Pigments of fungi. Part 50.[1] Structure, biosynthesis and stereochemistry of new dimeric dihydroanthracenones of the phlegmacin type from *Cortinarius sinapicolor* Cleland

Catherine Elsworth, Melvyn Gill,* Alberto Giménez, Nives M. Milanovic and Evelin Raudies

*School of Chemistry, The University of Melbourne, Parkville, Victoria 3052, Australia*

(3*S*,3′*S*,*P*)-Anhydrophlegmacin-9,10-quinone 8′-*O*-methyl ether **2** and its (2′*S*)-hydroxy derivative **3** are isolated from the bright yellow, glutinous fruiting bodies of the Australian toadstool *Cortinarius sinapicolor* and their structures and absolute stereochemistry are deduced by spectroscopic, chemical and isotopic labelling methods. The biosynthesis of the phlegmacin derivatives **2** and **3** in *C. sinapicolor* has been studied by feeding experiments using sodium [2-^{13}C]acetate, [*Me*-^{13}C]methionine and 6-*O*-[*Me*-^{13}C]-torosachrysone 8-*O*-β-D-gentiobioside **5**.

Introduction

In the preceding part of this series[1] we reported the isolation, structural elucidation and biosynthesis of dermocanarin 4 **1**, a unique naphthoquinone–dihydroanthracenone dimer, from the bright yellow fruiting bodies of the Australian toadstool *Cortinarius sinapicolor*. We report here the isolation from the same fungus of two pigments belonging to the phlegmacin class of coupled dihydroanthracenones,[2] namely (3*S*,3′*S*,*P*)-anhydrophlegmacin-9,10-quinone 8′-*O*-methyl ether **2** and its (2′*S*)-hydroxy derivative **3**. An octaketide biosynthesis for **2** and **3** involving the intermediacy of (*S*)-torosachrysone **4** is established by feeding experiments using sodium [2-^{13}C]acetate and the 8-*O*-β-D-gentiobioside **5** of 6-*O*-[*Me*-^{13}C]-(*S*)-torosachrysone. The absolute stereochemistry of **2** and **3** is deduced from the ^{1}H NMR and CD spectra and confirmed by both chemical degradation of **2** to (*S*)-torosachrysone 8-*O*-methyl ether **6** and the results of a feeding experiment involving **5**.

Results and discussion

Cortinarius sinapicolor is a common species throughout Australia.[3] The toadstools have been beautifully illustrated but erroneously identified as '*Cortinarius ochraceus*' by Fuhrer.[4] For our work, carpophores were collected from mixed *Eucalyptus* forest in the Kinglake National Park, Victoria and were extracted with ethanol either immediately or after storage at −20 °C.† The extractives were distributed between ethyl acetate and water and the organic soluble material was purified by a combination of silica chromatography and gel permeation (Sephadex LH-20) to afford three orange pigments. The most polar zone [R_f 0.10; toluene–ethyl formate–formic acid (50:49:1)] gave dermocanarin 4 **1** (2.83×10^{-2}% of the fresh weight of the fungus) as described previously.[1] Two less polar zones (R_f 0.32 and 0.25) gave the phlegmacinquinones **2** and **3** as orange, optically active powders in yields of 1.35 and 8.33×10^{-3}%, respectively. The mass spectrum of the pigment **2** shows a molecular ion at *m/z* 584, which corresponds by high resolution mass measurement to the molecular formula $C_{33}H_{28}O_{10}$. This was immediately suggestive of a coupled octaketide structure for **2** but the NMR spectroscopic data (Tables 1 and 2) showed clearly that **2** was not a member of the

