METABOLITES OF *DACTYLARIA LUTEA*
THE STRUCTURES OF DACTYLARIOL AND THE ANTIPROTOZOAL ANTIBIOTIC DACTYLARIN

ANNA MARIA BECKER, RODNEY W. RICKARDS,
KARL J. SCHMALZL and HO C. YICK

Research School of Chemistry, Australian National University,
Canberra, A.C.T. 2600, Australia

(Received for publication February 2, 1978)

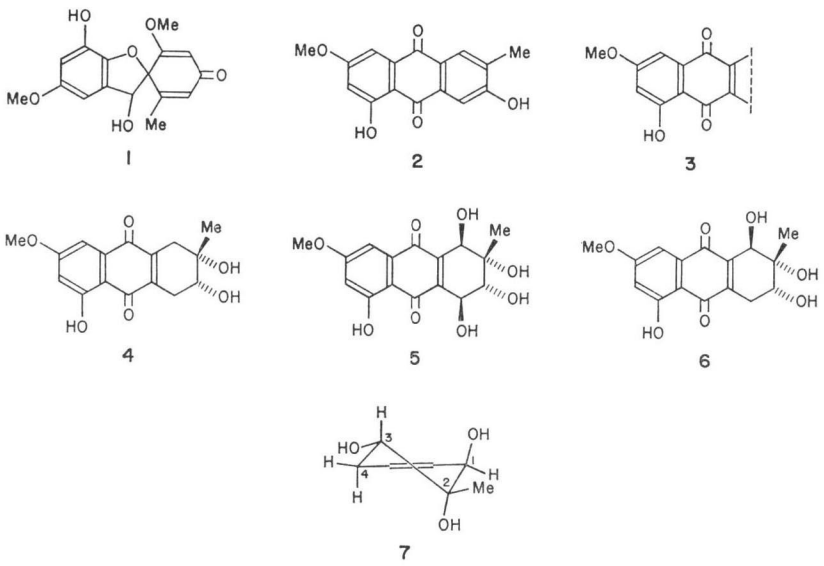
The metabolites of the predacious fungus *Dactylaria lutea* ROUTIEN include the anthraquinone macrosporin (**2**) and three hydroxylated 1,2,3,4-tetrahydro derivatives of this anthraquinone, altersolanol A (**5**), altersolanol B (**4**) and dactylariol (**6**). The structure and relative configuration of dactylariol are established from spectroscopic studies, and its absolute configuration is proposed as 1*R*, 2*R*, 3*R* by virtue of its co-occurrence with altersolanol B. Dactylarin, suggested by other authors to have the structure (**1**), is shown to be identical with altersolanol B (**4**).

Dactylaria lutea ROUTIEN is a predacious fungus[1] belonging to the order Moniliales of the Fungi Imperfecti. Following observations[2] of the activity of extracts of *D. lutea* against protozoa and nematodes, a red crystalline compound, dactylarin, was isolated from the culture medium[3] and from the mycelium[4] of this fungus. Dactylarin exhibited high antiprotozoal activity *in vitro* against *Leishmania braziliensis* and *Entamoeba invadens*[3], and was strongly cytotoxic to HeLa cells[5]. Its production from *D. lutea* was the subject of a German Patent in 1972[4]. On the basis of analytical and spectroscopic data, dactylarin was assigned the novel structure (**1**) in 1973 by KETTNER and coworkers[3].

In view of the interesting biological activities shown by the genus *Dactylaria*[1,2] and by dactylarin itself[3,5], we have investigated further the metabolites of *D. lutea* ROUTIEN. They are shown to include an anthraquinone and three hydroxylated tetrahydro derivatives of that anthraquinone. The structure (**1**) proposed by KETTNER *et al.*[3] for dactylarin is revised.

Metabolites of *D. lutea* and the Structure of Dactylariol

Growth of *D. lutea* ROUTIEN in shaken flasks on a malt extract medium as described by KETTNER *et al.*[3] produced red and yellow pigments in the culture medium and mycelium, which were extracted with ethyl acetate. The extract was washed successively with aqueous sodium bicarbonate, carbonate and hydroxide. The recovered acidic fractions were then subjected to gel permeation chromatography to remove lipids before final purification of the pigments themselves by preparative thin-layer chromatography. Four main pigments were present, closely related in structure. Mass spectrometry established their molecular formulae as $C_{16}H_{12}O_5$, $C_{16}H_{16}O_6$, $C_{16}H_{16}O_7$ and $C_{16}H_{16}O_8$ in order of increasing polarity. Their structures were established by consideration of their mass spectra, proton magnetic resonance spectra and electronic spectra, and were confirmed where possible by direct comparison of spectra and melting points with authentic specimens of known structure.



