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STRUCTURE OF ANTIFUNGAL AND PHYTOTOXIC PIGMENTS PRODUCED BY ALTERNARIA SPS.

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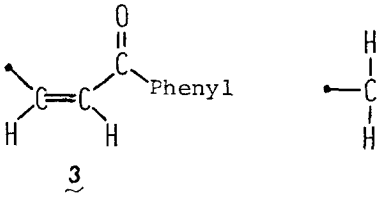
Abstract: New antifungal hydroxyhydroperylenediones have been isolated from Alternaria sps. and their structures have been shown to be 1 and 2.

Valsa ceratosperma is the pathogenic fungus responsible for the apple canker disease, which is one of the most harmful epidemics for apple trees in North Japan. In the course of studies on antibiotic substances against this fungus, we became interested in coloring matters produced by several unidentified strains of Alternaria sps.. In this paper we wish to report the structural determination of two new pigments alterperylenol 1 and dihydroalterperylenol 2, which at the concentration of 200 μg/ml show antifungal activity against V. ceratosperma and phytotoxicity of the growth inhibition of lettuce seedlings. The two reddish orange pigments were isolated by chloroform extraction of still culture broth and mycelia of the fungus. The crude extracts were chromatographed on silica gel and red oily fractions were re-chromatographed(silica gel, $CHCl_3$-EtOH) to give two kinds of crude crystals. Recrystallization from MeOH-$CHCl_3$ gave pure pigments. 1: mp 182-185°C; $[\alpha]_D^{26}$ +699° (c=0.26 acetone); mass(m/z) 350.0746(M^+, calcd for $C_{20}H_{14}O_6$, 350.0790), 332(M^+-H_2O), 314($M^+-2\times H_2O$); IR(ν_{max}^{nujol}) 3550-3150, 1658, 1603, 1225, 1030, 840 cm^{-1}; UV(λ_{max}^{MeOH}) 230, 252, 290, 380 nm; ^{1}H-NMR(400 MHz, DMSO-d_6, δ) 2.89(1H, dd, J=16.0, 11.7 Hz, A part of ABCX) and 2.97(1H, dd, J=16.0, 6.3 Hz, B part of ABCX), 3.10(1H, dd, J=9.7, 1.7 Hz, C part of ABCX), 4.50(1H, m, X part of ABCX) 5.73(1H, d, J=6.3 Hz, OH), 5.76(1H, d, J=1.7 Hz, OH), 6.35(1H, d, J=10.5 Hz), 6.96(1H, d, J=8.8 Hz), 7.06(1H, d, J=8.8 Hz), 7.89(1H, d, J=10.5 Hz), 8.02(1H, d, J=8.8 Hz), 8.08(1H, d, J=8.8 Hz), 12.36(1H, s), 12.72(1H, s); ^{13}C-NMR(25 MHz $CDCl_3$) Table 1. 2: mp 147-150°C; $[\alpha]_D^{26}$+380° (c=0.20 acetone); mass(m/z) 352.0968(M^+, calcd for $C_{20}H_{16}O_6$, 352.0946), 334(M^+-H_2O), 316($M^+-2\times H_2O$), 314($M^+-2\times H_2O$-2H); IR(ν_{max}^{nujol}) 3400(broad), 1630, 1235, 1060, 820 cm^{-1}; UV(λ_{max}^{MeOH}) 213, 257, 285, 355 nm; ^{1}H-NMR(400 MHz, $CDCl_3$, δ) 2.43(1H, dt, J=13.0, 3.0 Hz, A part of ABCD), 2.65(1H, dt, J=15.0, 3.0 Hz, C part of ABCD), 2.94(1H, dd, J=16.0, 11.7 Hz), 3.07(1H, dd, J=16.0, 5.6 Hz), 3.09(1H, d, J=8.8 Hz), 3.17(2H, m, BD part of ABCD), 4.78(1H, oct, J=11.7, 8.8, 5.1 Hz), 6.92(1H, d, J=8.8 Hz), 7.01 (1H, d, J=8.8 Hz), 7.82(1H, d, J=8.8 Hz), 7.87(1H, d, J=8.8 Hz), 12.37(1H, s), 12.74(1H, s). ^{13}C-NMR(15 MHz, acetone-d_6) 34.5(t), 36.0(t), 48.4(t), 52.8(d),

