

Some Observations on the Metabolites of *Phoma* Species

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Fifteen strains of *Phoma* species were examined for their production of toxic metabolite(s). Although three of them showed the toxicity for cultured HeLa cells and mice and the metabolites were not identical with the known metabolites, attempts to isolate toxic principle(s) were abandoned due to their instability. In the course of the work cynodontin²⁾ (3), 6-methylsalicylic acid, an unidentified phenolic of mp 191—193°, and a chromanone (4) were isolated. The chromanone (4) was proved to be (2*R*)-5-hydroxy-6-(2'-hydroxyethyl)-7-methoxy-2-methylchromanone.³⁾

In our course of screening programme of mycotoxin-producing fungi⁴⁻⁶⁾ we encountered some strains of *Phoma* species (Sphaeropsidales, Fungi Imperfecti) exhibiting cytotoxicity on HeLa cells and acute toxicity on mice. *Phoma* is a fungus genus consisting of more than 1700 species that are worldwide in their distribution. Most species of *Phoma* parasitize host plants, including many kinds of agricultural crops. Some species of the genus are isolated commonly from cereal grains as a seed-borne origin. As far as the authors are aware, there has been no mycotoxin-producing species belonging to the genus hitherto described.^{7,8)} As for the metabolites of *Phoma* spp. several anthraquinones,^{2,9)} phomazarine,¹⁰⁾ epoxydone¹¹⁾ (phyllosinol),¹²⁾ phomalactone,¹³⁾ phomin (1)¹⁴⁾ (cytochalasin B¹⁵⁾), 5-dehydrophomin (2)¹⁴⁾ (cytochalasin A¹⁵⁾), gentisyl alcohol,¹⁶⁾ toluhydroquinone,¹⁶⁾ and chlorogentisyl alcohol¹⁶⁾

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have been reported but none of them seems to be causative agent for the toxicity except **1** and **2**, which have shown to exhibit remarkable cytotoxicity.^{14,15)}

In order to clarify the toxic metabolite(s) of the genus, fifteen strains of *Phoma* isolated from foodstuffs and identified in our laboratory (Table I) were examined. After 3 weeks' incubation on potato-dextrose medium, the mycelium and the filtrate were separated and the filtrate and chloroform extract of the mycelium were applied for toxicity tests for mice and HeLa cells by the methods reported previously.⁴⁻⁶⁾ The filtrate was successively extracted with chloroform and ethyl acetate and the three extracts thus obtained were examined by thin-layer chromatography (TLC) using phomin (**1**), dehydrophomin (**2**), and phomalactone as the standards.

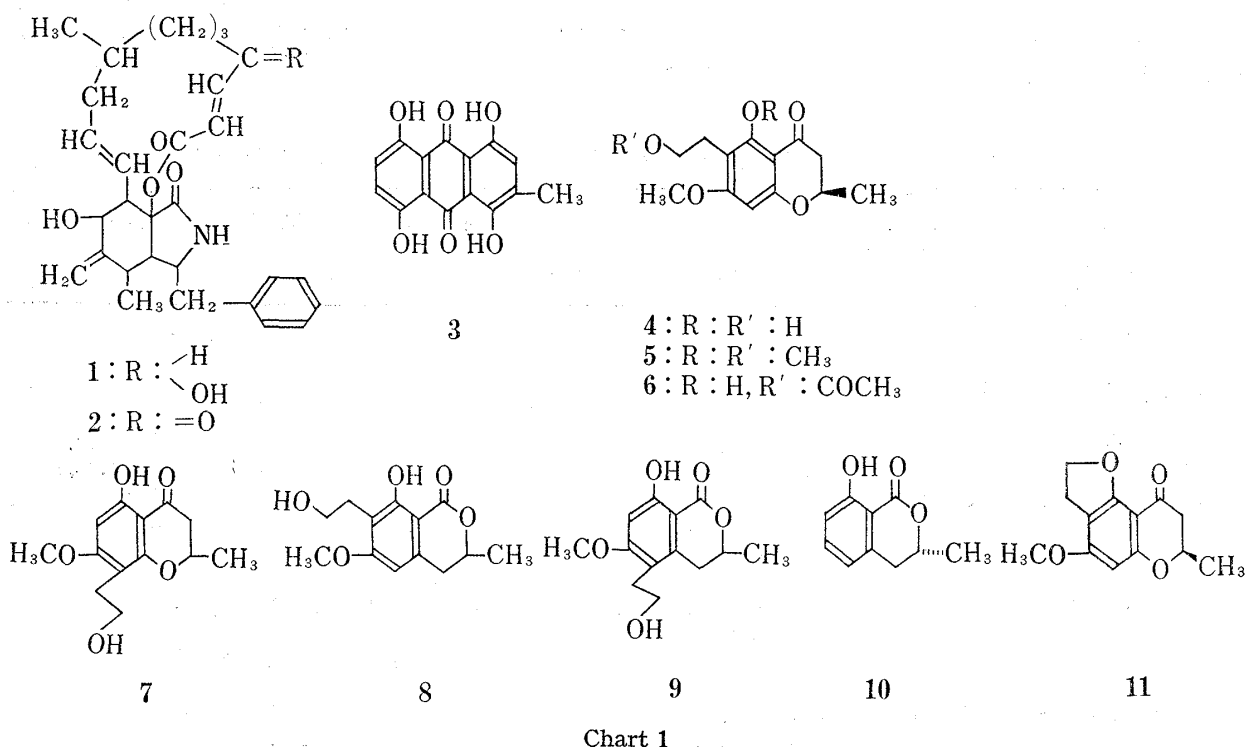
TABLE I. Source of Strains of *Phoma* species and Their Toxicity on HeLa Cells and on Mice

