran was tentatively deduced as (**Ia**) by spectroscopic, chemical and biosynthetic considerations, an authentic synthesis was necessary to confirm the structure.

Hereafter, we wish to describe the structure determination of eriobofuran (**Ia**) by comparison with a synthetic specimen. Although several synthetic methods of dibenzofuran ring system[9] have been reported so far, all of them have some disadvantages in yield or substitution varieties. Recently, Ames *et al.*[10] reported the synthesis of dibenzofuran derivatives by intramolecular cyclization of the corresponding diphenyl ethers with palladium (II) acetate as catalyst in fairly good yield.

As a more closely related compound to eriobofuran (**Ia**), Kemp *et al.*,[11] isolated α- and



Scheme 1 Synthesis of eriobofuran ethylether (**Ib**).

β-pyrufuran from the wood of *Pyrus comminis* L. and deduced their structures to be 1,3,4-trimethoxy-2-hydroxydibenzofuran (**IIIa**) and 1,2,4-trimethoxy-3-hydroxydibenzofuran (**IIIb**), respectively. In the same report, the authors confirmed their structures by comparison with the synthetic specimens.

2,4-Dimethoxy-3-ethoxydibenzofuran (**Ib**, eriobofuran ethyl ether) was synthesized by the route shown in Scheme 1. 2,6-Dimethoxyphenol (**IV**) was brominated with NBS to give 3-bromo-2,6-dimethoxyphenol (**V**) according to the reported procedure.[12] The hydroxy group of **V** was ethylated with ethyl iodide and NaH to give 2,4-dimethoxy-3-ethoxybromobenzene (**VI**) in almost quantitative yield. The biphenyl ether (**VII**) was prepared from the bromobenzene (**VI**) by a solvent-assisted copper (I) catalysed Ullman ether synthesis with phenol.[13] Then, the corresponding dibenzofuran (**Ib**) was obtained by palladium (II) acetate promoted cyclisation of the biphenyl ether (**VII**). Kemp *et al.*[11] had reported that in this cyclisation reaction the nature of solvent is important in determining both the rate of reaction and overall yield. They selected trifluoroacetic acid or trifluoroacetic acid–acetic acid as the best solvent systems in this type of reaction. In the present case, the reaction in acetic acid was completed after 9 hr reflux and the yield was 70.2%, however in trifluoroacetic acid–acetic acid (1 : 1 v/v), the maximum yield of (**Ib**) was only *ca.* 5% in several reaction conditions.

On the other hand, the natural eriobofuran was converted to its ethyl ether derivative which was identical in all respects (^{1}H NMR, MS, GC and UV) to those of the synthetic specimen (**Ib**). Thus, the structure of eriobofuran was established unequivocally to be 2,4-dimethoxy-3-hydroxy-dibenzofuran (**Ia**).

The antifungal activities of eriobofuran against several kinds of plant pathogenic fungi are given in Table 1. Eriobofuran strongly inhibited the spore germination of *P. funerea*, a host-pathogenic fungus of loquat tree as well as its spore germ tube growth. Meanwhile, eriobofuran did not inhibit significantly the germination or germ tube growth of non-host pathogenic fungi, such as *A. kikuchiana*, *C. lindemuthianum*, *G. cingulata*, *B. cinerea*, *F. solani* sp. *pisi*, *C. miyabeannus* and *C. fulvum*. Thus, the difference between the activity of eriobofuran against host-pathogenic and non-host pathogenic fungi is quite interesting from a view point of the resistance mechanism of loquat tree against fungal attack. Biological role of eriobofuran as phytoalexin in loquat tree are now under investigation and will be reported elsewhere in the near future.

EXPERIMENTAL

1. Apparatus

A Hitachi GC-163 with FID was used for GC-analysis. NMR spectra were recorded on a Varian XL-200 (200 MHz for ^{1}H) and a Hitachi R-900 (90 MHz for ^{1}H and 22.6 MHz for ^{13}C). IR spectra were obtained on a Hitachi 270-30 spectrophotometer. Mass spectra were measured on a Hitachi M-80 GC-MS spectrometer (ionization energy, 70 eV). Melting points were determined on a Mitamura melting point apparatus and uncorrected.

Table 1 Antifungal activities of eriobofuran (**Ia**) against several kinds of plant pathogenic fungi (16 hr after inoculation).