1

2

3

4 R = H
5 R = gent
6 R = Me

gent =
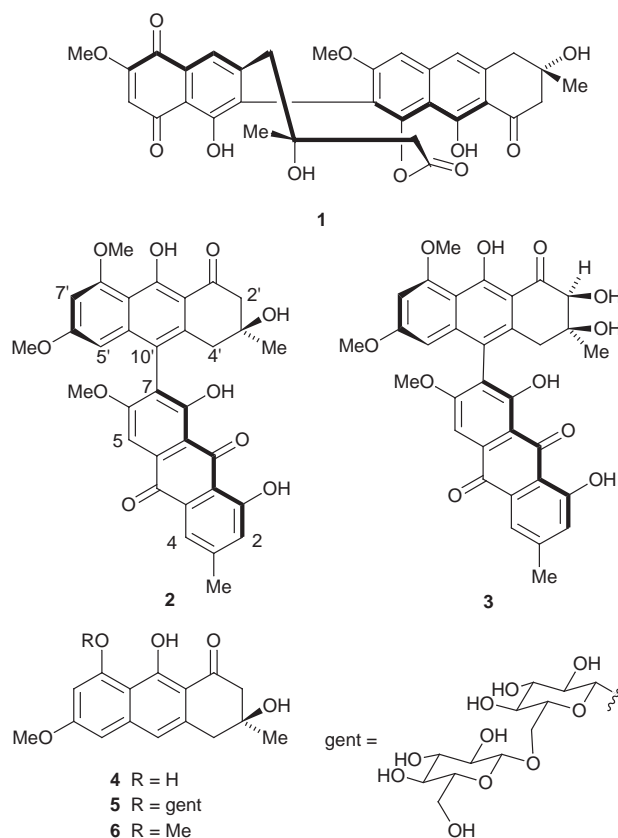
dermocanarin class. Instead, a comparison of the ^{1}H NMR spectrum of **2** with the corresponding data from the anthraquinone physcion **7** and the 8-*O*-methyl ether **6** of the dihydroanthracenone torosachrysone **4** (Table 1) shows that pigment **2** is a composite of the subunits **A** and **B** (Fig. 1) connected *via* a biaryl bond between C-7 in the the quinone **7** and C-10′ in the dihydroanthracenone **6**. Thus, the spectrum of **2** contains signals from three hydrogen bonded phenolic hydroxy groups, two methylene groups, two pairs of *meta* coupled and one isolated aromatic proton(s) together with one aliphatic and one aromatic C-methyl group. Only the signal from the exchangeable proton of the tertiary hydroxy group is absent. Nevertheless, its presence in **2** is clear from the ^{13}C NMR spectrum (Table 2) in which C-3′ appears as a singlet at δ 70.5. The 7–10′ (phlegmacin[2]) connectivity between the fragments **A** and **B**

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† Voucher specimens of the material examined are lodged in the herbarium of the Royal Botanic Garden, Edinburgh, UK under accession number WAT 24272.

Table 1 ¹H NMR data (CDCl₃, 400 MHz) for the pigments **2** and **3**, physcion **7** and (S)-torosachrysone 8-O-methyl ether **6**

Proton	Chemical shift (δ), multiplicity and coupling constant (J/Hz)			
	2	**3**	**7**	**6**
2-H	7.13 (br s)	7.12 (br s)	7.08 (br s)	—
3-Me	2.49 (s)	2.49 (s)	2.45 (s)	—
4-H	7.70 (br s)	7.70 (br s)	7.64 (br s)	—
5-H	7.60 (s)	7.60 (s)	7.37 (d, 2.6)	—
7-H	—	—	6.69 (d, 2.6)	—
6-OMe	3.90 (s)	3.89 (s)	3.94 (s)	—
1-OH	12.05 (s)	12.05 (s)	12.12 (s)	—
8-OH	12.38 (s)	12.45 (s)	12.32 (s)	—
2′-H$_{ax}$	2.85 (d, 17.1)	4.34 (s)	—	2.82 (d, 16.5)
2′-H$_{eq}$	2.88 (dd, 17.1 and 1.5)		—	2.84 (d, 16.5)
3′-Me	1.33 (s)	1.41 (s)	—	1.42 (s)
4′-H$_{ax}$	2.83 (d, 16.1)	2.93 (d, 17.6)	—	3.06 (2H, d, 16.5)
4′-H$_{eq}$	2.66 (dd, 16.1 and 1.5)	2.73 (d, 17.6)	—	
5′-H	6.12 (d, 2.4)	6.11 (d, 2.4)	—	6.56 (d, 2.2)
7′-H	6.49 (d, 2.4)	6.48 (d, 2.4)	—	6.43 (d, 2.2)
10′-H	—	—	—	6.84 (br s)
6′-OMe	3.66 (s)	3.66 (s)	—	3.91 (s)
8′-OMe	4.01 (s)	4.00 (s)	—	3.98 (s)
9′-OH	15.45 (s)	14.20 (s)	—	15.08 (s)

Table 2 ¹³C NMR data (CDCl₃, 100 MHz) and long-range heteronuclear correlations for compounds **2** and **3**