Strain No.	Isolate No.	Samples isolated:		Toxicity on HeLa cells ^{a)}		Toxicity on mice	
		Source	Locality	Filtrate	Mycelium	Filtrate	Mycelium
NHL7003	69-SA-12	fermented pickles	Saku-shi, Nagano-ken	—	—	—	—
7004	69-SA-13	fermented pickles	Saku-shi, Nagano-ken	—	+	± (liver)	—
7005	69-SA-180	wheat flour	Saku-shi, Nagano-ken	—	—	—	+ (kidney, thymus)
7006	69-SA-181	milled rice	Saku-shi, Nagano-ken	±	±	—	± (proliferating cells)
7007	69-SA-182	cowpea	Saku-shi, Nagano-ken	—	±	—	—
7008	68-AO-70	milled rice	Amami-oshima Is., Kagoshima-ken	+++	—	+++ (acute death, thymus, proliferating cells)	—
7009	68-GO-161	adzuki bean	Goto Is., Nagasaki-ken	+++	—	+++ (acute death, thymus, proliferating cells)	—
7011	68-GO-164	adzuki bean	Goto Is., Nagasaki-ken	+	—	+ (thymus, proliferating cells)	± (thymus)
7014	70-HO-66	wheat flour	Shizunai-machi, Hidaka-gun, Hokkaido	—	—	—	—
7015	FF-16-C	frozen vegetables	Tokyo	—	±	—	± (thymus, lymph node)
7016	FF-21-C	half-cooked frozen product	Tokyo	±	±	—	—
7017	FF-33-C	frozen shrimp	Tokyo	±	—	± (liver)	—
7018	FF-34-C	frozen oyster	Tokyo	±	±	± (liver, kidney)	+ (stomach)
7019	FF-42-C	frozen dasheen	Tokyo	—	—	—	± (proliferating cells)
7020	70-NU-20	peanut	Kebil, Chimbu Dist., Papua and New Guinea	±	+	—	+ (thymus)

a) Criteria employed for estimating the degree of toxicity is same as the previous paper.⁴⁾

Although none of the strains showed the production of these known metabolites, the filtrates of three strains (NHL 7008, 7009, and 7011) showed toxicity both on mice and HeLa cells as follows: The mice died after 1 to 3 intraperitoneal injections of the culture filtrates (0.8 ml/20 g body weight) on successive days, though histological changes were rather poor

and inconstant. Thymus cells were invariably damaged showing karyorrhexis. Non-suppurative peritonitis was an occasional finding, even in the mice which were not fatal. HeLa cells were led to necrosis after 3-days' treatment with 3.2% (NHL 7011) or 0.32% (NHL 7009 and 7008) of the respective filtrates. The morphological appearance of moderately damaged cells was not characteristic; irregularity in cell size, formation of multinuclear cells, and others. Mitotic figures were preserved. The extract of the mycelium did not show noticeable toxicity (Table I).