Fungi (typical host plant)	Germination rate[a] (%)			Germ tube length[b] (m)		
	Concentration[c] (ppm)			Concentration[c] (ppm)		
	0[d]	14.4	43.2	0[d]	14.4	43.2
Pestalotia funerea (loguat)	100	43	0	228.6	92.2	0
Alternalia kikuchiana (pear)	100	95	79	394.5	307.9	117.5
Colletotrichum lindemuthianum (kidney bean)	100	95	69	132.2	106.2	29.1
Glomerella cingulata (tea, apple)	100	98	56	511.7	237.1	48.1
Botrytis cinerea (kidney bean)	100	88	27	209.5	139.5	23.7
Fusarium solani sp. *pisi* (pea)	100	85	64	105.5	85.9	65.9
Cochliobolus miyabeanus (rice)	100	94	38	574.3	422.6	139.1
Cladosporium fulvum (tomato)	100	79	14	163.5	87.0	22.1

[a] 600 spores were counted (100: no effect, 0: complete inhibition).
[b] Mean lengths of germ tubes were measured (120 spores each).
[c] In water-ethanol (98: 2, v/v).
[d] Control (treated with 2% ethanol only).

*2. Isolation and Purification of Eriobofuran (**Ia**)*

Loquat leaves (10.0 g) infected with *Entomosporium eriobotryae* were washed with water and extracted with 80% aq. EtOH (90 ml) for 24 hr at room temperature. After filtration, the extract was concentrated (35°C) *in vacuo* to 10 ml. To the concentrate, 80 ml of water was added and centrifugalized (14,000 rpm, 45 min). The supernatant was adjusted to pH 3.0 with dil. HCl and extracted with equal amount of petroleum ether (3 times). The combined petroleum ether layer was concentrated *in vacuo* to give 27.8 mg of crude phytoalexin. TLC analysis was performed using silica gel GF_{254} plate with fluorescent indicator (Merck, 20×20 cm, 0.25 mm). The solvent system was *n*-hexane: ethyl acetate: methanol [60:40:1 (v/v)]. An antifungal zone was detected at *Rf* 0.65–0.70 by bioautography employing *Cochliobolus miyabeanus* as test organism. Spores of *C. miyabeanus* which were collected from culture medium, were washed with distilled water and dispersed in water with a homogenizer. A portion (5 ml) of the resulting spore suspension (concentration *ca.* 1.0×10^5 cells/ml) was sprayed on the TLC plate. Thereafter, 5.0 ml of PSA culture medium was sprayed on the spore-treated plate (20×20 cm). White or discoloured spots due to local growth inhibition were developed after 7 to 10 days' incubation at 25°C under humid condition. The band at *Rf* 0.65–0.70 was purified by preparative TLC to give 21 mg of crystalline compound.

*3. Eriobofuran (**Ia**)*

mp, 157–158 °C; UV λ_{max}^{EtOH} nm (ϵ), 300 (17,000); GC 5% SE-30, 2 m, 275°C, t_R 2.30 min; GC-MS, *m/z* (rel. int.) 244 (100, M^+), 229 (75), 201 (11), 185 (30), 183 (50), 155 (13), 130 (20), 122 (14), 113 (20), 102 (45), 88 (18) and 75 (20); ^{1}H NMR $\delta_{TMS}^{CCl_4}$ ppm: 7.71 (1 H, d, d J=8.0, 1.5 Hz), 7.46 (1 H, dd, J=8.0, 1.5 Hz,) 7.37–7.10 (2 H, m), 7.00 (1 H, s), 5.48 (1 H, br.s), 4.21 (3 H, s) and 3.99 (3 H, s); ^{13}C NMR $\delta_{TMS}^{CDCl_3}$ ppm: 156.3 (s), 145.1 (s), 143.2 (s), 137.9 (s), 132.8 (s), 125.8 (d), 125.0 (s), 122.8 (d), 119.8 (d), 116.2 (s), 111.7 (d), 96.6 (d), 61.1 (q) and 56.9 (q); IR ν_{max}^{KBr} cm^{-1}: 3420, 1476, 1448, 1354, 1318, 1286, 1258, 1220, 1192, 1152, 1114, 1096, 1022, 1004, 880, 740 and 650.

*4. 3-Acetyleriobofuran (**Ic**)*

^{1}H NMR $\delta_{TMS}^{CDCl_3}$ ppm: 7.90 (1 H, dd, J= 8.0, 1.5 Hz), 7.65–7.20 (3 H, s), 7.17 (1 H, s), 4.23 (3 H, s), 3.94 (3 H, s) and 2.40 (3 H, s).

