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The Structures of Toxic Metabolites of *Aspergillus candidus*. I. The Compounds A and E, Cytotoxic *p*-Terphenyls

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The metabolites, compounds A—E, were isolated from a toxic strain of *Aspergillus candidus* and the structures of the characteristic cytotoxic compounds A (terphenyllin) and E(deoxyterphenyllin) were elucidated to *p*-terphenyl derivatives (**1** and **24**) by spectral data, oxidation reactions, and synthesis of compound A trimethyl ether. The cytotoxicity of the compounds and the derivatives was also discussed.

In our course of screening program of toxic mold metabolites[2,3] one strain (69-SA-156= NHL 5107) of *Aspergillus candidus* Link isolated from wheat flour was noticed by the cytotoxicity to cultured HeLa cells. Further works revealed that the mold grown on rice grain exhibited noticeable pathological changes on experimental animals.[4] This paper concerns with the isolation of the metabolites tentatively named compounds A—E of the strain and the structural elucidation of the compounds A and E, *p*-terphenyl derivatives. Among these metabolites compounds A and B are major products and the former has been proved to be the main causative agent for the specific cytotoxicity on HeLa cells, while the latter for the pathological changes on animals.[5]

Although the metabolites were first isolated from the mold grown on the liquid potato-dextrose medium,[3] the culture on polished rice grain was employed for the isolation of the metabolites in further works. The extraction of the molded rice grain with chloroform and methanol successively followed by the separation by silica-gel chromatography afforded five metabolites: Compound A (2.1 g from 10 kg rice), mp 244—245°, B (0.8 g), mp 165—170° (decomp.), C (0.1 g), identified with ergosterol, D (0.1 g), mp 90—91°, and E (0.2 g), mp 230—231°. Cytotoxicity to cultured HeLa cells of the isolated metabolites was shown in Table I.

The compound A (**1**) has a molecular formula, $C_{20}H_{18}O_5$ (by a high resolution mass spectrum) and the ultraviolet (UV) and infrared (IR) spectral data showed a typical phenolic nature of the compound. The compound A (**1**) forms triacetate (**2**). Methylation of **1** with diazomethane followed by separation by preparative thin-layer chromatography gave two monomethyl ethers (**3** and **4**), two dimethyl ethers (**5** and **6**), and trimethyl ether (**7**) (the position of the methoxyl groups in partially methylated products will be discussed later). Spectral data of these derivatives especially of nuclear magnetic resonance (NMR) spectra (Table II) disclosed that the molecule of the compound is composed of two *p*-substituted phenyl rings (two pairs of *o*-coupled protons, each corresponding to two protons) and one penta-substituted

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2) M. Saito, T. Ishiko, M. Enomoto, K. Ohtsubo, M. Umeda, H. Kurata, S. Udagawa, S. Taniguchi, and S. Sekita, *Japan. J. Exptl. Med.*, **44**, 63 (1974).

3) M. Umeda, T. Yamashita, M. Saito, S. Sekita, C. Takahashi, K. Yoshihira, S. Natori, H. Kurata, and S. Udagawa, *Japan. J. Exptl. Med.*, **44**, 83 (1974).

4) K. Ohtsubo, M. Enomoto, T. Ishiko, M. Saito, F. Sakabe, S. Udagawa, and H. Kurata, *Japan. J. Exptl. Med.*, **44**, 477 (1974).

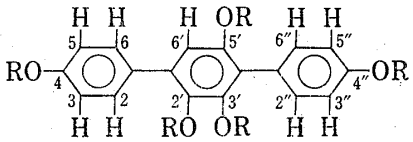
5) C. Takahashi, K. Yoshihira, S. Natori, M. Umeda, K. Ohtsubo, and M. Saito, *Experientia*, **30**, 529 (1974).

Table I. Cytotoxicity to HeLa Cells of the Metabolites of *Aspergillus candidus* and the Derivatives[a]

Compound	100 (μg/ml)	32 (μg/ml)	10 (μg/ml)	3.2 (μg/ml)	1.0 (μg/ml)	Morphological characteristics[b]
Compound A (**1**)	4	3	2	1		R
B	4	4	4	1		
D	4	2	0	0		
E (**24**)	4	2	0	0		S
A-Acetate (**2**)	4	2	1	0		R
A-Monomethyl ether (**3**)	4	4	4	2	0	S
(**4**)	4	3.5	3	2	0	R
A-Dimethyl ether (**5**)	4	4	3.5	2.5	1	R
(**6**)	0	0	0	0		
A-Trimethyl ether (**7**)	0	0	0	0		
Pentahydroxy-*p*-terphenyl (**8**)	4	4	0	0		
Trihydroxy-*p*-terphenylquinone (**9**)	4	2	0	0		
Triacetoxy-*p*-terphenylquinone (**10**)	4	4	0	0		
Dianisyl-*p*-benzoquinone (**15**)	4	4	2	0		
Triacetoxydimethoxy-*p*-terphenyl (**16**)	4	4	3	0		A
Diacetoxydimethoxy-*p*-terphenyl (**17**)	4	4	3.5	2.5	0	A
Dimethoxymonohydroxy-*p*-terphenyl-quinone (**18**)	4	4	4	3	1	A
Trimethoxy-*p*-terphenylquinone (**19**)	4	4	4	4	2	A
Atromentin (**20**)	0	0	0	0		
Atromentin dimethyl ether (**21**)	1	0	0	0		
E-Acetate (**25**)	1	1	0	0		

a) The degree of the cytotoxicity was estimated on scale 0 (no cellular damage) through 4 (complete cytolysis).[2,3]
b) See the text.