From these findings further attempts to isolate toxic principle(s) were carried out using the strain NHL 7008. However in the course of an incubation in large scale or subculturing, we encountered the loss of acute toxicity on mice, though the cytotoxicity on HeLa cells had been retained (3.2 $\mu\text{g}/\text{ml}$ for CHCl_3 extract). Furthermore, when the filtrate was treated successively with CHCl_3 , EtOAc, and charcoal chromatography, none of the separated fractions showed cytotoxicity, probably due to the decomposition in the course of separation. Under such instability of the toxic principle(s) further attempt was abandoned.

In the course of studies one of the strains NHL 7017 was noticed by pigment formation. From chloroform extract of the mycelium cynodontin²⁾ (3) was isolated and identified. From the culture filtrate of the toxic strain NHL 7008, 6-methylsalicylic acid and an unidentified phenolic with no toxicity, mp 191—193°, $\text{C}_{15}\text{H}_{16}\text{O}_7$, were isolated.

From the filtrate of another strain NHL 7018 a phenolic (4), mp 190—191°, $[\alpha]_{\text{D}}^{18.5} +24.8^\circ$ (MeOH), $\text{C}_{13}\text{H}_{16}\text{O}_5$, was isolated. It forms dimethyl ether (5) (by methyl iodide) and monoacetate (6). From the ultraviolet spectrum (UV) ($\lambda_{\text{max}}^{\text{MeOH}}$: 240, 288, 325 nm) and infrared spectrum (IR) ($\nu_{\text{max}}^{\text{KBr}}$: 3320, 1640, 1590 cm^{-1}) (in 5, $\nu_{\text{max}}^{\text{KBr}}$: 1685, 1600 cm^{-1}) the compound was assumed to be an *o*-hydroxyarylketone. Nuclear magnetic resonance (NMR) spectra of 4, 5, and 6 including decouplings disclosed the presence of one each of hydroxyethyl, phenolic hydroxyl, methoxyl, ring hydrogen, and $-\text{CH}_2-\text{CH}(\text{O})-\text{CH}_3$ groups in the compound (Table II). The four structures 4, 7, 8, and 9 suffice these observations.¹⁷⁾ In order to distinguish the chromanone (4, 7) and the 3,4-dihydroisocoumarin (8, 9) structures, the circular dichroism (CD)

17) The methoxyl group was placed to *meta* to the hydroxyl group from biogenetical back ground.

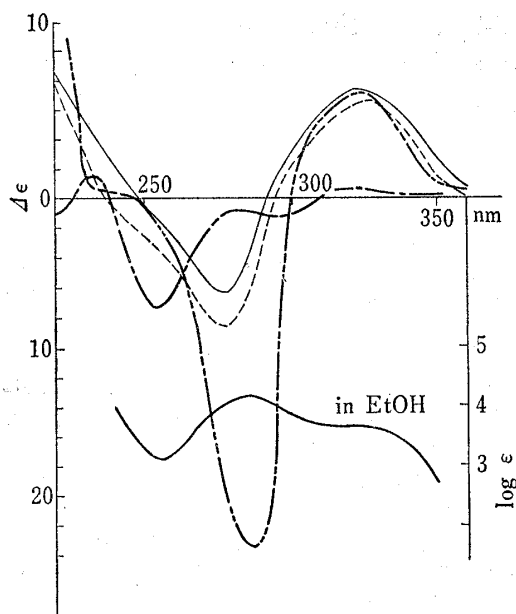


Fig. 1. CD (in MeOH) and UV of the Chromanone 4 and the Related Compounds

—: the chromanone (4)
 - - - : dimethyl ether of 4 (5)
 ···· : mellein
 - · - · : (2S) sakuranetin

curves of **4** and **5** were compared with mellein (**10**) and flavanones.¹⁸⁾ As shown in Fig. 1, the compounds (**4** and **5**) showed a negative Cotton effect at the $\pi \rightarrow \pi^*$ transition and a positive effect at $n \rightarrow \pi^*$ transition superimposable with 2S-flavanones. Thus the compound was established to be a chromanone and at the same time the absolute configuration at 2-position was shown to be *R*.