Position	Chemical shift (δ$_C$), multiplicity^a and coupling constant (J/Hz)		^2J, ^3J{¹³C–¹H}
	2	**3**	
1	162.7, d, 5.9	162.7, d, 5.9	2-H, 1-OH
1-OH	—	—	—
2	124.8, D, 155.8	124.8, D, 160.0	3-Me, 4-H, 1-OH
3	148.9, q, 5.9	148.8, q, 5.9	3-Me
3-Me	22.2, Qt, 127.7, 4.4	22.2, Qt, 127.6, 4.4	2-H, 4-H
4	121.5, Dm, 165.8	121.5, Dm, 167.2	2-H, 3-Me
4a	133.0, s	133.0, s	—
5	103.5, d, 165.8	103.5, d, 167.2	—
6	164.1, m	164.2, m	6-OMe, 5-H
6-OMe	56.7, Q, 145.3	56.8, Q, 145.2	—
7	110.8, t, 5.9	111.2, t, 5.9	5-H, 8-OH
8	162.0, d, 4.4	162.1, t, 5.9	8-OH
8-OH	—	—	—
8a	120.9, t, 5.9	120.5, t, 4.8	5-H, 8-OH
9	191.2, s	191.3, s	—
9a	113.6, t, 5.9	113.6, t, 5.9	2-H, 4-H, 1-OH
10	182.0, t, 4.4	182.1, t, 4.4	4-H, 5-H
10a	135.2, s	135.2, s	5-H
1′	202.1, t, 5.9	200.7, d, 3.0	2′-H
2′	51.7, d, 4.4	77.8, D, 136.5	3′-Me
3′	70.5, m	73.1, m	3′-Me, 4′-H
3′-Me	29.7, Q, 124.7	26.7, Q, 124.7	—
4′	41.7, T, 129.1	38.4, T, 129.1	3′-Me
4a′	135.4, t, 5.9	134.6, t, 5.9	4′-H
5′	97.5, Dd, 159.2, 4.4	97.4, Dd, 159.2, 5.9	7′-H
6′	162.7, m	162.6, m	5′-H, 7′-H, 6′-OMe
6′-OMe	55.2, Q, 145.3	55.2, Q, 145.2	—
7′	97.7, Dd, 159.9, 5.9	97.8, Dd, 159.0, 4.4	5′-H
8′	161.9, m	161.9, m	8′-OMe
8′-OMe	56.4, Q, 145.3	56.4, Q, 145.4	—
8a′	111.2, t, 5.9	110.5, q, 5.9	5′-H, 7′-H, 9′-OH
9′	166.4, d, 4.4	165.3, d, 4.4	9′-OH
9′-OH	—	—	—
9a′	109.4, t, 4.4	108.2, t, 4.4	4′-H, 9′-OH
10′	117.7, d, 4.4	117.8, q, 4.4	5′-H
10a′	140.5, s	141.1, s	—

^{a} Capital letters refer to one-bond multiplicities and lower case letters to two and three bond couplings. All direct couplings were confirmed in the proton coupled spectrum and by the results of NOE, NOESY and HMQC experiments.

that constitute **2** was deduced from the NMR data. Thus, there is no signal in the ¹H NMR spectrum of **2** that can be assigned to a proton at C-10′ [the corresponding proton in the spectrum of torosachrysone **4** appears at δ 6.84] while 5-H in the spectrum of **2** resonates as a singlet (δ 7.60) rather than as a doublet (δ 7.37) as in the spectrum of physcion **7**. The 7–10′ connectivity in **2** is borne out by the ¹³C NMR data (Table 2)

[*cf.* chemical shift and multiplicity of C-5′, C-10′, C-5 and C-7 in **2**] and the results of HMBC experiments. The structure **2** for this pigment is also in accord with the UV and IR data, which are recorded in the Experimental section. The absolute stereochemistry at the stereogenic biaryl axis and at the C-3′ chiral centre in **2** will be addressed later in this paper.

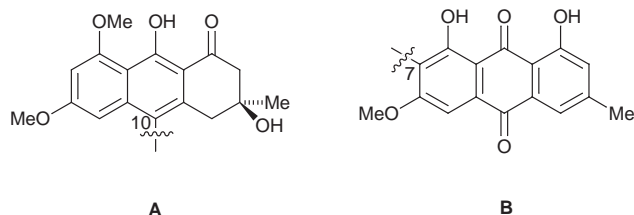The most abundant pigment from *Cortinarius sinapicolor* is

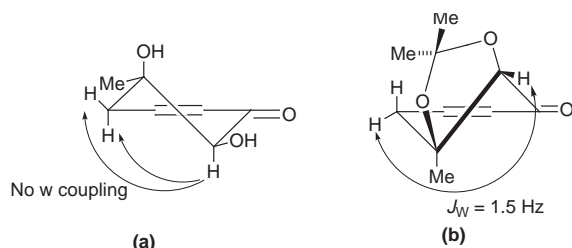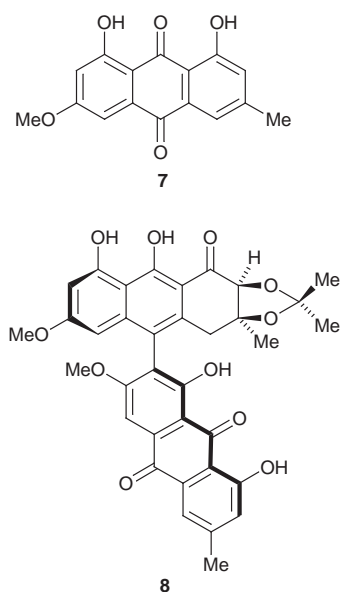**Fig. 1** Partial formulae **A** and **B** that constitute the structure of pigment **2**.