Table II. Chemical Shifts of NMR Spectra of Terphenyl Derivatives
(δ values in ppm from TMS in $CDCl_3$ unless otherwise specified)



Compound	2′-OCH_3 or -$OCOCH_3$	3′-H, -OH, -OCH_3 or -$OCOCH_3$	5′-OCH_3 or -$OCOCH_3$	6′-H	4-OH, -OCH_3 or -$OCOCH_3$	4″-OH, -OCH_3 or -$OCOCH_3$	2- and 6-H[a]	2″- and 6″-H[a]	3- and 5-H[a]	3″- and 5″-H[a]
Compound A(**1**)[b]	3.38	8.29	3.70	6.47	8.29	8.29	7.50	7.24	6.92	6.84
Compound A(**1**)[c]	3.40	—	3.70	6.54	—	—	7.56	7.31	6.95	6.90
A-Acetate (**2**)	3.41	2.05	3.73	6.82	2.31	2.31	7.66	7.33	7.16	7.12
A-Acetate (**2**)[c]	3.40	2.00	3.74	6.90	2.30	2.30	7.65	7.29	7.18	7.07
A-Monomethyl ether (**3**)	3.42	—	3.70	6.36	3.83[d]	—[d]	7.50	7.26	6.90	6.80
A-Monomethyl ether (**4**)	3.42	—	3.70	6.36	—[d]	3.80[d]	7.45	7.32	6.90	6.80
A-Dimethyl ether (**5**)	3.58[d]	3.60[d]	3.70	6.60	—[d]	3.83[d]	[e]	[e]	6.90	6.80
A-Dimethyl ether (**6**)	3.42	—	3.70	6.36	3.83[d]	3.80[d]	7.50	7.34	6.88	6.88
A-Trimethyl ether (**7**)	3.55[d]	3.57[d]	3.60	6.56	3.80	3.80	7.42	7.24	6.85	6.85
Triacetoxydimethoxy-*p*-terphenyl (**16**)	2.06	1.96	1.96	7.02	3.78	3.78	7.34	7.34	6.84	6.82
Diacetoxydimethoxy-*p*-terphenyl (**17**)	2.08	7.12	2.08	7.12	3.79	3.79	7.34	7.34	6.90	6.90
Compound E (**24**)	3.34	—	3.64	6.40	[f]	—	[f]	7.15	[f]	6.73
E-Acetate (**25**)	3.37	2.03	3.71	6.80	[f]	2.28	[f]	7.33	[f]	7.12

a) each 2H, d, J=9 Hz b) in acetone-d_6 *c*) in CD_3OD *d*) These assignments may be reversed.
e) 7.2—7.6 (4H) *f*) 7.2—7.5 (5H)

phenyl ring (one singlet proton appearing in higher field) with two methoxy and three hydroxyl groups.

The oxidation of the compound A (**1**) with hydrogen peroxide in alkali and of the dimethyl ether (**6**) with chromium trioxide afforded *p*-hydroxybenzoic acid and *p*-anisic acid, respectively. Demethylation of compound A (**1**) by boron tribromide gave an unstable phenol (**8**), which was oxidized by ferric chloride or chromium trioxide to a quinone (**9**), $C_{18}H_{12}O_5$. The quinone forms a triacetate (**10**). The UV spectra of the compounds (**9**, $\lambda_{max}^{dioxane}$ 255, 390 nm, **10**, $\lambda_{max}^{dioxane}$ 345 nm) showed a close similarity to those reported for *p*-terphenylquinones.[6]

Three formulae (**1**, **11**, and **12**) are possible as far as the nucleus is *p*-terphenyl and the results were preliminary reported.[5,7]