Although there existed no decisive evidence to distinguish the structures **4** and **7**, we rather favoured the latter from the observation of nuclear Overhauser effects (*cf.* Table II) of **4** and **5** and proposed the structure.¹⁹⁾ Just after that isolation of three novel chromanones, LL-D253 α , - β , and - γ from *Phoma pigmentivora* was reported and the structures were elucidated as **4**, **6**, and **11** respectively.³⁾ Direct comparison of our sample and the acetate with LL-D253 α and - β respectively showed identity. The American chemists discriminate the structure (**4**) from **7** for their LL-D253 α by the fact that the compound forms

TABLE II. NMR Spectra of the Chromanone (4) and the Derivatives

Compound solvent	4 CD ₃ OD	5 CDCl ₃	6 CD ₃ OD	Coupling
CH ₃	1.45 ^{a)}	1.45	1.45	d, <i>J</i> =6
-O-CH	4.44 ^{a)}	4.35	4.40	qt, <i>J</i> =6, 8
CH ₂	2.52 ^{a)}	2.56	2.52	d, <i>J</i> =8
-OR	R=H	R=CH ₃	R=H	
	—	3.88	—	s
OR'	R'=H	R'=CH ₃	R'=COCH ₃	
	—	3.35	1.97	s
CH ₂	3.64 ^{a)}	3.42	4.10	t, <i>J</i> =8
CH ₂	2.84 ^{a)}	2.85	2.85	t, <i>J</i> =8
-OCH ₃	3.76	3.86	3.73	s
-H (aromatic)	6.08 ^{b)}	6.01	6.00	s

a) Decoupling experiments were performed on these protons and the couplings were confirmed.

b) The irradiation at the proton resulted in the increase (9.7%) of the signal at δ 3.76.

11 on cyclisation by sulfuric acid treatment.³⁾ Although the optical activity of the naturally occurring compound LL-D253 γ (**11**) was recorded, that derived from LL-D253 α was not shown.³⁾ It is a well known fact that chromanones undergo the Wessely-Moser rearrangement in acidic condition. Thus there still remained the possibility of the formation of **11** from a compound having the structure **7**. In order to confirm the problem the same reaction was repeated for our compound and the product was proved to be identical with **11** retaining the same optical activity (determined by CD). Thus the structure of the chroma-

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19) C. Takahashi, S. Sekita, K. Yoshihira, and S. Natori, the paper presented at the 92nd Annual Meeting of Pharmaceutical Society of Japan, Osaka, April 1972.

none was firmly established as (2*R*)-5-hydroxy-6-(2'-hydroxyethyl)-7-methoxy-2-methyl-chromanone. Recently another example of an optically active chromanone of fungal origin was reported.²⁰⁾

Experimental

Culture and Culturing Conditions—Fifteen strains of *Phoma* species, listed in Table I, were used for this study. The fungi were 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). The inoculated flasks were incubated at 25° for 3 weeks under stationary conditions. The mycelium and the filtrate were separated by filtration, the mycelium was immediately homogenized with CHCl₃ in a warring blender, and the solvent was evaporated.

Toxicity Tests using HeLa Cells and Mice—The methods were the same as those reported in the previous papers.⁴⁻⁶⁾ The filtrate and dimethyl sulfoxide solution of the mycelial extract were used for the tests. The results were summarized in Table I.

Examination of the Metabolites by Thin-Layer Chromatography—The filtrate was extracted successively with CHCl₃ and EtOAc and the two extracts and the mycelial extract were used for the tests. Silica gel HF₂₅₄ was employed for the absorbant, mixtures of CHCl₃-EtOAc (1:1) and CHCl₃-MeOH (10:1) were used as the developing solvents, UV lamps (254 and 365 nm) and sulfuric acid were used for the detection, and phomin (1), dehydrophomin (2), and phomalactone¹⁹⁾ were used for the standards. All fifteen strains showed several spots but none of them were identical with the standards.

Isolation of Cynodontin from the Strain NHL 7017—The chloroform extract of the mycelium (from 5 liter of medium) was passed over a silica gel column and a red-colored zone was collected. The fraction was dissolved in benzene and partitioned with 5% NaOH. The alkaline layer was separated, acidified, and extracted with benzene. The benzene layer was washed with 5% NaHCO₃ and 5% Na₂CO₃ and evaporated. The residue (ca. 50 mg) was recrystallized from CHCl₃ to dark red crystals of mp 245°, UV $\lambda_{\text{max}}^{\text{CHCl}_3}$ nm (log ϵ): 295, 490, 514, 524, 550, 564 (3.99, 4.07, 4.25, 4.32, 4.30, 4.37), IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 1580, 1440, 1390, 1270, 1250, 1170, 1080, 1020, 970, 790. The identity with the authentic sample of cynodontin (3) was confirmed by TLC and IR. It forms tetraacetate, yellow needles of mp 221–222° from benzene.