Fig. 2 Preferred half-chair conformation of the dihydroanthracenone ring of (a) 2′-hydroxyphlegmacin **3** and (b) the acetonide **8**.

Fig. 3 CD spectrum of the phlegmacinquinones **2** (——) and **3** (-----) in methanol.

smoothly and in high yield. The ^{1}H NMR spectrum of **8** confirms the presence of a dimethyl acetal (δ_H 1.41 and 1.45). The observation of W-coupling between the proton at C-2′ and the equatorial proton at C-4′ in **8** shows that the dihydroanthracenone ring prefers the half-chair conformation shown in Fig. 2(b). Unfortunately, the acetonide **8** did not provide crystals suitable for X-ray crystallographic analysis and therefore its formation was useful only in the determination of the relative, rather than the absolute, configuration at C-2′ and C-3′ in **3**.

The CD spectra of coupled pre-anthraquinones are dominated by a bisignate Cotton effect couplet centred between 250 and 300 nm that originates from exciton coupling between the asymmetrically disposed long axes of the two aromatic chromophores.[6,7] Consequently, there exists a direct relationship between the shape of the CD spectrum of one of these molecules and its absolute axial configuration.[7] Thus, exciton chirality theory predicts that a binaphthol that contains aromatic chromophores that are related by an anticlockwise helical twist should exhibit a negative Cotton effect at longer wavelength and a positive one at shorter wavelength (a so-called A-type curve[2]).[8] The alternative situation (one in which the chromophores describe a clockwise twist) gives rise to a CD spectrum in which the signs of the Cotton effects are inverted (known as a B-type curve[2]). The CD spectra of the pigments **2** and **3** are shown in Fig. 3, from which it is evident that (i) the natural products **2** and **3** have the same axial stereochemistry, and (ii) the Cotton effect centred at 275 nm accords with an anticlockwise twist between the napthalenoid rings, as is shown in structures **2** and **3**. Subsequent application of the Prelog–Helmchen rules[9] then leads to the assignment of the (*P*) axial configuration to **2** and **3**.‡

With the axial chirality of **2** and **3** established we addressed the question of the configuration at the various stereogenic centres. Inspection of the ^{1}H NMR spectrum of the phlegmacins **2** and **3** (Table 1) and comparison of the chemical shifts and coupling constants of 4′-H_{ax} and 4′-H_{eq} with the corresponding data for other phlegmacins from plant and fungal sources[2,10–12] reveals an empirical relationship between the difference ($\Delta\delta$) in the chemical shift of these methylene protons and the relative configuration between the C-3′ stereocentre and the biaryl axis. Thus, in those phlegmacins in which the C-3′ hydroxy group is on the same face of the dihydroanthracenone ring as the bulk of the C-10′-dihydroanthracenone (or anthraquinone) ring [see Fig. 4(a) to see how this applies to **2** and **3**] then there tends to be a large chemical shift difference ($\Delta\delta = 0.15$–0.25 ppm) between 4′-H_{ax} and 4′-H_{eq}. On the other hand, when the C-3′ hydroxy group and the C-10′-dihydroanthracenone (or anthraquinone) ring are on opposite sides of the molecule [see Fig. 4(b)] then the chemical

the *cis*-2-hydroxy derivative **3** of **2**. The mass spectrum of **3** contains a molecular ion at *m/z* 600 from which the formula $C_{33}H_{28}O_{11}$ followed from high resolution mass measurement. This formula differs from that of pigment **2** by one additional oxygen atom. That this extra atom is part of a hydroxy group located at C-2′ in the dihydroanthracenone part of the molecule followed from the ^{1}H and ^{13}C NMR data (Tables 1 and 2, respectively) along the following lines. The ^{1}H NMR spectra of the pigments **2** and **3** are very similar, differing significantly only in the chemical shift and multiplicity of the signal(s) due to the proton(s) at C-2′ in each case. Thus, 2′-H resonates in the spectrum of **3** as a one proton singlet at δ 4.34 as opposed to the AB quartet (*J* 17.1 Hz) centred close to δ 2.87 due to the protons of the C-2′ methylene group seen in the spectrum of pigment **2**. Significantly, 2′-H in the spectrum of **3** shows no W-coupling with the equatorial proton at C-4′ [*cf.* J_W 1.5 Hz in the spectrum of **2**] suggesting that the C-2′ hydroxy group occupies an equatorial configuration in the preferred conformation for **3** [Fig. 2(a)]. If it is assumed that, as is usual in dihydroanthracenones of this type,[5] the C-3′ methyl group occupies an equatorial configuration, then this conformation places the C-2′ and C-3′ hydroxy groups on the same face of the tetrahydoaromatic ring in the pigment **3**.