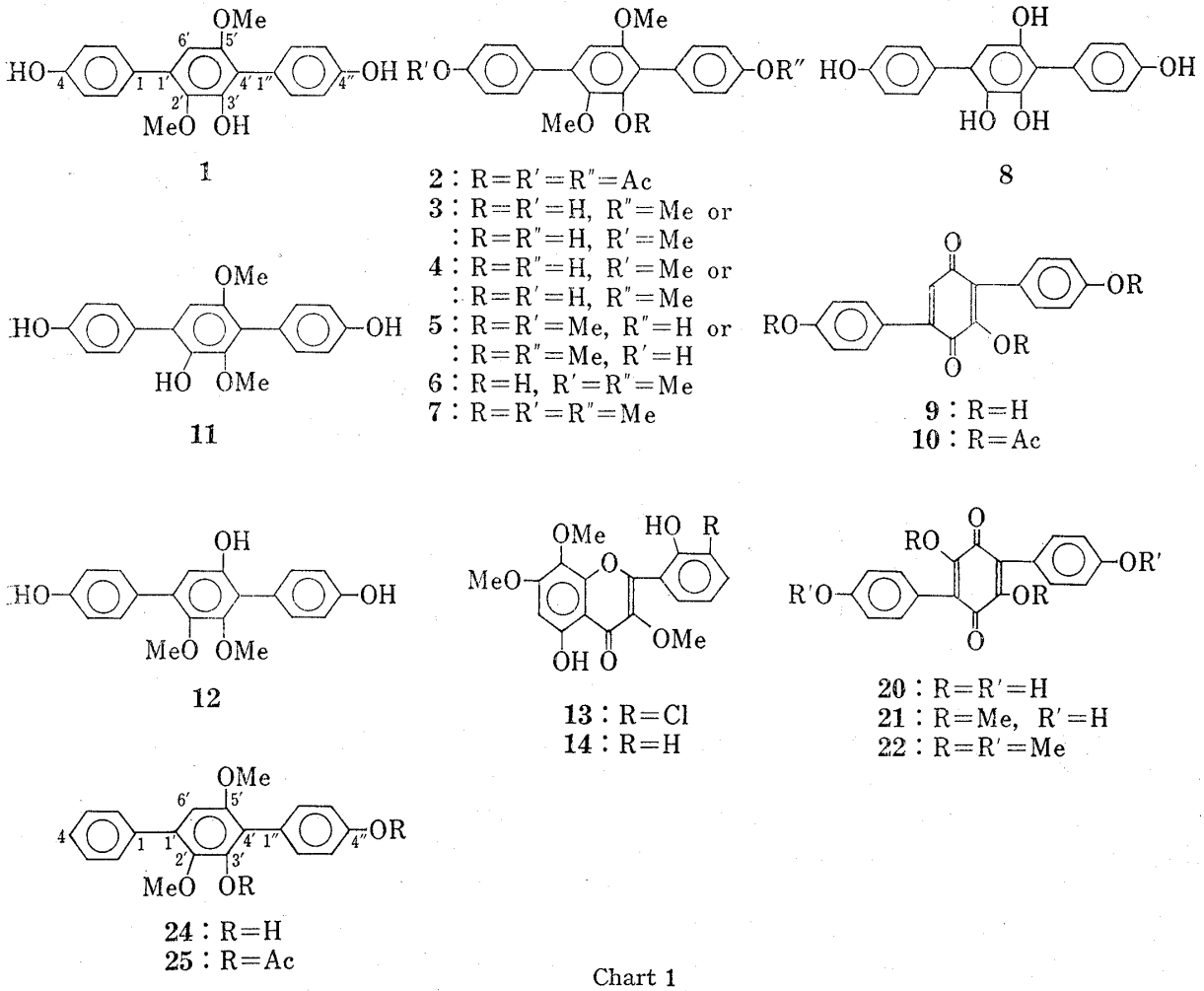


Chart 1

At this stage of our work Marchelli and Vining[8] reported the isolation of a terphenyl assumed to be identical with the compound A[9] from a strain of *Aspergillus candidus* in their course of studies on biosynthesis of chlorflavonin (**13**) along with dechlorochlorflavonin (**14**) and proposed the structure (**1**) for the terphenyl. Their proposal was based on the spectral

6) J. Gripenberg, *Acta Chem. Scand.*, **12**, 1762 (1958).
7) S. Natori, S. Sekita, C. Takahashi, and K. Yoshihira, "The paper presented at 24th IUPAC Congress," Abstracts of Papers, Hamburg, Sept. 1973, p. 188.
8) R. Marchelli and L.C. Vining, *J. Chem. Soc., Chem. Comm.*, **1973**, 555.
9) The identity has now been established by the direct comparison with the sample provided by Prof. L.C. Vining. He informed us that a trivial name 'terphenyllin' was given for the compound and a note describing the isolation of the metabolite was submitted to *J. Antibiotics* (April 1975). The paper has been published (R. Marchelli and L.C. Vining, *J. Antibiotics*, **28**, 328 (1975)).

data including an observation on the presence of a long range coupling between one of the two methoxyl groups and the isolated ring hydrogen, on the positive Gibbs' reaction and on the biogenetical consideration but was lacking in any other evidences.

In order to establish the *p*-terphenyl nucleus beyond doubt following synthesis was carried out: The Thiele acetylation of 2,5-di-*p*-anisyl-*p*-benzoquinone[10] (**15**) gave 4,4″-dimethoxy-2′,3′,5′-triacetoxy-*p*-terphenyl (**16**), which was derived to 2,4′,4″-trimethoxy-*p*-terphenylquinone (**19**) as shown in Chart 2. The catalytic hydrogenation of the quinone followed by the methylation with diazomethane gave 4,2′,3′,5′,4″-pentamethoxy-*p*-terphenyl which was proved to be identical with the trimethyl ether (**7**) of the compound A.