Examination of Metabolites of the Strain NHL 7008—The filtrate (from 10 liter of medium) was successively extracted with CHCl₃ and EtOAc. The aqueous layer after extractions was absorbed on a charcoal column and successively eluted with water, MeOH, acetone, and benzene. Each fraction was tested for cytotoxicity but none showed the activity. The EtOAc extract (7.6 g) was chromatographed using silica gel and eluted successively with benzene, benzene-EtOAc (8:2), benzene-EtOAc (1:1), CHCl₃-EtOAc (1:1), EtOAc, and MeOH. From benzene-EtOAc (8:2) eluate 6-methylsalicylic acid (81 mg), mp 164°, was obtained and identified by IR and NMR.

From EtOAc fraction colorless needles (55 mg) of mp 191–193° was obtained; IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3550, 1700, 1680, 1617, 1580, 1465, 1440, 1355, 1300, 1250, 1225, 1190, 1170, 1090, 1055; mass spectrum (*m/e*): observed, 308.089; Calcd. for C₁₅H₁₆O₇: 308.089. The methyl ether obtained by CH₃I-Ag₂O showed mp 128–130° (from AcOEt).

Isolation of the Chromanone (4) from the Strain NHL 7018—The culture filtrate (from 3 liter medium) was extracted with EtOAc and the extract was passed over silica gel using CHCl₃, CHCl₃-EtOAc, EtOAc, and MeOH successively as the eluting solvents. From CHCl₃-EtOAc fraction the chromanone (4) was obtained as colorless needles (450 mg), mp 190–191°, after recrystallization from EtOAc, $[\alpha]_{\text{D}}^{25} + 24.8^\circ$ (*c*, 0.01, MeOH) (lit.³⁾ mp 188–189°, $[\alpha]_{\text{D}}^{25} + 25.2 \pm 0.42^\circ$ (MeOH); UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (log ϵ): 240, 288, 325 (4.07, 4.21, 3.70), $\lambda_{\text{max}}^{\text{EtOH}}$ nm (log ϵ): 253, 328 (3.86, 4.34); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3320, 1640, 1590, 1460, 1390, 1340, 1290, 1210, 1160, 1120, 1100, 1030; Mass Spectrum *m/e*: 252.096 (M⁺, Calcd for C₁₃H₁₆O₅: 252.100), 221 (C₁₂H₁₃O₄), 179 (C₉H₇O₄).

Dimethyl Ether (5): The chromanone (4) (50 mg) in CHCl₃ (2 ml) was treated with CH₃I (0.7 ml) and Ag₂O (0.2 g) at room temperature for 20 hr. After working up usual and purification by preparative layer chromatography colorless needles (5) (28 mg) were obtained, mp 95° (from hexane), IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 1685, 1600, 1575, 1475, 1460, 1355, 1265, 1210, 1130, 1080; Mass Spectrum *m/e*: 280.126 (M⁺, Calcd. for C₁₅H₂₀O₅: 280.131).

Monoacetate (6): The chromanone (4) (35 mg) was treated with Ac₂O (2 ml)-pyridine (2 ml) at room temperature for overnight. Recrystallization from EtOAc gave colorless crystals (6) (21 mg) of mp 207–208° (lit.³⁾ mp 207–208°), IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3180, 2950, 1740, 1645, 1600, 1500, 1450, 1420, 1390, 1360, 1300, 1240, 1210, 1155, 1120, 1090, 1030; Mass Spectrum *m/e*: 294.111 (M⁺, Calcd. for C₁₅H₁₈O₆: 294.110).

Formation of the Dihydrofuran (11) from the Chromanone (4): The chromanone (4) (6.3 mg) was dissolved in conc. H₂SO₄ (0.1 ml). After standing at room temperature overnight, the solution was diluted with water

20) Ch. Tamm, B. Böhner, and W. Zürcher, *Helv. Chim. Acta*, **55**, 510 (1972).

and neutralized. The EtOAc extract was then purified by preparative layer chromatography. The spot showing the identical *R_f* value with LL-D253 γ (**11**) was collected and the CD curves are compared.

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