66.3(d), 69.5(s), 113.6(s), 116.8(d), 117.8(s), 119.0(d), 124.5(s), 125.8(s), 133.4(d), 138.6(s), 141.4(s), 162.3(s), 162.7(s), 204.6(s).

The ^{1}H-NMR and ^{13}C-NMR spectra of 1 showed the presence of one carbonyl, one α,β-unsaturated carbonyl, one secondary hydroxyl, one tertiary hydroxyl, one methylene, and one methine group and two benzene rings, each of which bears two protons, a hydroxyl and a carbonyl group. Decoupling experiments revealed that the two aromatic protons forms an AB system in both benzene rings, and their values indicated that proton A is o or p to the aromatic hydroxyl group and proton B is placed at an o or p position of the carbonyl group. The signals at δ 5.73, 5.76, 12.36 and 12.72 were attributable to hydroxyl protons because they disappeared by addition of D_2O. The last two signals should be due to the phenolic hydroxyl groups with a strong intramolecular hydrogen bond. The signals at δ 6.35 and 7.89 were assigned to olefinic protons in an α,β-unsaturated carbonyl system 3. Further decoupling experiments revealed also the interrelation of the methylene, methine and secondary hydroxyl groups(ABCX, δ 2.89, 2.97, 3.10 and 4.50) and accordingly partial structure 4 was inferred. Taking into consideration all the above facts, formula 1 was suggested for alterperylenol. In order to confirm the structure 1, an X ray crystallographic study was carried out.



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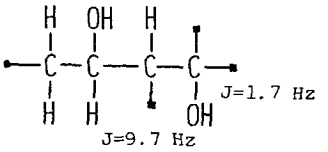

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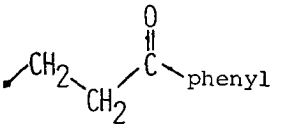

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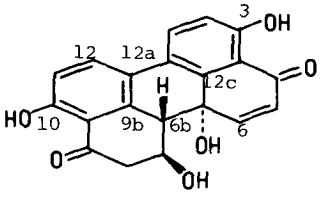
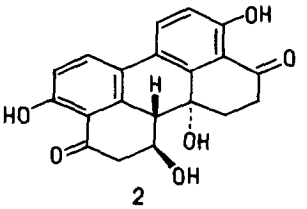


1

(6aR,6bS,7S)-3,6a,7,10-Tetrahydroxy-4,9-dioxo-4,6a,6b,7,8,9-hexahydroperylene



2

3,6a,7,10-Tetrahydroxy-4,9-dioxo-4,5,6,6a,6b,7,8,9-octahydroperylene

The crystal data for the methanol solvate of alterperylenol were as follows: $C_{20}H_{14}O_6$ CH_3OH, orthorhombic, space group $P2_12_12_1$, a=11.466(8), b=20.926(8), c=7.037(3) Å, Z=4, D_c=1.504 g cm^{-3}. The intensities of 1715 independent reflections with $2\theta<135°$ were collected on an automated fourcircle diffractometer with Ni-filtered Cu K radiation. The structure was solved by the Monte Carlo direct method,[2] using the 30 strongest reflections as the starting set. The 51st random phase set led to the correct solution; an E-map based on 412 phases revealed the locations of all the non-hydrogen atoms. The structure obtained was refined by the block-diagonal least-squares method with

anisotropic temperature factors. After all the hydrogen atoms had been located in a difference Fourier map, further least-squares refinements were carried out including the hydrogen atoms.[3] The final R value was 0.057. The absolute configuration was determined by using the anomalous dispersion of oxygen and carbon atoms for Cu K radiation.[4] For the application of the Bijoet method,[5] 20 sets of reflections with large values of $\|F_c(hkl)| - |F_c(\bar{h}\bar{k}\bar{l})\| / \sigma(F_o)$ were selected. The results are summarized in Table 2. The signs of the corresponding F_o and F_c values are in agreement with each other for all the 20 sets of reflections. Thus, the complete structure of alterperylenol including the absolute configuration has been established as shown in Fig. 1.