This conclusion was verified when treatment of the natural product **3** with 2,2-dimethoxypropane gave the acetonide **8**
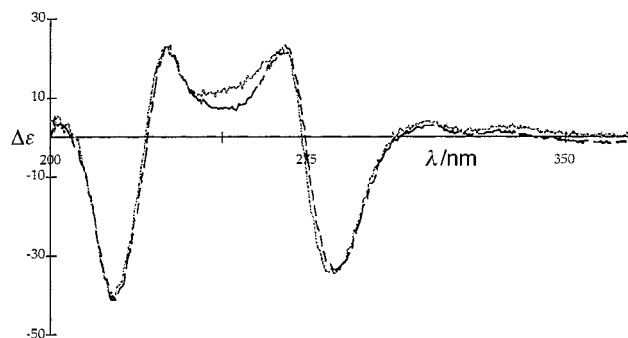
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‡ The assignment depends on an order of priority based on atomic number (as in the Cahn–Ingold–Prelog rules) and is not governed by the helicity of the chromophores.
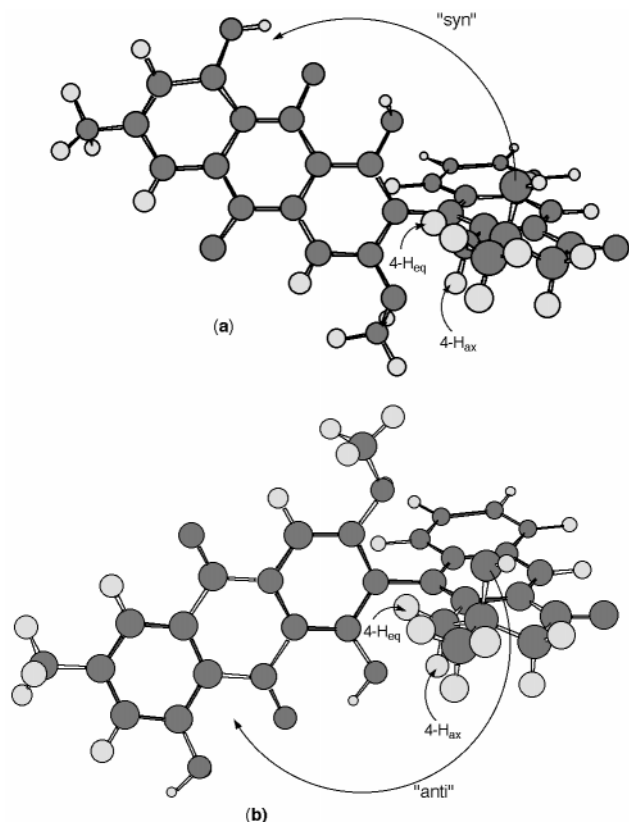
Fig. 4 Relationships between the C-3′ hydroxy group, the C-4′ methylene protons and the C-10 anthraquinone ring in (a) a dimer with (3′S,P) stereochemistry, and (b) a dimer with (3′S,M) stereochemistry.

shift difference is often much smaller ($\Delta\delta \leq 0.08$ ppm).§ This phenomenon and its application were first documented by Oertel,[10] who introduced the terms 'syn' and 'anti' to describe the relative orientations shown in Fig. 4(a) and 4(b), respectively. Consequently, inspection of both the CD and the ¹H NMR spectra of molecules of this type allows the assignment of their absolute configuration, especially in cases where more than one diastereoisomer is known. In the case of **2** and **3** (Fig. 3 and Table 1) the large $\Delta\delta$ between 4′-H_{ax} and 4′-H_{eq} (0.17 ppm for **2** and 0.20 ppm for **3**) indicates a 'syn' arrangement [Fig. 4(a)] and therefore the absolute configuration at C-3′ in **2** must be (S). Similarly, the configuration of the hydroxyphlegmacinquinone **3** must be (2′S,3′S).