The least polar metabolite, a yellow anthraquinone $C_{16}H_{12}O_5$, was identified as the known macrosporin, 3,5-dihydroxy-7-methoxy-2-methyl-anthraquinone (**2**)[6]. The remaining three red pigments had similar naphthoquinonoid rather than anthraquinonoid chromophores, and p.m.r. spectra indicative of the system (**3**). They were shown to be 1,2,3,4-tetrahydro derivatives of the anthraquinone (**2**). The compound $C_{16}H_{16}O_6$ and the most polar metabolite $C_{16}H_{16}O_8$ were identified as altersolanols B[7] and A[7,8] respectively, the known 2,3,5-trihydroxy (**4**) and 1,2,3,4,5-pentahydroxy (**5**) derivatives of 7-methoxy-2-methyl-1,2,3,4-tetrahydroanthraquinone.

The final pigment $C_{16}H_{16}O_7$, here named dactylariol, was intermediate in oxidation level between the tetrahydro-anthraquinones (**4**) and (**5**), and had a similar electronic spectrum. Its p.m.r. spectrum in CD_3CO_2D showed, in addition to the methoxyl singlet at δ 3.88 and the *m*-coupled aromatic protons at δ 6.68 and 7.16 ($J=2$ Hz) characteristic of the system (**3**), singlets at δ 1.47 and δ 4.74 (broad) due to CH_3-C2 and H-Cl, and an ABX system corresponding to $ArCH_2CHOD$. The components of this ABX system were centred at δ 2.59 (dd, $J=9$ and 20 Hz), δ 3.08 (dd, $J=6$ and 20 Hz) and δ 4.12 (dd,

Table 1. Proton resonances of 1,2,3,4-tetrahydro-anthraquinones[a]

Compound	H–Cl	CH_3–C2	H–C3	H–C4	H–C6	H–C8	CH_3O–C7
Altersolanol B (**4**)[7]	2.38~2.73	1.20	3.55[b]	2.38~2.73	6.71,	6.92	3.88
Altersolanol A (**5**)[7]	4.38	1.31	3.68[c]	4.54[c]	6.72,	6.93	3.87
Dactylariol (**6**)[d]	4.38	1.32	3.79	~2.3[e], 2.86	6.79,	7.06	3.90
Dactylarin[f]	Unresolved	1.18	3.60	Unresolved	6.78,	6.99	3.88

[a] Chemical shifts in $(CD_3)_2SO$ as δ values relative to internal tetramethylsilane.

[b] STOESSL[7] quotes δ 3.25. In our hands authentic altersolanol B shows H–C3 at δ 3.55, with H_2O impurity at δ 3.27.

[c] These two resonances were not specifically allocated by STOESSL[7] to H–C3 or H–C4. However, their assignments must be as shown.

[d] Multiplicities and coupling constants were the same as in CD_3CO_2D solution (see text).

[e] This doublet of doublets partly obscured by solvent absorption at δ 2.54.

[f] Values are those reported[3] shifted 0.25 ppm to higher field (see text).

$J=6$ and 9 Hz). The use of $(CD_3)_2SO$ as p.m.r. solvent gave rise to large solvent shifts of the alicyclic protons relative to the values in CD_3CO_2D, and considerable line broadening which may be due to partial ionisation of the phenolic hydroxyl in addition to hydrogen bonding. This line broadening could be overcome, without significantly changing chemical shifts, by the addition of a trace of CD_3CO_2D to the $(CD_3)_2SO$ solution. The spectrum of dactylariol could then be directly compared, as in Table 1, with those reported[7] for altersolanol A (**5**) and altersolanol B (**4**) in $(CD_3)_2SO$. The chemical shifts and multiplicities of the alicyclic protons in dactylariol unequivocally establish its structure (without regard to stereochemistry) as the 1,2,3,5-tetrahydroxy derivative (**6**) of 7-methoxy-2-methyl-1,2,3,4-tetrahydroanthraquinone.