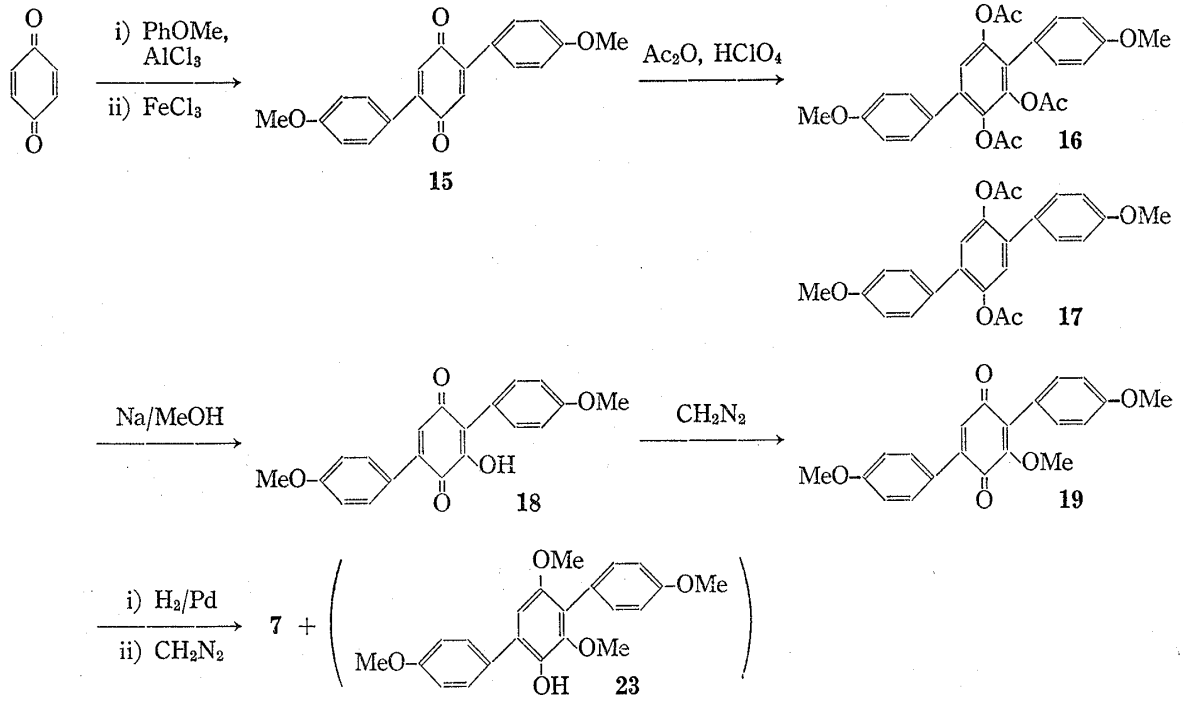


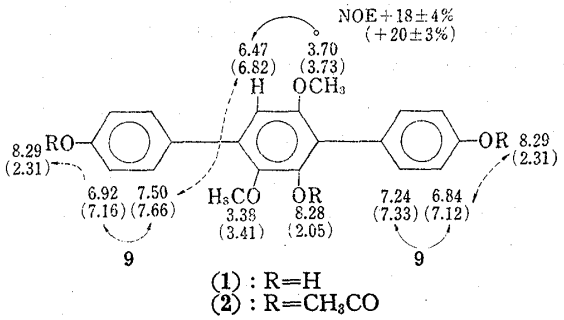
Chart 2

A choice between the three formulae (**1**, **11**, and **12**) is a next problem. Precise examinations of NMR spectra of the compound A and the acetate (**2**) were performed and the results were shown in Chart 3. The nuclear Overhauser effects were clearly observed on the isolated ring proton when the methoxyl methyl group in lower field was irradiated, the fact excluding the formula (**12**) for the compound A. The decoupling experiments also made clear the assignments of the aromatic protons on the *p*-substituted phenyl rings at the both ends (Chart 3 and Table II). Since the positive Gibbs' test of the compound A favours the formula **1** than **11**, the Fremi oxidations of the compound A and the dimethyl ether (**6**) were attempted expecting the direct correlation to atromentin dimethyl ether[11] (**21**) and tetramethyl ether[11] (**22**) respectively. However several attempts were in vain in recoverring the starting materials or formation of intractable materials. However the observation does not preclude the formula (**1**), since negative results of the reaction at sterically hindered positions have been known.[12]

As shown in Table II the methoxy methyl signals at 4 and 4″ positions appear in lower fields than those on the middle ring.[13] Thus the monomethyl ethers (**3** and **4**) and the dimethyl

10) R. Pummerer and E. Prell, *Ber.*, **55**, 3105 (1922).
11) F. Kögl and H. Becker, *Ann.*, **465**, 211 (1928).
12) H. Ishii, *Yukigoseikagaku Kyokaishi*, **30**, 922 (1972).
13) D. Bartle and D.W. Jones,"Advances in Org. Chem.," (ed. E.C. Tayler), Vol. 8, Wiley, New York, 1972, p. 317; J.W. Hooper, W. Marlow, W.B. Whalley, A.D. Borthwick, and R. Bowden: *J. Chem. Soc.* (*C*), **1971**, 3580.

ethers (**5** and **6**) are respectively assigned as shown in Chart 1. The Gibbs test[14] were compared on the compound A and the derivatives, in which those retaining a free hydroxyl group at the middle ring (**1**, **3**, **4**, **6**) showed positive, while the compounds in which the hydroxyl group had been methylated (**5**, **7**) negative. The examination by thin-layer chromatography of the reaction mixture of the reductive methylation of the quinone (**19**) to pentamethoxy-*p*-terphenyl (**7**) showed a presence of a by-product assumed to be 4,3′,5′,4″-tetramethoxy-2′-hydroxy-*p*-terphenyl (**23**), which did not coincide with the dimethyl ether (**6**) of compound A. The comparison of the chemical shifts of the singlet ring proton in **1** with that in the acetate (**2**) (Table II) showed remarkable low shift.[15] All these facts suggest that the free hydroxyl group in **1** must not be to *meta* but to *para* to the ring hydrogen. Thus the structure of the compound A has been proved to be 2′,5′-dimethoxy-4,3′,4″-trihydroxy-*p*-terphenyl (**1**).



(**1**) : R=H
(**2**) : R=CH_3CO

Chart 3. NMR Spectra of Compound A (**1**) and the Acetate (**2**)

Chemical shifts are δ values in ppm from TMS in acetone-d_6 for **1** and in $CDCl_3$ for **2** (in parenthesis).

: vicinal coupling and coupling constant (in Hz)

: long range coupling

: NOE

The compound E (**24**) is a minor metabolite of the fungus. The UV and IR spectra are quite similar to those of the compound A (**1**). It shows a positive Gibbs' reaction. The molecular formula, $C_{20}H_{18}O_4$ (by a high resolution mass spectrum), suggested the compound might be a deoxy derivative of the compound A. The compound E forms diacetate (**25**). The NMR spectra (Table II) of **24** and **25** clearly disclosed that one of the two *p*-hydroxyl groups at 4- and 4″-positions in **1** is replaced by a hydrogen. Although their exists no decisive evidence, the chemical shifts of the remaining pair of *o*-coupled protons showed closer values to those of the phenyl group flanked by one methoxyl and one hydroxyl group appearing in higher field than those of the other ring flanked by one methoxyl group in the compound A[13] (Table I, see also Chart 3). Thus the structure (**24**), 3′,4″-dihydroxy-2′,5′-dimethoxy-*p*-terphenyl, was put forward for the compound E.[16]

Terphenyl compounds are rare as natural products and terphenylquinones have chiefly been isolated from Basidiomycetes,[17] in which the compounds originated from C_6–C_3 units occur more frequently. The species, *Aspergillus candidus*, is assumed to be unique as *Aspergillus*, since it forms terphenyls (**1**, **24**) and flavones (**13**, **14**) of C_6–C_3 origin.[8,18]