The (S) absolute configuration at C-3′ in **2** was confirmed by reductive cleavage of the natural product **2** with alkaline sodium dithionite.[15] The reaction mixture so obtained was purified by gel permeation and HPLC to afford physcion **7** and (3S)-torosachrysone 8-O-methyl ether **6**, which was identified by spectroscopic comparison with an authentic sample of **6**.[16] Chiral HPLC analysis of **6** obtained by degradation of **2** confirmed the (S) stereochemistry of the major product [(3S)-torosachrysone 8-O-methyl ether and its enantiomer were separated by chiral HPLC from a sample of 66% ee (S)-**6**[16] and their identities confirmed by CD spectroscopy] but also revealed the presence of a small amount (16.5%) of the (R) enantiomer of **6**.[17] Since we have no evidence to suggest that the natural product **2** is accompanied by an equivalent quantity of its C-3′ epimer, the implication of this observation is that

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§ The relationship of the chemical shift of C-4′$_{ax}$ and C-4′$_{eq}$ to the chirality at the axis and C-3′ centre is also evident in the spectra of dimers of the atrovirin[10,13] and pseudophlegmacin types.[10,14] We consider that it may arise in the case of the phlegmacins from a combination of the differential levels of anisotropic shielding of 4′-H_{ax} and 4′-H_{eq} by the C-10′ naphthalene system and the counterbalancing (deshielding) influence of the C-6 methoxy group [see Fig. 4(a) and (b)].

Table 3 Levels of enrichment in ¹³C content in phlegmacinquinone **3** as a result of feeding sodium [2-¹³C]acetate to *Cortinarius sinapicolor*

| Carbon	Atom% enrichment[a]	Carbon	Atom% enrichment[a]
1	—	1′	—
2	0.7	2′	0.8
3	—	3′	—
3-Me	0.8	3′-Me	1.0
4	0.8	4′	0.9
4a	—	4a′	—
5	0.8	5′	0.8
6	—	6′	—
7	0.8	7′	0.9
8	—	8′	—
8a	0.6	8a′	0.7
9	—	9′	—
9a	0.7	9a′	0.8
10	0.7	10′	Obscured
10a	—	10a′	—

[a] Enrichment refers to level over and above natural abundance. Data was obtained by comparison of the enriched and natural abundance spectra after normalisation.

the quinone **2** is produced in *Cortinarius sinapicolor* as an anisochiral mixture[18] in which the (3S,3′S,P) enantiomer **2** predominates to the extent of 67% ee. This result is interesting but not entirely unexpected. The diastereoisomeric phlegmacins A₁ **9** and B₁ **11** [which are derived biosynthetically from (R)-torosachrysone (see below)] occur in admixture to varying degrees in several *Cortinarius* species[2] and their enantiomers are found together in the medicinal plant *Cassia torosa*.[11] Torosachrysone (first found in *C. torosa*)[19] is itself known to occur in fungi in anisochiral mixtures of variable composition.[20] Nevertheless, this is the first report in which the enantiomeric purity of a coupled pre-anthraquinone of any sort has been determined. The degradative technique used here, when coupled with chiral HPLC analysis, should be widely applicable to the determination of the stereochemistry of these natural systems at the milligram level.

The biosynthesis of phlegmacins 2 and 3

The biosynthesis of the phlegmacins **2** and **3** in *Cortinarius sinapicolor* has been studied by feeding ¹³C-labelled putative precursors to the fruiting bodies growing in their natural habitat. By harvesting the toadstools and isolating the quinone **3** in the usual way the sites and levels of incorporation of label could be determined from the NMR spectra. The results of feeding sodium [2-¹³C]acetate (Table 3) are entirely consistent with an octaketide origin for both halves of the phlegmacin molecule, with the carbon atoms of the C-6, C-6′ and C-8′ methoxy groups coming from methionine {0.6, 0.8 and 0.9 atom% enrichment, respectively, from [Me-¹³C]methionine}.