Stereochemistry of *D. lutea* Metabolites

Direct comparisons of optical rotation clearly establish that the present isolates of altersolanol B and altersolanol A from *D. lutea* have the same absolute configuration as the original isolates from *Alternaria solani*. The absolute configuration of altersolanol B has been established as 2*S*, 3*R* (as in **4**) by X-ray diffraction studies on its mono-*p*-bromobenzoate[9,10]. Altersolanol A by virtue of its co-occurrence with altersolanol B in *A. solani*, has been suggested[10] to have the same absolute configuration at C2 and C3, and thus from p.m.r. studies[11] has been tentatively assigned[10] the 1*R*, 2*S*, 3*R*, 4*S* configuration as in **5**. This proposed correspondence of configuration between altersolanol A and altersolanol B is now reinforced by their co-occurrence in a different microorganism.

This correspondence of relative and absolute configuration would be expected to extend also to dactylariol, which would thus have the 1*R*, 2*R*, 3*R* configuration as in **6**. Support for this conclusion as far as relative configuration is concerned comes from the precise correspondence of resonance positions of H-Cl and CH_3-C2 in altersolanol A and dactylariol, which reflects the identical surroundings of these protons in the two compounds. The resonance of H-C3 in dactylariol does not closely match that in either altersolanol B or altersolanol A. This is to be expected, as can be seen from diagram (**7**), which represents the probable preferred conformation of the cyclohexene ring in dactylariol. Dactylariol either has an additional β-hydroxyl at Cl compared to altersolanol B, or lacks the β-hydroxyl at C4 of altersolanol A, either of which would influence the shielding experienced by the β-oriented H-C3. The half-chair conformation (**7**) of dactylariol is deduced from the vicinal coupling constants of 6 and 9 Hz between H-C3 and the two protons at C4, which define one essentially *trans*-diaxial relationship[12], together with consideration of non-bonded interactions which would destabilise a possible boat conformation. This conformation is similar to that previously established for altersolanol A[11], and also to that suggested for the triacetate of altersolanol B[10].

A Revised Structure for Dactylarin

The spectroscopic data presented by other authors[3] for the antiprotozoal antibiotic dactylarin do not lead to the proposed structure (**1**). Several features are particularly difficult to reconcile. Thus dactylarin was red, with an absorption maximum at 435 nm (ε 3,720), and lacked optical activity, although structure (**1**) contains no extended chromophore and has two potentially chiral centres. The mass spectrum, in addition to the expected molecular ion at *m/e* 304 corresponding to $C_{16}H_{16}O_6$, also contained an intense ion at *m/e* 284 assigned[3] to an "M-20" fragment. Discrepancies are also apparent in the published pmr assignments.

The reported data[3] for dactylarin can, however, be readily accommodated if dactylarin is in fact the $C_{16}H_{16}O_6$ compound altersolanol B (**4**), which we have now shown to be a metabolite of *D. lutea*. The electronic and infrared absorption characteristics of dactylarin match those of altersolanol B. The mass spectrum presented for dactylarin closely resembles that of altersolanol B, with minor variations in the relative intensities of the major ions. An exception is the intensity of the ion at *m/e* 284 in the spectrum of dactylarin, which is extremely weak in the case of altersolanol B. This is probably the anthraquinonoid ion $C_{16}H_{12}O_5^+$ arising either from some macrosporin (**2**) present as an impurity in the Czech isolate, or by dehydration and dehydrogenation of altersolanol B, the extent of which would depend upon the conditions under which the spectrum is obtained. The pmr spectrum of dactylarin in $(CD_3)_2SO$ as reported[3] does not correspond to that of altersolanol B, but if shifted 0.25 ppm to higher field the agreement is good (*cf.* Table 1). The doublet and singlet at δ 4.80 and δ 4.43 (corrected values) in the spectrum of dactylarin now match the exchangeable secondary and tertiary hydroxyl resonances (d, δ 4.72 and s, δ 4.38) of altersolanol B in the same solvent[12]. The signal from dactylarin at δ 2.53 (corrected), assigned to a second methoxyl group overlaid by solvent absorption[3], is attributable to solvent isotopic impurity CD_3SOCD_2H flanked by allylic methylene absorption. We are unable to account for the reported optical inactivity of dactylarin, except to note that rotational measurements on strongly chromophoric compounds present some difficulties due to light absorption.