The cytotoxicity of the compounds A and E and the derivatives (**1**—**10**, **24**, **25**) along with the synthetic intermediates (**15**—**19**) and atromentin derivatives (**20**, **21**) is shown in Table I. Wide range of the compounds of terphenyls and terphenylquinones showed cytotoxicity to cultured HeLa cells at the level of 3—10 μg/ml. However there were differences in the induced morphological changes. As reported in the previous paper[5] the compound A produced slightly enlarged cells with faintly stained cytoplasm, evenly distributed chromatin and relatively small nucleoli ("R" type changes[2]). This specific morphology was observed in the acetate (**2**), the monomethyl ether (**4**), and the dimethyl ether (**5**). Although the structures

14) F.E. King, T.J. King, and L.C. Manning, *J. Chem. Soc.*, **1957**, 563.
15) J.A. Ballantine and C.T. Pillinger, *Tetrahedron*, **23**, 1961 (1967).
16) Since the compound A has been designated terphenyllin,[9] the compound E will hereafter be called 4-deoxyterphenyllin.
17) R.H. Thomson, "Naturally Occurring Quinones," Academic Press, London and New York, 1971, p. 153.
18) R. Marchelli and L.C. Vining, *Can. J. Biochem.*, **51**, 1624 (1973).

are similar, the compound E (**24**) and the monomethyl ether (**3**) induced a different change, *i.e.* spindle-shaped small cells ("S" type change[2]). The dimethyl ether (**6**) and the trimethyl ether (**7**) showed little toxicity to HeLa cells. The observation may suggest that the methylation or the removal of the 4-hydroxyl group leads to the loss of the typical "R" type change exhibited by the compound A. The synthetic intermediates (**16**—**19**) produced another type of change, *i.e.* irregularity in cell size, polynuclear cells and abnormal mitosis ("A" type change[2]). Antitumor activities of some terphenylquinone derivatives had been reported before.[19]

The further works on the compound B, the characteristic toxin for animals, and the compound D will be reported in a forthcoming paper.

Experimental[20]

Culture and Culturing Condition——The strain NHL 5107 (69-SA-156)[2-4] was isolated from wheat flour collected at Saku-shi, Nagano Prefecture, in 1969. The fungus was grown in 1000 ml Roux flasks containing 200 ml each of potato-dextrose medium (potato 300 g, glucose 50 g, and deionized water 1 liter) or 200 g each of polished rice grain after washing with water. The inoculated flasks were incubated at 25° for 3 weeks in the case of the former medium and for 2 weeks of rice grain. In the latter case the flasks were shaken occasionally.

Extraction and Isolation of the Metabolites——a) From Potato-dextrose Broth: The mycelium and the filtrate were separated and the former was homogenized in chloroform and filtered. The mycelium was then extracted with methanol. The culture filtrate was extracted with ethyl acetate. The three extracts were examined by thin-layer chromatography (TLC) and the toxicity test using HeLa cells.[3] The chloroform extract of the mycelium and the ethyl acetate extract of the filtrate showed the presence of compound A. The former also showed a slight spot corresponding to compound B.

b) From Rice Culture: The molded rice (28 kg) was sterilized by steaming and extracted by chloroform for three times at a room temperature. The combined chloroform extract was evaporated to one fifths of the original volume and the precipitate thus formed was separated (16 g, fraction 1). The mother liquor was evaporated to dryness (124 g, fraction 2). The residual rice grain was then extracted with methanol for three times at a room temperature. The methanol extract containing a rather large amount of water was evaporated and then extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness (fraction 3). The aqueous layer after extraction with ethyl acetate (fraction 4) was applied for column chromatography through charcoal. The fraction 1 was chromatographed through a column of silica gel (1 kg). The column was eluted with graduent amount of chloroform–ethyl acetate. From the fraction eluted by chloroform–ethyl acetate (4: 1) the compound B (2.43 g) and then the compound E (0.02 g) were obtained. From the fraction eluted by chloroform–ethyl acetate (1: 1) the compound A (1.46 g) was obtained. The fraction 2 was chromatographed in the same manner (3 kg of silica gel) and the fraction eluted by ethyl acetate afforded the compound C (0.21 g), the compound E (0.45 g), and the compound A (0.67 g) in that order. The fraction 3 was also treated in the same way (0.8 kg of silica gel) and the compound C (0.05 g) and the compound A (1.14 g) were obtained from the fractions eluted by chloroform–ethyl acetate (4: 1). Each fraction was checked by thin-layer chromatography and, if necessary, by the cytotoxicity test, and the fractions containing same compounds were combined and rechromatographed if necessary. The fractions originated from fractions 2 and 3, from which bulk of the compound A was removed, were combined and rechromatographed to afford the compound D (0.27 g) and further amount of the compound A (2.62 g). The eluates of the charcoal chromatography of the fraction 4 did not show any noticeable cytotoxicity and were abandoned.

The compound A was recrystallized from benzene–ethanol (10: 1) to colorless crystals of mp 244—245°. The compound B was recrystallized from chloroform to pale yellow needles of mp 165—170° (decomp.). The compound C was recrystallized from ethanol to colorless leaflet of mp 152—154° and identified with ergosterol by a mixed fusion, IR, and NMR. The compound D was recrystallized from chloroform to yellow crystals of mp 90—91°. The compound E was recrystallized from ethyl acetate to colorless crystals of mp 230—231°.