Proof that the dimeric pigments **2** and **3** are formed in the fungus *via* oxidative phenolic coupling between two molecules of (S)-torosachrysone **4** was obtained by feeding the water soluble 8-O-β-D-gentiobioside **5** of (S)-torosachrysone in which the 6-O-methyl carbon had previously been enriched (10% over and above natural abundance) by feeding [Me-¹³C]methionine to fruiting bodies of *Cortinarius* sp. WAT 20880 and harvesting the major pigment.[20,21] As a result, the 6- and 6′-O-methyl carbons in the ¹³C NMR spectrum of **3** (Fig. 5) *but not the carbon of the 8′-O-methyl group* were enriched (0.8 and 0.7%, respectively) compared to the natural abundance level. The incorporation of the gentiobioside **5** into **3** is an important result on two counts. Not only does it prove for the first time that dimeric dihydroanthracenones are indeed derived by coupling of precursors of the torosachrysone type, but it also confirms the stereochemistry at C-2′ and C-3′ in **3** as (S), as
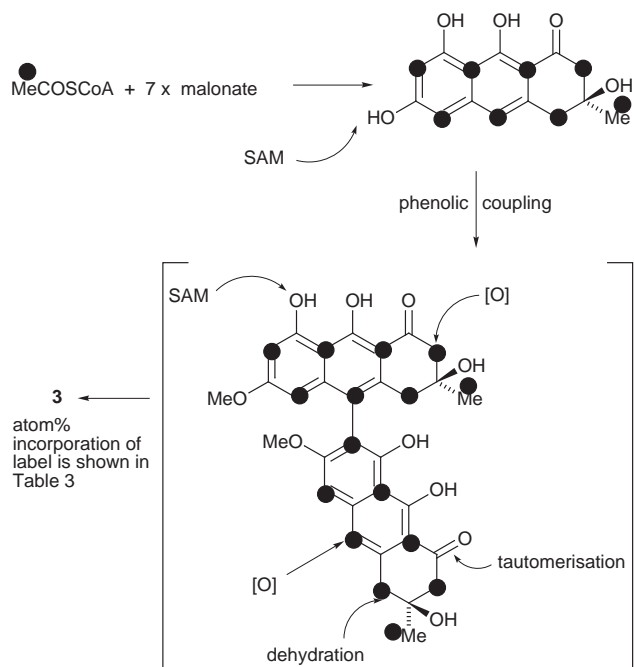
Scheme 1 Labelling pattern and proposed biosynthesis of *cis*-2′-hydroxyanhydrophlegmacin-9,10-quinone 8′-*O*-methyl ether **3**.

was deduced earlier on spectroscopic and chemical grounds.¶ A biosynthetic pathway to **3** that accommodates these results is shown in Scheme 1.

(2′*S*,3*S*,3′*S*,*P*)-2′-Hydroxyanhydrophlegmacin-9,10-quinone 8′-*O*-methyl ether **3** is the first fungal dihydroanthracenone known that contains a hydroxy group at C-2′ in the dihydro-aromatic ring. Other hydroxylated pre-anthraquinones that are known from *Cortinarius* and *Dermocybe* have the additional hydroxy group at C-4′.[2,22] Pigment **3** is therefore reminiscent of olivin aglycone **13** and members of the chromomycinone group of glycosidic antitumour antibiotics.[23] Pigments of the phlegmacin group, *e.g.* the phlegmacins **9** and **11** and their respective methyl ethers **10** and **11**, have been found before in *Cortinarius* subgen. *Phlegmacium* where they are of consider-able taxonomic significance.[2,12] Structurally, the phlegmacins are closely related to the toxins present in the toxic Mexican shrub *Karwinskia humboldtiana*, ingestion of which causes segmented demyelination of peripheral nerves.[24] Mixtures of isomeric pigments of undefined relative stereochemistry for which an anhydrophlegmacin-9,10-quinone 8′-*O*-methyl ether A structure has been suggested are known from several *Cortinarius* species.[2] Although it is impossible using the data available and without authentic materials to draw any firm conclusions, it nevertheless seems probable that the pigment isolated from *C. percomis*[10,12,25] and *C. nanciencis*[10,12] has the structure and stereochemistry shown in **2**.