A Taxonomic Note

Macrosporin (**2**) has previously been isolated from *Macrosporium porri*[6] (syn. *Alternaria porri*), *A. bataticola*[14], *A. cucumerina*[7], *A. solani*[7] and *Phomopsis juniperovora*[15]. Altersolanol A (**5**) has been obtained from the last two organisms[7,8,15], whilst altersolanol B (**4**) has previously been found only in *A. solani*[7]. The occurrence of these related quinones, together with the new tetrahydroanthraquinone dactylariol (**6**), in *Dactylaria lutea* is of taxonomic interest, since *Alternaria, Phomopsis* and *Dactylaria* are not regarded as related genera.

Experimental

General

Melting points are uncorrected. Preparative t.l.c. was run on methanol-washed silica gel in chloroform - ethyl acetate - methanol - acetic acid (60: 30: 10: 3). Optical rotations for solutions in ethanol were obtained on a Bendix NPL Automatic Polarimeter 143C. Ultraviolet spectra were recorded on a Unicam SP-800 spectrometer. P.m.r. data were obtained on a Varian HA-100 instrument, using tetramethylsilane as internal reference. Mass spectra were run on a GEC-AEI MS902 spectrometer operating at 70 eV ionising voltage.

Production and isolation of metabolites

Dactylaria lutea ROUTIEN (American Type Culture Collection number 12831) was maintained on corn-meal agar slopes. Inoculum cultures, prepared from slope macerates, were grown for 5~6 days on malt extract medium (Cornwell, 4%, 80 ml/flask) adjusted to an initial pH 6.5 in Erlenmeyer flasks (250 ml), shaken at 250 r.p.m. at 28°C. These cultures were used to inoculate the production medium, fermentation of which was carried out under the same conditions for 5~10 days.

The culture filtrate was acidified to pH 2 with dilute sulphuric or hydrochloric acid and extracted with ethyl acetate. The combined extracts were washed successively with water, 2% aqueous sodium bicarbonate, 2% aqueous sodium carbonate, and 2% aqueous sodium hydroxide. The basic solutions were acidified, and re-extracted with ethyl acetate. Evaporation gave altersolanols A and B and

dactylariol from the bicarbonate solution, altersolanol B and dactylariol from the carbonate solution, and altersolanol B from the hydroxide solution. The respective residues were extracted with petroleum ether several times to remove lipids, and then chromatographed twice on Sephadex LH-20 in chloroform - methanol (1: 1). In each case fractions containing pigments were combined, and the individual pigments separated by repeated t.l.c.

Continuous ether extraction of the culture filtrate, after extraction with ethyl acetate, yielded after purification macrosporin and further altersolanol A.

The mycelium was freeze-dried, ground, and extracted in a Soxhlet apparatus with slightly acidic ethyl acetate for 2 days. Analytical t.l.c. showed that this extract contained mainly lipids, together with some macrosporin and only traces of other pigments.

The yields of pigments varied substantially between fermentations. One 9-liter fermentation gave altersolanol A (480 mg), altersolanol B (15 mg), dactylariol (80 mg), and macrosporin (50 mg).

Macrosporin (**2**)

Macrosporin, purified by gel filtration on Sephadex LH-20, was recrystallised from methanol and then from pyridine, m.p. (sealed capillary) 297~301°C dec. (sublimation commencing at 240°C) undepressed on mixing with authentic material (lit. records m.p. 308~316°C dec.[7], 300~302°C[16]). M.s. *m/e* 284 (M^+), 283 (M−H, m* 282.0), 268, 255, 241, 226, 213, 198, 185; p.m.r. δ (CF_3CO_2H) 8.16 (s, H-Cl), 7.71 (s, H-C4), 7.52 (d, *J* 3 Hz, H-C8), 6.87 (d, *J* 3 Hz, H-C6), 4.01 (s, CH_3O), 2.45 (s, CH_3C). Authentic macrosporin exhibited identical mass and p.m.r. spectra.

Altersolanol B (**4**)

The t.l.c. purified pigment was recrystallised from ethanol, m.p. (sealed capillary) 227~231°C (lit.[7] records m.p. 228~230°C) undepressed when mixed with authentic altersolanol B. $[\alpha]_D^{26}$ −63±10° (*c* 0.046), authentic altersolanol B $[\alpha]_D^{26}$ −57±10° (*c* 0.043); λ_{max} (EtOH) 266.5 (ε 14,620), 287.5 (9,800), 424 nm (3,420); m.s. *m/e* 306 (M+2H, *cf.* APLIN and PIKE[17]), 304 (M^+), 286 ($M-H_2O$, m* 269.0), 275.0915 (M−CHO, m* 248.8, $C_{15}H_{15}O_5$ requires 275.0919), 268 ($M-2H_2O$), 233.0811 (275−C_2H_2O, m* 197.4, $C_{13}H_{13}O_4$ requires 233.0814), 231.0660 ($M-C_3H_5O_2$, $C_{13}H_{11}O_4$ requires 231.0657), 203 (231−CO, m* 178.4). Electronic, mass, and p.m.r. spectra were identical with those of authentic altersolanol B.