Compound A (1)——mp 244—245° from benzene–ethanol (10: 1). The Gibbs reaction, positive. UV λ_{max}^{EtOH} nm (log ε): 222, 275 (4.50, 4.46). IR ν_{max}^{KBr} cm^{-1}: 3250, 1605, 1560, 1510, 1480, 1400, 1355, 1295, 1220, 1165, 1110, 1075, 995, 895, 830. NMR (Table II). Mass Spectrum *m/e*: 338.109 (Calcd. for $C_{20}H_{18}O_5$, 338.115, M^+), 323.089 (Calcd. for $C_{19}H_{15}O_5$, 323.092, M^+-CH_3), 308.066 (Calcd. for $C_{18}H_{12}O_5$, 308.068, $M^+-2\times$

19) J.F. Burton and B.F. Cain, *Nature*, **184**, 1326 (1959); B.F. Cain: *J. Chem. Soc.*, **1961**, 936; **1963**, 356; **1964**, 5472; *J. Chem. Soc. (C)*, **1966**, 1041.

20) The melting points were determined on a Yanagimoto melting point apparatus and not corrected. For thin-layer chromatography silica gel HF_{254} and for column chromatography silicic acid (100 mesh, Mallinckrodt) were used unless otherwise specified.

CH_3), 292.073 (Calcd. for $C_{18}H_{12}O_4$, 292.074, $M^+-CH_3-CH_3O$), 263.070 (Calcd. for $C_{17}H_{11}O_3$, 263.071), 229.055 (Calcd. for $C_{13}H_9O_4$, 229.050).

Compound A Triacetate (2)——Compound A (**1**) (62 mg) was treated with acetic anhydride (1 ml) and pyridine (1 ml) at a room temperature overnight. Recrystallization from hexane gave colorless prisms (72 mg) of mp 148—149°. IR ν_{max}^{KBr} cm^{-1}: 1765, 1605, 1515, 1480, 1420, 1390,1370, 1200, 1165, 1105, 1080, 1000, 910,850, 810. NMR (Table II). Mass Spectrum *m/e*: 464.145 (Calcd. for $C_{26}H_{24}O_8$, 464.147, M^+), 422 ($C_{24}H_{22}O_7$), 380 ($C_{22}H_{20}O_6$), 367 ($C_{21}H_{19}O_6$), 338 ($C_{20}H_{18}O_5$), 323 ($C_{19}H_{15}O_5$).

Compound A Monomethyl Ethers (3,4), Dimethyl Ethers (5,6), and Trimethyl Ether (7)——Compound A (200 mg) in dioxane (8 ml) was treated with an excess amount of diazomethane in ether (10 ml) overnight in a refrigirator. The reaction mixture was separated by preparative layer chromatography using chloroform–ethyl acetate (4: 1) as the developer. Seven bands thus obtained were recovered separately. The first band from the top (7 mg) was recrystallized from hexane to colorless prisms of trimethyl ether (**7**), mp 163—164°. The Gibbs reaction, negative. IR ν_{max}^{KBr} cm^{-1}: 1610, 1582, 1515, 1462, 1385, 1292, 1242, 1182, 1112, 1085, 1022, 1002, 934, 824. NMR (Table II). Mass Spectrum *m/e*: 380.1629 (Calcd. for $C_{23}H_{24}O_5$, 380.1624, M^+), 365 ($C_{22}H_{21}O_5$), 334 ($C_{21}H_{18}O_4$), 190 ($C_8H_{14}O_5$), 149 ($C_8H_5O_3$).

The second fraction (19 mg) gave dimethyl ether (**6**), colorless needles, mp 181—182° (from benzene). The Gibbs reaction, positive. IR ν_{max}^{KBr} cm^{-1}: 3400, 1610, 1590, 1520, 1450, 1395, 1365, 1320, 1285, 1235, 1175, 1120, 1070, 1020, 992, 840, 815. NMR (Table II). Mass Spectrum *m/e*: 366.1535 (Calcd. for $C_{22}H_{22}O_5$, 366.1467, M^+), 351 ($C_{21}H_{19}O_5$), 243 ($C_{14}H_{11}O_4$), 183 ($C_9H_{11}O_4$), 161 ($C_8H_2O_4$), 150 ($C_7H_3O_4$).

The third fraction (25 mg) gave dimethyl ether (**5**), colorless prisms, mp 170—180° (from benzene). The Gibbs reaction, negative. IR ν_{max}^{KBr} cm^{-1}: 3350, 1614, 1555, 1520, 1460, 1440, 1392, 1292, 1250, 1230, 1178, 1115, 1085, 1035, 1020, 1005, 932, 835. NMR (Table II). Mass Spectrum *m/e*: 366. 1514 (Calcd. for $C_{22}H_{22}O_5$, 366.1466, M^+), 351 ($C_{21}H_{19}O_5$), 320 ($C_{20}H_{16}O_4$).

The fourth fraction (34 mg) gave monomethyl ether (**4**), colorless prisms, mp 186—189° (from benzene–methanol). The Gibbs reaction, positive. IR ν_{max}^{KBr} cm^{-1}: 3400, 1610, 1565, 1520, 1488, 1465, 1400, 1362, 1290, 1264, 1240, 1180, 1120, 1076, 1000, 902. 822. NMR (Table II). Mass Spectrum *m/e*: 352.1355 (Calcd. for $C_{21}H_{20}O_5$, 352.1311, M^+), 337 ($C_{20}H_{17}O_5$), 306 ($C_{19}H_{14}O_4$).

The fifth band (43 mg) gave monomethyl ether (**3**), mp 196—198° (from benzene–methanol). The Gibbs reaction, positive. IR ν_{max}^{KBr} cm^{-1}: 3380, 1610, 1582, 1562, 1515, 1490, 1452, 1435, 1398, 1360, 1230, 1174, 1116, 1070, 1025, 1005, 904, 835, 802, 770. NMR (Table II). Mass Spectrum *m/e*: 352. 1357 (Calcd. for $C_{21}H_{20}O_5$, 352.1311, M^+), 337 ($C_{20}H_{17}O_5$), 306 ($C_{19}H_{14}O_4$).