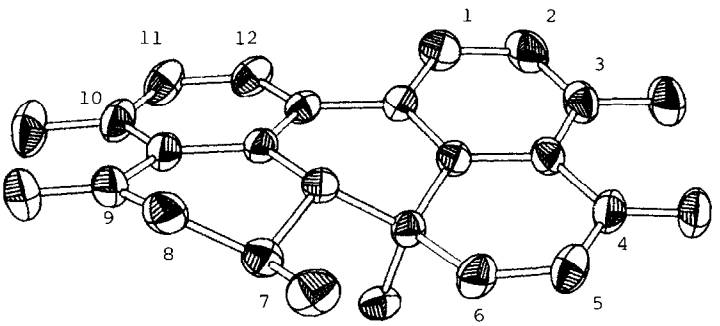
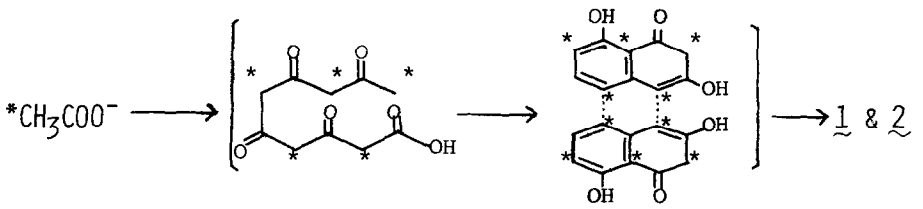


Fig.1. Perspective drawing with hydrogens omitted.

The structure of 2 was determined by comparison of its spectral properties with those of 1. In the ^1H spectrum, no olefinic proton signals were observed and instead signals due to four methylene protons newly appeared at δ 2.43, 2.65, 2.94 and 3.07. Decoupling experiments disclosed that these methylene groups were adjacent to each other and their values indicated they were contained in 5. Therefore pigment 2 was formulated as dihydro compound of 1. The ^{13}C spectrum also supported the above conclusion. The mass spectrum provided an additional proof for formula 2, since fragmentation patterns of both compounds were quite similar and in particular the peak due to the 3,10-dihydroxy-4,9-perylenequinone ion was observed at m/z 314 in both spectra. These new, partly reduced perylene derivatives were very unstable and changed gradually into dark purple amorphous powder insoluble in conventional solvents.

These pigments seemed to be biosynthetically formed by oxdative coupling of two molecules of a tetralone derivatives, which in turn was synthesized from a pentaketide derivative in vivo.[6] In order to confirm these hypothesis, incorporation experiment of 2-^{13}C labelled sodium acetate in to 1 was carried out. The ^{13}C spectrum of the labelled 1 was measured to investigate the labelling pattern. The enhancement of the peaks as compared with those of the unlabelled 1 was summerized in Table 1. Unequivocal enhancement of ten carbon signals were observed for the expected carbons and it was therefore concluded that 1 was a dimerized product of an octalone derivative. A possible

biosynthetic route may be depicted as shown in Scheme 1.



Scheme 1. A possible biosynthetic path way of 1 and 2.