*5. 2,4-Dimethoxy-3-ethoxybromobenzene (**VI**)*

Sodium hydride dispersed in nujol (60%)

(0.66 g, 16.5 mmol) was added in portions to 3-bromo-2,6-dimethoxyphenol (**V**) (3.50 g, 15.0 mmol) in dry DMSO (30 ml) and kept for 1 hr at room temperature with stirring. To the reaction mixture was added 2.57 g (16.5 mmol) of ethyl iodide dropwisely over 1 hr and kept for another 3 hr at room temperature with stirring. The reaction mixture was poured into water (50 ml) and extracted with toluene (30 ml×3). The toluene layer was washed with 10% HCl aq., sat. $NaHCO_3$ aq. and sat. NaCl aq. solution successively and dried (Na_2SO_4). After evaporation of the solvent, 4.01 g of crude **VI** was obtained. Distillation (105–108°C/2.0 mmHg) yielded 2,4-dimethoxy-3-ethoxybromobenzene (**VI**): 3.29 g (84.2%), UV λ_{max}^{EtOH} nm (ϵ): 228 nm (6400) and 276 (1100); GC 5% XE-60, 1 m, 200–300°C programmed linearly by 10°C/min, t_R 2.15 min; MS, *m/z* (rel. int.) 262 and 260 (each 14, M^+), 234 (100), 232 (100), 219 (64), 217 (64), 188 (28), 173 (36), 124 (48), 109 (72), 95 (48), 77 (40), 66 (68) and 53 (100); ^{1}H NMR $\delta_{TMS}^{CCl_4}$ ppm: 7.10 (1 H, d, J=9.0 Hz), 6.46 (1 H, d, J=9.0 Hz), 4.00 (2 H, q, J=7.0 Hz), (3.85 3 H, s), 3.80 (3 H, s) and 1.34 (3 H, t, J=7.0 Hz); IR ν_{max}^{film} cm^{-1}: 2976, 2938, 1576, 1467, 1416, 1386, 1295, 1233, 1217, 1091, 1030, 925 and 791.

6. *2,4-Dimethoxy-3-ethoxy-1-phenoxybenzene* (***VII***)

Under dry nitrogen, freshly cut sodium (0.33 g, 14.0 mmol) was added to dry MeOH (20 ml), followed by dry benzene (20 ml) and phenol (2.82 g, 30.0 mmol). The mixture was heated to boiling and bromobenzene (**VI**) (3.92 g, 15.0 mmol) and anhydrous copper (I) chloride (0.1 g) were added. The mixture was refluxed for 40 hr. The reaction mixture was poured into water (150 ml) and the solvent layer was washed with 10% HCl aq., sat. $NaHCO_3$ aq., and sat. NaCl aq. successively. After evaporation of the solvent, the residue (3.78 g) was purified by preparative thin layer chromatography ($CHCl_3$, *Rf* 0.53) to give 2.98 g (77.6%) of pure crystals of (**VII**): mp, 50–51°C; UV λ_{max}^{EtOH} (ϵ), 243 nm (2600), 269 (2200) and 276 (2200); GC 5% XE-60, 1 m, 170°C, t_R 3.25 min; MS, *m/z* (rel. int.) 274 (48, M^+), 246 (22), 231 (20), 213 (7), 199 (15), 185 (10), 171 (13), 131 (100), 114 (12), 91 (12), 83 (12), 77 (42), 69 (12) and 51 (28); ^{1}H NMR $\delta_{TMS}^{CCl_4}$ ppm: 7.30–6.70 (4 H, m), 6.66 (1 H, d, J=10.0 Hz), 6.48 (1 H, d, J=10.0 Hz), 4.02 (2 H, q, J=7.0 Hz), 3.78 (3 H, s), 3.72 (3 H, s) and 1.34 (3 H, t, J=7.0 Hz); ^{13}C NMR $\delta_{TMS}^{CCl_4}$ ppm: 158.9 (s), 150.1 (s), 146.9 (s) 142.0 (s), 142.7 (s), 129.1 (d), 121.5 (d), 116.0 (d), 107.0 (d), 68.6 (t), 60.4 (q), 55.9 (q) and 15.6 (q); IR ν_{max}^{KBr} cm^{-1}: 2984, 2944, 1602, 1592, 1484, 1426, 1390, 1252, 1216, 1166, 1092, 1046, 1016, 914, 826, 756 and 692.

7. *2,4-Dimethoxy-3-ethoxydibenzofuran* (***Ib***)

A mixture of biphenyl ethyl ether (**VII**) (2.74 g, 10.0 mmol) and palladium (II) acetate (2.47 g, 11.0 mmol) in acetic acid (30 ml) was kept at reflux under nitrogen for 9 hr. The solvent was removed by distillation. The residue was dissolved to diethyl ether (40 ml) and washed with water, sat. $NaHCO_3$ aq., and sat. NaCl aq. successively and dried (Na_2SO_4). The solvent was removed *in vacuo* and the crude product was purified by preparative thin layer chromatography (toluene, *Rf* 0.34) to yield 1.91 g (70.2%) of pure (**Ib**) as yellow oil. UV λ_{max}^{EtOH} nm (ϵ): 224 (20,300), 255 (8700), 289 (11,600), 296 (10,200) and 316 (4400); GC 5% XE-60, 1 m, 200°C, t_R 3.50 min; MS, *m/z* (rel. int.) 272 (100, M^+), 244 (40), 243 (88), 229 (16), 215 (6), 200 (11), 185 (8) and 183 (18); ^{1}H NMR $\delta_{TMS}^{CCl_4}$ ppm: 7.72 (1 H, dd, J=8.0, 1.5 Hz), 7.54–7.15 (3 H, m), 6.96 (1 H, s), 4.10 (3 H, s), 4.03 (2 H, q, J=7.0 Hz), 3.80 (3 H, s) and 1.35 (3 H, t, J=7.0 Hz); ^{13}C NMR $\delta_{TMS}^{CCl_4}$ ppm: 156.4 (s), 150.9 (s), 140.9 (s), 139.8 (s), 125.8 (d), 124.7 (s), 122.2 (d), 119.8 (d), 115.2 (s), 111.4 (d), 97.3 (d), 69.0 (t), 60.4 (q), 56.3 (q) and 15.6 (t); IR ν_{max}^{film} cm^{-1}: 2980, 2940, 1595, 1530, 1460, 1428, 1358, 1305, 1250, 1232, 1195, 1130, 1050, 925, 893, 820, 750 and 650.