The sixth band (13 mg) was found to be a mixture of uncharacterized products. From the seventh band the starting material (59 mg) was recovered.

Oxidation of Compound A (1)——To the compound A (**1**) (103 mg) in 0.1 N NaOH (40 ml) and methanol (4 ml) 30% hydrogen peroxide (12 ml) was added and kept at a room temperature overnight. The reaction mixture was acidified and extracted with ether. The ethereal extract was shaken with sodium hydrogen carbonate solution, which was acidified and extracted with ether to give a residue (30 mg), showing an identity with *p*-hydroxybenzoic acid by TLC. The residue was then acetylated by the usual manner and the acetate thus formed, mp 170—174°, showed identity with the sample prepared from authentic *p*-hydroxybenzoic acid by IR, TLC, and a mixed fusion.

Oxidation of Compound A Dimethyl Ether (6)——To the dimethyl ether (**6**) (10 mg) in acetic acid (10 ml) chromium trioxide (12 mg) in water was added at a room temperature. After warming on a water bath iced water was added to the reaction mixture and extracted with ethyl acetate. The ester layer was extracted with sodium hydroxide solution. The alkaline layer was acidified and the precipitate was recrystallized from water to colorless crystals (4 mg) of mp 180—184°. The comparison with the authentic sample of *p*-anisic acid showed an identity by IR, TLC, and a mixed fusion.

Demethylation of Compound A and the Formation of the Terphenylquinone (9)——The compound A (575 mg) was treated with boron tribromide (1.2 g) in dichloromethane (50 ml) for 4 hr. The reaction mixture was poured into iced water and the precipitate thus formed was taken up in ether. The evaporation of the solvent gave the phenol (**8**) (*ca.* 500 mg), NMR (Table II), which is unstable in air and used for the following steps directly. The phenol (**8**) (10 mg) in 20% methanol was oxidized with ferric chloride (0.6 g) in diluted hydrochloric acid at a room temperature. The precipitate formed by the addition of water was collected and washed well with water. Dark brown crystals (**9**) (5 mg) thus obtained showed mp 315—316°. The phenol (**8**) (50 mg) was also treated with chromium trioxide in acetic acid and the same compound (**9**) (20 mg) was obtained. UV λ_{max}^{EtOH} nm: 260, 400: $\lambda_{max}^{dioxane}$ nm: 255, 390. IR ν_{max}^{KBr} cm^{-1}: 3230, 1655, 1620, 1515, 1455, 1370, 1345, 1312, 1290, 1260, 1235, 1180, 1112, 1008, 903, 830, 818. NMR δ (in CD_3OD): 6.6—6.9 (5H), 7.22 (2H, d, J=9 Hz), 7.42 (2H, d, J=9 Hz). Mass Spectrum *m/e*: 308.0679 (Calcd. for $C_{18}H_{12}O_5$, 308.0684, M^+), 280 ($C_{17}H_{12}O_4$), 223 ($C_{15}H_{11}O_2$). The quinone (**9**) (70 mg) was derived to the diacetate (**10**) by the usual manner and crystallized from chloroform to yellow needles (47 mg) of mp 206—207°, UV $\lambda_{max}^{CHCl_3}$ nm: 355; $\lambda_{max}^{dioxane}$ nm: 345. IR ν_{max}^{KBr} cm^{-1}: 1755, 1675, 1650, 1610, 1505, 1420, 1375, 1328, 1292, 1200 (br.), 1090, 1015, 915, 862, 805. NMR δ (in $CDCl_3$): 2.22 (3H, s), 2.30 (6H, s), 6.90 (1H, s), 7.0—7.3 (4H), 7.30 (2H, d, J=9 Hz), 7.52 (2H, d, J=9 Hz). Mass Spectrum *m/e*: 434.0978 (Calcd. for $C_{24}H_{18}O_8$, 434.1000, M^+), 392 ($C_{22}H_{16}O_7$), 350 ($C_{20}H_{14}O_6$), 308 ($C_{18}H_{12}O_5$), 280 ($C_{17}H_{12}O_4$).

The Fremi Oxidation of Compound A (1) and the Dimethyl Ether (6)——The oxidation in acetone, methanol, dimethylformamide, or dioxane was carried out in recovering the starting materials or obtaining intractable products, which showed no spot identical with atromentin dimethyl ether[11] (**21**) or tetramethyl ether[11] (**22**) by TLC.

Synthesis of Compound A Trimethyl Ether (7)——To the solution of 2,5-di-*p*-anisyl-*p*-benzoquinone[10] (**15**) (10.0 g) in acetic anhydride (270 ml) 72% perchloric acid (1.4 ml) was added gradually at around 40° and, after 20 hr, the reaction mixture was poured onto iced water. The precipitate thus formed was collected and passed through a column of silica gel. Each fraction was monitored by thin-layer chromatography and spectroscopic methods. 4,4″-Dimethoxy-2′,3′,5′-triacetoxy-*p*-terphenyl (**16**) (2.1 g) was obtained as colorless crystals of mp 187—188°. IR ν_{max}^{KBr} cm^{-1}: 1765, 1615, 1580, 1525, 1470, 1440, 1405, 1370, 1295, 1250, 1180, 1095, 1035, 915, 840. NMR (Table II). Mass Spectrum *m/e*: 464.1445 (Calcd. for $C_{26}H_{24}O_8$, 464.1470, M$^+$), 422 ($C_{24}H_{22}O_7$), 380 ($C_{22}H_{20}O_6$), 339 ($C_{20}H_{19}O_5$), 338 ($C_{20}H_{18}O_5$). The fraction eluted before the objective compound gave 2,5-diacetoxy-4,4″-dimethoxy-*p*-terphenyl (**17**) (0.4 g), mp 205—207°. IR ν_{max}^{KBr} cm^{-1}: 1765, 1615, 1580, 1525, 1470, 1440, 1405, 1370, 1295, 1250, 1180, 1095, 1035, 915, 840. NMR (Table II). Mass Spectrum *m/e*: 406 ($C_{24}H_{22}O_6$, M$^+$).

