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PERYLENEQUINONES FROM CUCUMBER SEEDLINGS INFECTED WITH *CLADOSPORIUM CUCUMERINUM**

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Key Word Index—*Cladosporium cucumerinum*; Deuteromycetes; perylenequinones; structural elucidation; stereochemistry.

Abstract—The structure of cladochrome A, a perylenequinone pigment isolated from etiolated cucumber seedlings infected with fungal spores of *Cladosporium cucumerinum*, has been revised and established as that of a diester of 3-hydroxybutyric acid with *ent*-isophleichrome. Another pigment from the same source, cladochrome B, is the corresponding ester of *ent*-isophleichrome with 3-hydroxybutyric and benzoic acids.

INTRODUCTION

Cladosporium cucumerinum Ell. et Arth. is a fungus responsible for an economically important scab disease of many Cucurbitaceae plants. The ultrastructure of the damaged cells and the mechanism of the resistance of cucumbers have been investigated [1, 2].

In 1967 Overeem *et al.* [3] found that a wine-red discolouration occurred on cotyledons and hypocotyls of susceptible etiolated cucumber seedlings four to five days after infection of the seedlings with *C. cucumerinum* spores. The same Authors recognized at least five pigments responsible for this typical pigmentation. The major pigment, although only present in small amounts due to the particular conditions of production, was named cladochrome A and was partially characterized as a perylenequinone derivative having a close relationship with the elsinochromes [4].

More recently, other perylenequinones of the same class (cercosporins and phleichromes and their analogues, hypocrellins) have been isolated from fungal cultures and their structures have been elucidated. [5, 6]. Compounds of this class are forced into an helical shape by the steric constraints of the side chains, with the consequence that they possess axial chirality and strong optical rotation. If the side chains contain asymmetric carbons, the existence of diastereoisomers with opposite helicity is possible. So far fungi have been reported to produce only one of the two possible diastereoisomers (see ref. [6] for a discussion of these aspects and of their biosynthetic relevance).

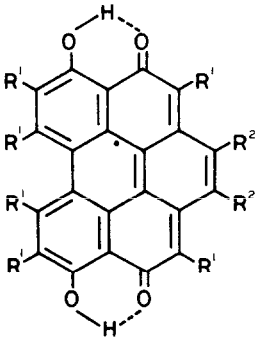
Interest in the biological activity of these perylenequinones has arisen as they act as fungal toxins causing disruption of membranes and enzyme inactivation through photo-sensitized formation of toxic singlet oxygen and therefore of photooxidation products [7].

Cladochromes are peculiar, as they are produced by the fungus only when it grows on its host. After unsuccessful attempts to grow the fungus on various media, we cannot but confirm the results of Overeem *et al.* [3] in this respect, the reasons for this behaviour remaining unknown.

RESULTS AND DISCUSSION

Two pure pigments (**1** and **2**) were isolated in small amounts from infected seedlings. We believe that **1** is identical with cladochrome A [3] for the reasons outlined below, and **2** we have named cladochrome B.

High resolution mass spectroscopy indicates a molecular formula for **1** of $C_{38}H_{42}O_{14}$, which is consistent with the microanalytical data for cladochrome A of Overeem *et al.* [3], who were however forced to suppose that the compound was the dihydrate of $C_{38}H_{38}O_{12}$, in order to satisfy their interpretation of NMR and IR data, which led to the structural proposal A.



R¹ = 4 OMe + 2H
R² = CHMe – $COCH_2$ – CHOH – Me

A

*Part 20 in the Series, 'Secondary Mould Metabolites'. For part 19 see Arnone, A., Cardillo, R., Nasini, G. and Meille, S. V. (1988) *J. Chem. Soc. Perkin Trans. I* (in press).

A comparison of the ^{1}H NMR chemical shifts of **1** with those reported for Cladochrome A [3] shows that they are very similar, except for the presence in **1** of two additional high-field methylene protons resonating at δ1.38 and 0.67. Moreover, it must be noted that the decoupling experiments previously reported for Cladochrome A [3] can be rationalized in terms of the new structure (**1**) if the multiplets resonating at δ4.85 and 3.04 and attributed to CH_2–CH OH–Me and >CH–Me methine protons are more properly assigned to CH_2–CH (OCOR)Me and CH_2–CH OH–Me methine protons (see below).

All these pieces of evidence strongly suggest that **1** is the same compound isolated by Overeem *et al.* [3], although a direct comparison with the original sample was not possible due to depletion of the stock of Cladochrome A (Overeem, J. C. personal communication, 1987).