Table 1. ^{13}C-NMR spectral data of 1 and [2-^{13}C]acetate derived 1.

a)	b)	c)	a)	b)	c)
204.2 s	C-9	0.60	125.3 s,d	C-5 and C-12a or C-12b	2.53*
191.3 s	C-4	0.96			
162.5 s	C-3 or C-10	1.10	118.4 s	C-2 or C-11	2.38*
161.7 s	C-3 or C-10	0.89	117.6 d	C-9a or C-3a	2.14*
153.4 d	C-6	1.00	116.8 d	C-2 or C-11	2.67*
140.7 s	C-9b or C-12c	1.12	113.5 s	C-9a or C-3a	2.50*
138.1 s	C-9b or C-12c	1.00	67.2 s	C-6a	1.89*
132.6 d	C-1 or C-12	0.98	65.9 d	C-7	0.90
132.3 d	C-1 or C-12	1.00	52.0 d	C-6b	2.33*
126.4 s	C-12a or C-12b	2.04*[d]	48.0 t	C-8	2.36*

a) Chemical shift and multiplicity. b) Assignment. c) Relative intensity. The values were calculated from the labelled and unlabelled spectra measured under NNE mode.

d) *Carbons anticipated to be derived from [2-^{13}C]acetate.

Table 2. Bijvoet inequalities

h k l	F_o[a)]	F_c[a)]	ΔF_o[b)]	ΔF_c[b)]	h k l	$\bar{F}_o$	$\bar{F}_c$	ΔF_o	ΔF_c
4 5 1	18.00	16.79	-0.28(2)	-0.25	8 1 3	10.67	10.67	-0.28(4)	-0.20
2 2 3	18.83	20.29	-0.16(2)	-0.20	1 7 3	35.49	35.20	+0.19(3)	+0.11
2 4 1	16.36	16.23	-0.11(2)	-0.16	1 1 1	16.02	14.83	+0.15(2)	+0.06
2 1 3	23.02	23.69	-0.26(2)	-0.19	3 1 3	23.12	22.01	+0.12(3)	+0.10
110 2	37.01	35.81	-0.09(3)	-0.19	416 4	17.57	17.62	+0.22(4)	+0.15
2 6 1	12 31	12.01	-0.23(2)	-0.15	7 4 2	12.31	12.63	+0.18(4)	+0.14
9 1 1	33.22	32.90	-0.18(3)	-0.20	2 2 2	54.21	56.48	+0.20(2)	+0.07
3 6 1	13.64	14.09	-0.15(2)	-0.13	1 6 1	41.54	44.40	+0.16(2)	+0.07
5 2 4	27.05	26.86	+0.04(3)	+0.16	2 3 1	40.68	38.39	+0.12(2)	+0.06
3 5 3	28.59	28.17	-0.19(3)	-0.13	414 2	39.14	39.69	+0.33(3)	+0.12

a) $\bar{F} = \{|F(hkl)| + |F(h\bar{k}l)| + |F(\bar{h}kl)| + |F(\bar{h}\bar{k}l)| + |F(\bar{h}k\bar{l})| + |F(h\bar{k}\bar{l})| + |F(\bar{h}\bar{k}\bar{l})| + |F(hk\bar{l})|\}/8$

b) $\Delta F = \{|F(hkl)| + |F(h\bar{k}\bar{l})| + |F(\bar{h}k\bar{l})| + |F(\bar{h}\bar{k}l)| - |F(h\bar{k}l)| - |F(\bar{h}kl)| - |F(\bar{h}\bar{k}\bar{l})| - |F(hk\bar{l})|\}/4$

References and Notes

1) The intensity measurement was made at the High Brilliance X-Ray Diffraction Laboratory of Hokkaido University.

2) A. Furusaki, Acta Crystallogr., Sect. A, 35, 220 (1979).

3) The tables of the atomic coordinates and bond distances have been deposited with the Cambridge Crystallographic Data Centre.

4) International Tables for X-Ray Crystallography, Vol. IV, p. 149, The Kynoch Press, Birmingham, England (1974).

5) J. M. Bijvoet, A. F. Peerdeman, and A. J. van Bommel, Nature, 168, 271(1951)

6) A. Okubo, S. Yamasaki and K. Fuwa, Agr. Biol. Chem., 39 1173 (1975).

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