Altersolanol A (**5**)

The t.l.c. purified material was recrystallised twice from dioxane and dried at 60°C/2 mm for 2 days. Its melting process was complex: melting and sublimation commenced at 192°C, the bulk melted at 203~204°C, sublimed material melted at 240°C, with some reformed crystals still remaining at 300°C. Authentic altersolanol A, in our hands, showed identical behaviour, either alone or admixed with the present isolate (lit. records m.p. 218~218.5°C[7], 218°C (dec.)[8], 218~220°C[15]). $[\alpha]_D^{26}$ −200±10° (*c* 0.063), authentic altersolanol A $[\alpha]_D^{26}$ −185±10° (*c* 0.053) [lit. records −292° (*c* 0.25, pyridine)[7,8], −154° (*c* 0.071, EtOH)[15]]; λ_{max} (EtOH) 269 (ε 14,540), 427 nm (4,670); m.s. *m/e* 338 (M+2H, *cf.* APLIN and PIKE[17]), 336 (M^+), 320 ($M+2H-H_2O$), 318 ($M-H_2O$), 300 ($318-H_2O$, m* 283.0), 284, 262, 234; p.m.r. δ (CD_3CO_2D) 7.17 (d, *J* 2.5 Hz, H-C8), 6.69 (d, *J* 2.5 Hz, H-C6), 4.92 (d, *J* 7.5 Hz, H-C4), 4.65 (s, H-C1), 4.06 (d, *J* 7.5 Hz, H-C3), 3.90 (s, CH_3O), 1.51 (s, CH_3C). Electronic, mass and p.m.r. spectra were identical with those of authentic altersolanol A.

Dactylariol (**6**)

Dactylariol purified by t.l.c. was recrystallised from ethanol as red prisms, which were dried at 35°C/2 mm for 3 days. Its melting process was complex: the bulk melted at 219~223°C, with partial change of crystal structure at 190°C to needles which finally melted at 304~306°C. $[\alpha]_D^{26}$ −33±10° (*c* 0.032); λ_{max} (EtOH) 269 (ε 15,110), 286 (infl., 9,270), 423 nm (3,605); m.s. *m/e* 322 (M+2H, *cf.* APLIN and PIKE[17]), 320.0890 (M^+, $C_{16}H_{16}O_7$ requires 320.0896), 302 ($M-H_2O$, m* 285.0), 284.0691 ($M-2H_2O$, $C_{16}H_{12}O_5$ requires 284.0685), 268, 259, 247.0604 ($M-C_3H_5O_2$, $C_{13}H_{11}O_5$ requires 247.0607), 234, 231, 219 (247−CO, m* 194.2); p.m.r. δ (CD_3CO_2D) 7.16 (d, *J* 2 Hz, H-C8), 6.68 (d, *J* 2 Hz, H-C6), 4.74 (bs, H-C1), 4.12 (dd, *J* 6 and 9 Hz, H-C3), 3.88 (s, CH_3O), 3.08 (dd, *J* 6 and 20 Hz, H-C4), 2.59 (dd, *J* 9 and 20 Hz, H-C4), 1.47 (s, CH_3C); p.m.r. δ (CD_3SOCD_3+

CD_3CO_2D) 7.06 (d, *J* 2 Hz, H-C8), 6.79 (d, *J* 2 Hz, H-C6), 4.38 (s, H-C1), 3.90 (s, CH_3O), 3.79 (dd, *J* 6 and 9 Hz, H-C3), 2.86 (dd, *J* 6 and 20 Hz, H-C4), ~2.3 (dd, *J* 9 and 20 Hz, H-C4 partly obscured by solvent), 1.32 (s, CH_3C).

Anal.: Calcd. for $C_{16}H_{16}O_7$, C, 60.00, H, 5.04; Found, C, 60.09, H, 4.81.