The structure of Cladochrome A (**1**) and B (**2**) follow from a comparison of their ^{1}H NMR data with those of the previously elucidated isophleichrome [8], which suggests that they contain a 4,9-dihydroxyperylene-3,10-quinone nucleus and differ in the nature of the substituents at C-14 and C-21. Specifically, the downfield shift exhibited by the carbinol methine protons H-14 and H-21 in cladochrome A and B with respect to the corresponding protons in isophleichrome ($\Delta\delta$ 1.11–1.30 ppm, see later) can be explained by assuming that the geminal 14 and 21 hydroxy groups are esterified. This hypothesis, also supported by the presence of other signals at high field (Table 1), was confirmed by mild hydrolysis of

Table 1. ^{1}H NMR chemical shifts for compounds **1**–**3** in $CDCl_3$

Proton	1	2		3
5, 8	6.61	6.50[a]	6.32[a]	6.44
13a, 20a	3.51	3.57,	3.61	3.54
13b, 20b	2.91	2.95,	3.13	2.93
14, 21	4.85	4.85,	5.04	3.74
15, 22	1.10	1.10,	1.28	0.95
17a, 24a	1.38	1.34		
17b, 24b	0.67	0.62		
18, 25	3.04	2.99,	6.87	
19, 26	0.68	0.64,	6.93	
27			7.26	
28			6.93	
29			6.87	
4-, 9-OH	15.69	15.79[b],	15.74[b]	15.85
14-, 21-OH				
18-, 25-OH	1.60	1.80		
2-, 11-OMe	4.28	4.30[c],	4.31[c]	4.22
6-, 7-OMe	4.05	3.96[d],	3.88[d]	3.99

[a–d]Values may be interchanged.

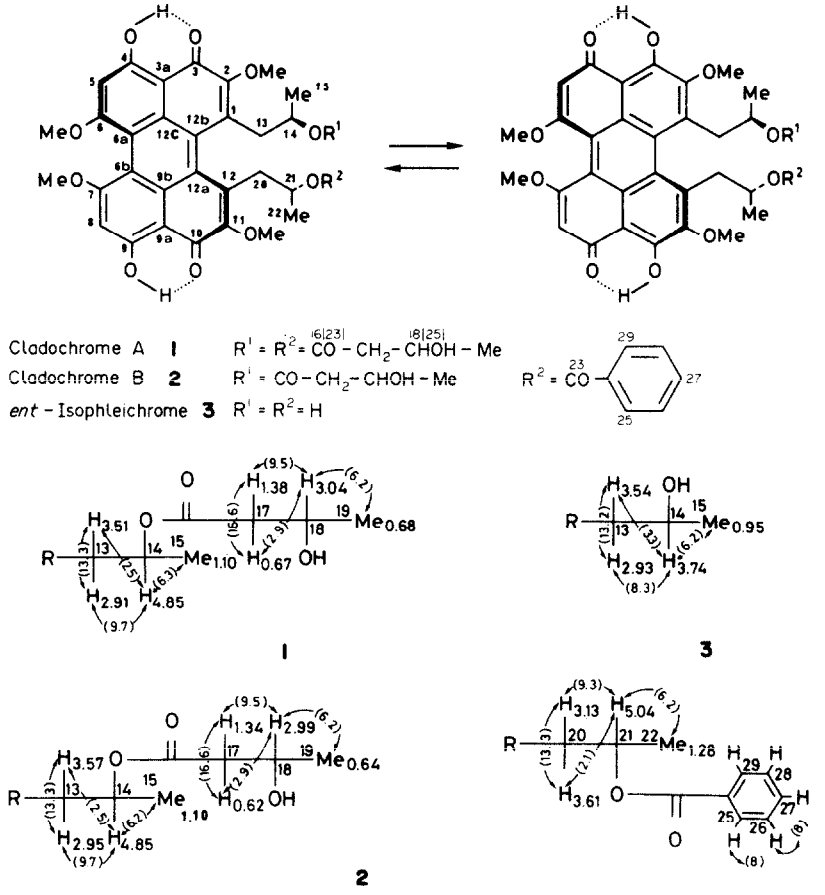


Fig. 1. Chemical shifts (δ) and J (Hz) of the protons of the side-chains of cladochrome A (**1**) and B (**2**), and *ent*-isophleichrome (**3**).

cladochrome A and B with diluted NaOH, which gave, in each case, a compound (**3**), indistinguishable from isophleichrome by chromatography and NMR.