8. *Determination of Fungicidal Activities of Eriobofuran* (***Ia***)

Fungicidal activities of eriobofuran were determined by spore containing agar block method.[7] Organisms tested here were *Pestalotia funerea* as a pathogenic fungus of loquat tree and seven other fungi such as *Alternaria kikuchiana, Colletotrichum lindemuthianum, Glomerella cingulata, Botrytis cinerea, Fusarium solani* sp. *pisi, Cochliobolus miyabeanus* and

Cladosporium fulvum as non pathogenic ones. Germination rate (%) and germ tube lengths of these fungal spores were assessed after 14 hr incubation at 25°C.

ACKNOWLEDGEMENTS

We are grateful to Dr. Kenzo Ueda of this Research Center for his encouragement throughout this work. We also wish to express our deepest thanks to Dr. Noritada Matsuo and Mr. Kazunori Tsushima of this Research Center for their helpful suggestions in synthetic works.

REFERENCES

1) I. A. M. Cruickshank: "Plant Disease," ed. by J. G. Horsfall & E. B. Cowling, Vol. V, Academic Press, New York, p. 247, 1980
2) K. Watanabe, Y. Ishiguri, F. Nonaka & A. Morita: *Agric. Biol. Chem.* **46**, 567 (1982)
3) K. Watanabe, M. Miyakado, N. Ohno, F. Nonaka & A. Morita: unpublished results
4) A. Morita, F. Nonaka & K. Makizumi: *Ann. Phytopathol. Soc. Jpn.* **49**, 587 (1983)
5) F. Nonaka, K. Makizumi & A. Morita: *Bull. Fac. Agric. Saga Univ.* **53**, 27 (1982)
6) A. Morita & F. Nonaka: *Ann. Phytopathol. Soc. Jpn.* **49**, 407 (1983)
7) Z. Kiraly, Z. Klement, F. Solymosy & J. Voros: "Methods in Plant Pathology," Elsevier Scientific Publishing Company, Amsterdam, p. 309, 1974
8) A. I. Scott: "Interpretation of the Ultraviolet Spectra of Natural Products," Pergamon Press, Oxford, p. 138, 1964
9) M. V. Sargent, P. O. Stransky, V. A. Patrick & A. H. White: *J. Chem. Soc. Perkin Trans. I* **1983**, 231
10) D. E. Ames & A. Opalko: *Synthesis* **1983**, 234
11) M. S. Kemp, R. S. Burden & R. S. T. Loeffler: *J. Chem. Soc. Perkin Trans. I* **1983**, 2267
12) D. Friedman & D. Grinsburg: *J. Org. Chem.* **23**, 16 (1957)
13) A. L. Williams, R. E. Kinney & R. F. Bridger: *J. Org. Chem.* **32**, 2501 (1967)

要　　約

ビワ葉からの新規なジベンゾフラン系ファイトアレキシン，エリオボフランの単離と構造決定

宮門正和，渡辺敬介，大野信夫
野中福次，森田　昭

ゴマ色斑点病菌 (*Entomosporium eriobotryae*) に感染したビワの葉 (*Eriobotrya japonica* L.) (バラ科) から，新規なジベンゾフラン系の抗菌性物質（ファイトアレキシン）を結晶状で単離し，エリオボフランと命名した．各種スペクトル，化学反応ならびに生合成経路の考察から，その構造を 2,4-ジメトキシ-3-ヒドロキシベンゾフランと推定した．その構造は，別途合成した標品 (2,4-ジメトキシ-3-エトキシジベンゾフラン)とエリオボフランのエチルエーテル誘導体とが完全に一致することより決定した．エリオボフランはビワの寄生菌である灰斑病菌 (*Pestalotia funerea*) の生育を強く阻害したが，各種類の非寄生菌の生育にはほとんど影響を与えなかった．