Acknowledgements

We are indebted to Dr. A. STOESSL, Canada Department of Agriculture, London, Ontario, for kindly providing authentic samples of macrosporin and altersolanols A and B. We thank Mrs. M. G. ANDERSON and Mrs. J. ROTHSCHILD for microbiological work. Mass spectra were recorded by Mr. M. CHAPMAN, p.m.r. spectra by Mr. C. ARANDJELOVIC and Mr. B. PAAL. Microanalysis was carried out by the A.N.U. Analytical Service Unit.

References

1) DUDDINGTON, C. L.: Predacious fungi and nematodes. Experientia 18: 537~592, 1962
2) NEMEC, P.; L. KRIŽKOVÁ, J. BALAN, J. BALANOVÁ & M. KUTKOVÁ: Incidence of antiprotozoal and antivermal antibiotics in fungi. I. Class Fungi imperfecti. J. Antibiotics 22: 345~350, 1969
3) KETTNER, M.; P. NEMEC, Š. KOVÁČ & J. BALANOVÁ: Dactylarin, a new antiprotozoal antibiotic from *Dactylaria lutea*. J. Antibiotics 26: 692~696, 1973
4) KETTNER, M.; P. NEMEC, J. BALAN, J. BALANOVÁ, J. DOBIAS & L. KRIZHKOVÁ: Verfahren zur Isolierung eines neuen Antibiotikums mit antiprotozoaler und kanzerostatischer Wirksamkeit. German Patent 2,211,299, Sept. 28, 1972
5) HORÁKOVÁ, K.; J. NAVAROVÁ, P. NEMEC & M. KETTNER: Effect of dactylarin on HeLa cells. J. Antibiotics 27: 408~412, 1974
6) SUEMITSU, R.; M. NAKAJIMA & M. HIURA: Studies on the metabolic products of *Macrosporium porri* ELLIOTT. III. Structure of macrosporin (group II). Bull. Agric. Chem. Soc. Japan 23: 547~551, 1959. IV. Structure of macrosporin (group III). *Ibid.* 25: 100~102, 1961
7) STOESSL, A.: Some metabolites of *Alternaria solani*. Canad. J. Chem. 47: 767~776, 1969
8) STOESSL, A.: Altersolanol A, a novel tetrahydroanthraquinone. J. Chem. Soc., Chem. Commun. 1967: 307~308, 1967
9) TAKENAKA, A.; A. FURUSAKI, T. WATANABE, T. NODA, T. TAKE, T. WATANABE & J. ABE: The crystal and molecular structure of bostrycin *p*-bromobenzoate, a derivative of bostrycin. Tetrahed. Lett. 1968: 6091~6094, 1968
NODA, T.; T. TAKE, T. WATANABE & J. ABE: The structure of bostrycin. Tetrahedron 26: 1339~1346, 1970
10) GORDON, M.; A. STOESSL & J. B. STOTHERS: Stereochemistry of some altersolanol B derivatives and their correlation with bostrycin. Canad. J. Chem. 50: 122~124, 1972
11) STOESSL, A.: Relative stereochemistry of altersolanol A. Canad. J. Chem. 47: 777~784, 1969
12) JACKMAN, L. M. & S. STERNHELL: Applications of nuclear magnetic resonance spectroscopy in organic chemistry. 2nd Edition. pp. 280~304, Pergamon Press, Oxford, 1969
13) CHAPMAN, O. L. & R. W. KING: Classification of alcohols by nuclear magnetic resonance spectroscopy. J. Am. Chem. Soc. 86: 1256~1258, 1964
14) NAKAJIMA, S.: Studies on the metabolites of phytotoxic fungi. I. Isolation of macrosporin and 6-methylxanthopurpurin 3-methyl ether from *Alternaria bataticola* IKATA *et* YAMAMOTO. Chem. Pharm. Bull. 21: 2083~2085, 1973
15) WHEELER, M. M.; D. M. S. WHEELER & G. W. PETERSON: Anthraquinone pigments from the phytopathogen *Phomopsis juniperovora* HAHN. Phytochemistry 14: 288~289, 1975
16) SUEMITSU, R.; Y. MATSUI & M. HIURA: Metabolic products of *Macrosporium porri*. I. Isolation and general properties of macrosporin, a new pigment. Bull. Agric. Chem. Soc. Japan 21: 1~4, 1957
17) APLIN, R. T. & W. T. PIKE: Mass spectra of 1,4-dihydroxybenzenes and *p*-benzoquinones. Quinol-quinone interconversion in the heated inlet system of a mass spectrometer. Chem. Ind. (London) 1966: 2009, 1966