However, the circular dichroism (CD) spectra of **1**–**3** show absorption peaks opposite in value to those of isophleichrome [8]. This fact indicates that both metabolites **1** and **2** contain the *ent*-isophleichrome (**3**) nucleus, having *S* axial chirality and *R* configuration at C-14 and C-21, and differ only in the RCOO-moieties, the molecular formulae of which are $C_8H_{14}O_6$ for cladochrome A and $C_{11}H_{12}O_5$ for cladochrome B ($C_{41}H_{40}O_{13}$ from mass spectra).

The presence of only half of the expected resonance signals in both ^1H and ^{13}C NMR spectra (Table 1) is indicative of a symmetrical structure for cladochrome A (**1**), which must have, like *ent*-isophleichrome (**3**), a C_2 symmetry axis.

The assignment of the structure of the two identical ester chains as 3-hydroxybutyrate moieties followed from coupling constant analysis, the results of which are reported in Fig. 1. The anomalous high-field position exhibited by 17- and 24-methylene protons (δ1.38 and 0.67 ppm) α to a carbonyl group, and by 19- and 26-methyl protons (δ0.68 ppm) can be attributed to the shielding effect exerted by the ring current of the perylenequinone nucleus if the 14- and 21-OR groupings are oriented inward,* so as to be positioned over the five-ring system, as happens in *ent*-isophleichrome [8] and other compounds of this class [6].

The different downfield shift experienced by H-14 and H-21 ($\Delta\delta$ 1.11 and 1.30 ppm) in the ^1H NMR spectrum of cladochrome B (**2**) when compared with the corresponding protons in *ent*-isophleichrome (**3**) (see Fig. 1) indicates that the 14- and 21-OH groups are esterified by two different acids.

The structures of these compounds, *viz.* 3-hydroxybutyric acid and benzoic acids, were readily determined on the basis of the mass spectral data and of the appropriate ^1H NMR data reported in Fig. 1. The high-field position displayed by 17-methylene, 19-methyl, and phenyl protons (in methyl benzoate the corresponding protons resonate *ca* 0.2–1.1 ppm downfield [9]) has to be attributed to the same effect described above for cladochrome A.

All these findings indicate that cladochrome A (**1**) is a diester of 3-hydroxybutyric acid with *ent*-isophleichrome (**3**) and that cladochrome B (**2**)† differs from (**1**) in that the two acid residues are those of 3-hydroxybutyric and of benzoic acid. Similar unsymmetrical esters are produced also in the case of cercosporin [10, 11].

Lack of material prevented the assignment of the absolute configuration of the asymmetric carbon atoms of the 3-hydroxybutyrate moieties. Attempts to convert cladochrome A into the diastereoisomer with opposite helicity by refluxing in xylene were unsuccessful.

*A detailed discussion of the keto–enol tautomerism, and of the preferred conformations of the side-chain groups in phleichromes and their analogues will appear in a forthcoming paper.

†Due to the presence of a C_2 symmetry axis in *ent*-isophleichrome, the interchange of the two side chains betwen position C-1 and C-12 affords the same molecule.

EXPERIMENTAL

Mps: uncorr. UV: 95% EtOH; flash chromatography: Merck silica gel (0.040–0.063 mm); TLC: Merck HF_{254} silica gel; MS: 70 eV; ^1H (300.13 MHz) and ^{13}C (75.47) NMR: TMS as int. standard.

Isolation and purification of the metabolites **1** *and* **2**. The methodology for production and purification of cladochromes given in ref. [3] has been improved and simplified. Cucumber seeds (cv. 'Bianco lungo di Parigi', Ingegnoli no. 121, 40 g) were washed in tap H_2O for 3 hr and sown in a plastic tank (55 × 49 × 19 cm) on a water-soaked cotton layer. The tank, protected with a black plastic film, was kept at 26° in the dark until etiolated seedlings reached 6 cm height. The seedlings were cut and placed in Petri dishes (23 × 23 cm) over a plastic net on moist filter paper. They were then infected with a suspension (20 ml) of spores (8×10^9 spores/ml) of the pathogenic strain *Cladosporium cucumerinum* CBS 109.08 (reisolated by us from infected cucumbers) and the dishes were placed in the dark at 26°. After four days the seedlings showed the typical red pigmentation and were dried *in vacuo* at 40° and extracted in a Soxhlet apparatus first with hexane and then with EtOAc. The EtOAc extract was dried with Na_2SO_4 and evapd *in vacuo* at 40°. The residue was dissolved in CH_2Cl_2 (5 ml) and hexane (20 ml) was added; the ppt. (20 mg) was collected and chromatographed on silica gel (added with 2% KH_2PO_4) using CH_2Cl_2–MeOH (30:1) as eluent to give Cladochrome B (**2**) (3 mg), and CH_2Cl_2–MeOH (15:1) as eluent to give Cladochrome A (**1**, 8 mg). Both metabolites were purified by precipitation with hexane from $CHCl_3$ solns.