The triacetate (**16**) (0.89 g) in methanol (50 ml) was added with sodium (0.15 g) and the reaction mixture was diluted with water and acidified. The precipitate was collected and purified by preparative layer chromatography using chloroform–ethyl acetate (4: 1) as the developer. The main fraction was recovered and recrystallized from chloroform to violet-red crystals (0.33 g) of 2,5-dianisyl-3-hydroxy-*p*-benzoquinone (**18**), mp 224—226°. UV λ_{max}^{EtOH} nm (log ε): 388, 256 (3.87, 4.49). IR ν_{max}^{KBr} cm^{-1}: 3280, 1660, 1630, 1615, 1580, 1515, 1465, 1415, 1370, 1350, 1305, 1285, 1250, 1180, 1110, 1030, 1020, 1005, 900, 825, 815. NMR δ (in $CDCl_3$): 3.84 (6H, s), 6.78 (1H, s), 6.93 (4H, d, J=9.0 Hz), 7.45 (2H, d, J=9 Hz), 7.55 (2H, d, J=9 Hz). Mass Spectrum *m/e*: 336.1027 (Calcd. for $C_{20}H_{16}O_5$, 336.0998), 308 ($C_{19}H_{16}O_4$).

The quinone (**18**) (80 mg) in chloroform (15 ml) was methylated with methyl iodide (1 ml) and silver oxide (1.5 g). The reaction product was purified by preparative layer chromatography to yield a dark orange crystals (38 mg) of the methyl ether (**19**), mp 155—156°. UV $\lambda_{max}^{dioxane}$ nm (log ε): 254, 385 (4.56, 3.97). IR ν_{max}^{KBr} cm^{-1}: 1660, 1645, 1610, 1575, 1515, 1450, 1325, 1295, 1275, 1250, 1205, 1175, 1100, 1030, 940, 925, 900, 860, 820. NMR δ (in $CDCl_3$): 3.82 (3H, s), 3.86 (6H, s), 6.83 (1H, s), 6.96 (4H, d, J=9 Hz), 7.34 (2H, d, J=9 Hz), 7.54 (2H, d, J=9 Hz). Mass Spectrum *m/e*: 350.1171 (Calcd. for $C_{21}H_{18}O_5$, 350.1154, M$^+$), 332 ($C_{21}H_{16}O_4$), 307 ($C_{19}H_{15}O_4$), 251 ($C_{17}H_{15}O_2$).

The quinone (**19**) (30 mg) in methanol was hydrogenated catalytically in the presence of palladized-charcoal. To the solution ethereal diazomethane was directly added and, after 20 hr, the solvent was evaporated. The residue was applied for thin-layer chromatography using chloroform–methanol (4: 1) as the developer. Three spots were observed and the upper spot was recovered (7 mg) and recrystallized from hexane to colorless needles of mp 165—166°. A mixed fusion and IR spectra showed the identity with compound A trimethyl ether (**7**). Other two spots showed identity with neither of the two compound A dimethyl ethers (**5** and **6**).

Compound E (24)——Colorless needles of mp 230—231° from ethyl acetate. The Gibbs reaction, positive. UV λ_{max}^{EtOH} nm (log ε): 225, 277 (4.48, 4.30). IR ν_{max}^{KBr} cm^{-1}: 3400, 1610, 1560, 1485, 1365, 1220, 1116, 1070, 990, 830, 775, 700. NMR (Table II). Mass Spectrum *m/e*: 322.122 (Calcd. for $C_{20}H_{18}O_4$, 322.120, M$^+$), 307.099 (Calcd. for $C_{19}H_{15}O_4$, 307.097, M$^+$–CH_3), 292.070 (Calcd. for $C_{18}H_{12}O_4$, 292.073, M$^+$–2×CH_3), 276.070 (Calcd. for $C_{18}H_{12}O_3$, 276.078, M$^+$–CH_3–CH_3O).

Compound E Diacetate (25)——Compound E (**24**) (30 mg) was acetylated by acetic anhydride (0.5 ml) and pyridine (0.3 ml). Recrystallization from ethanol afforded the acetate (**25**) (27 mg), mp 165—166°. IR ν_{max}^{KBr} cm^{-1}: 2970, 1762, 1602, 1512, 1478, 1390, 1372, 1200, 1100, 1080, 998, 915, 846, 804, 698. NMR (Table II). Mass Spectrum *m/e*: 406.1415 (Calcd. for $C_{24}H_{22}O_6$, 406.1415, M$^+$), 364 ($C_{22}H_{20}O_5$), 322 ($C_{20}H_{18}O_4$), 307 ($C_{19}H_{15}O_4$).

Cytotoxicity Tests——HeLa cells were grown in Eagle's minimun essential medium supplemented with 10% calf serum and antibiotics (100 u/ml penicillin and 100 μg/ml streptomycin). The modified panel method was employed for cytotoxicity bioassay. The test samples were dissolved in dimethyl sulfoxide and diluted in the medium at half log intervals. A round cover glass was placed in each cup of glass panels (Ikemoto Rika Kogyo Co., Ltd.), which receives each dilution of the samples and cells. After 3-days' incubation cells grown on cover glasses were fixed with Carnoy's fixative, stained with hematoxylin and eosin, and examined for cytotoxicity and the induced morphological changes. The degree of cytotoxicity was estimated on a scale ranging "0" (no cellular damage) through "4" (complete cytolysis or cell detachment) as described previously.[3]

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