The same methodology was used with *C. cucumerinum* on *Citrullus lanatus* (cv. 'Sugar Baby' and 'Crimson Sweet'), *Cucumis melo* (cv. 'Napoletano a buccia gialla' and 'Napoletano a buccia verde') and *Cucurbita pepo* (cv. 'Bianca della Virginia' and Faentina'). From melon and watermelon but not zucchini, red metabolites with the same R_f of cladochromes were observed.

Cladochrome A (**1**) had mp 95–100°; UV λ_{max} nm: 200, 220 sh, 265, 320 sh, 470, 535, and 580 (ε 39500, 35500, 22450, 4600, 15100, 8400, and 7800); CD (EtOH, *c* 0.028 nm). 289, 355, 442, 542, and 580 ($\Delta\varepsilon$ 23.0, −5.4, 7.7, −3.1, and −4.2); IR ν_{max}^{nujol} cm^{-1}: 1730 (OCOR) and 1610 (conj. CO); EIMS *m/z*: 722 $[M]^+$, 704 $[M-18]^+$, 678, and 634; $[M]^+$, 722.260 ± 0.004. $C_{38}H_{42}O_{14}$ requires $[M]^+$, 722.256. ^1H NMR data are reported in Table 1 and Fig. 1. ^{13}C NMR (75.47 MHz, $CDCl_3$): δ177.89, 175.34, and 171.19 (*s*, C-3, C-10, C-4, C-9, C-16, and C-23), 166.34 (*s*, C-6 and C-7), 152.17 (*s*, C-2 and C-11), 133.95, 128.48, and 126.22 (*s*, C-1, C-12, C-9b, C-12c, C-12a, and C-12b), 115.60 (*s*, C-6a and C-6b), 106.06 (*s*, C-3a and C-9a), 101.12 (*d*, C-5 and C-8), 71.63 and 63.50 (*d*, C-14, C-21, C-18, and C-25), 61.15 (*q*, OMe-2 and OMe-11), 56.32 (*q*, OMe-6 and OMe-7), 42.00 and 38.79 (*t*, C-13, C-20, C-18, and C-24), 21.89 and 20.77 (*q*, C-15, C-22, C-19, and C-26).

Cladochrome B (**2**) had mp 85–90°; UV λ_{max} nm: 200, 218sh, 255, 470, 535 sh, and 585 (ε 49000, 37500, 21250, 9000, 5500, and 4500); CD (EtOH; *c* 0.02) nm: 285, 352, 440, 544, and 580 ($\Delta\varepsilon$ 28, −8.5, 14, −4.4, and −7.4); IR ν_{max}^{nujol} cm^{-1}: 1710, (OCOR and OCOAr, *br*) and 1610 (conj. CO); EIMS *m/z*: 740 $[M]^+$, 722 $[M-18]^+$, 696 and 679; M^+, 740.244 ± 0.004. $C_{41}H_{40}O_{13}$ requires 740.247. ^1H NMR data are reported in Table 1 and Fig. 1.

Hydrolysis of the esters **1** *and* **2**. Cladochrome A (**1**) and B (**2**) (3 mg each) were treated with 0.5 N NaOH (0.2 ml) at 60° for 15 min. Acidification, extraction with CH_2Cl_2 and pptn with hexane from CH_2Cl_2 solns of the residue gave in both cases *ent*-isophleichrome (**3**), mp 208–210 (indistinguishable from isophleichrome [8] on TLC (benzene–Et_2O–HCOOH 50:50:1,

IH NMR and MS spectra); CD (EtOH; c 0.022) nm: 294, 360, 450, 540, and 580 (Ae 36, -7.4, 18, -5.2, -8.3); EIMS *re~z:*550 [-M] +. 1HNMR data are reported in Table 1 and Fig. 1. *ent-*Isophleichrome (3, 3 mg), dissolved in xylene (1 ml), was refluxed for 0.5hr, the conversion into the isomeric *ent-phleichrome* being monitored by TLC [-8]. No isomerization was observed when solns of the ester I were refluxed for 2hr in the same solvent.

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