Experimental

General

Melting points were determined on a hot-stage apparatus and are uncorrected. IR spectra were recorded using a Perkin-Elmer 983 G spectrophotometer for samples as potassium bromide discs. Electronic spectra were recorded on a Varian SuperScan 3 spectrophotometer using ethanolic solutions in a 10 mm quartz cell. NMR spectra were recorded with a JEOL JNM-GX-400 spectrometer (¹H at 399.65 MHz and ¹³C at
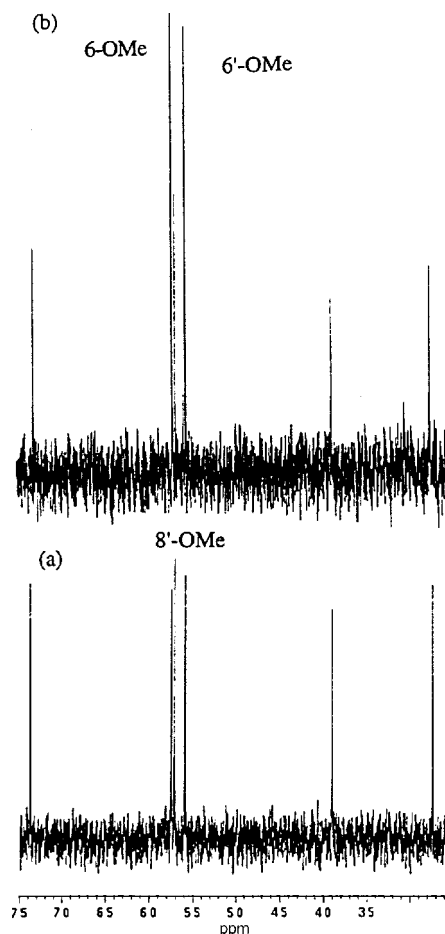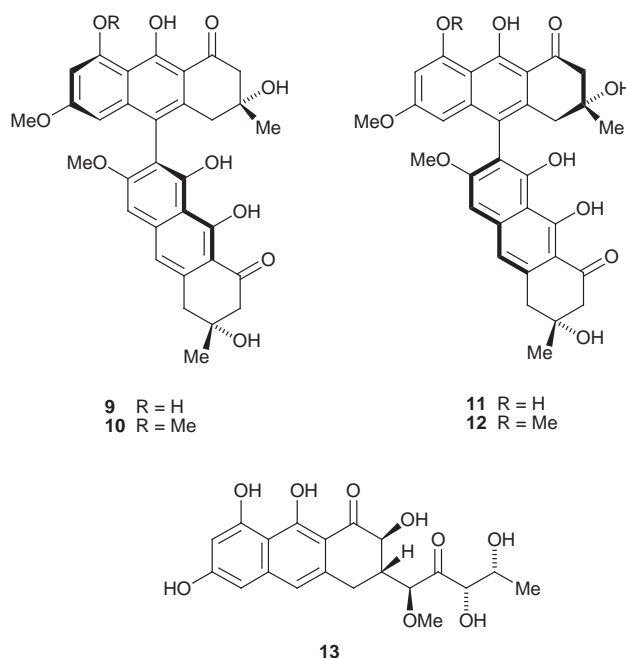
Fig. 5 Partial ¹³C NMR spectrum (CDCl₃, 100 MHz) of *cis*-2′-hydroxyanhydrophlegmacin-9,10-quinone 8′-*O*-methyl ether **3** (a) at natural abundance, and (b) enriched by incorporation of 6-*O*-[*Me*-¹³C]-(*S*)-torosachrysone 8-*O*-β-D-gentiobioside **5**.

9 R = H
10 R = Me

11 R = H
12 R = Me

13

100.4 MHz) for solutions in CDCl₃. Mass spectra were recorded on V. G. Micromass 7070F and JEOL JMS AX505H spectrometers at 70 eV (probe). Specific rotations were measured for chloroform solutions using a Perkin-Elmer 241MC polarimeter and are given in units of 10^{-1} deg cm² g⁻¹. CD spectra were obtained using an AVIV 62DS spectrometer for solutions in methanol.

Materials

Thin layer chromatography (TLC) and preparative TLC (PLC) were performed on Merck precoated silica gel 60 F_{254} and Merck Kieselgel 60 GF_{254} (20 g silica gel spread on 20 × 20 cm glass plates), respectively. Visualisation was under UV light (254 or 366 nm). R_f-values quoted for pure compounds were measured using toluene–ethyl formate–formic acid (50:49:1) as eluent. Gel permeation chromatography (GPC) employed a column (40 × 3.5 cm) of Sephadex LH-20 suspended in and eluted with methanol.

[*Me*-13C]Methionine (99.6 atom% 13C) and sodium [2-13C]acetate (99.5 atom% 13C) were used as purchased from Sigma-Aldrich. Deuteriochloroform (Cambridge Isotope Laboratories) was washed with water, dried (K_2CO_3), distilled, and stored in the dark prior to use.

Cortinarius sinapicolor was collected in the Kinglake National Park, Victoria, Australia from under mixed *Eucalyptus* and *Leptospermum* during May and June 1991 and in subsequent years. Voucher specimens are lodged in the herbarium of the Royal Botanic Garden, Edinburgh, under accession number WAT 24272 and were identified by Dr R. Watling, MBE (Edinburgh) and Professor E. Horak (ETH, Zurich).

Isolation of metabolites from *Cortinarius sinapicolor*

Fresh fruit bodies (1 kg) were macerated in ethanol (2 l) for 2 h at room temperature. The deep yellow extract was concentrated and the aqueous slurry was partitioned, in several portions, between ethyl acetate (500 ml) and water (500 ml). The organic phases were combined, dried and evaporated to afford an orange–brown residue (6.83 g) that was purified by PLC using toluene–ethyl formate–formic acid (50:49:1) as eluent to give two orange zones (R_f 0.34 and 0.20) and a more polar yellow zone (R_f 0.10). The most polar zone gave dermocanarin 4 **1** as discussed previously.[1]

The orange zone (R_f 0.34) was chromatographed [PLC, toluene–ethyl formate–formic acid (50:49:1)], and passed through a column of Sephadex LH-20 to afford *(3S,3'S,P)-anhydrophlegmacin-9,10-quinone 8'-O-methyl ether* **2** (13.5 mg, 1.35×10^{-3}% fr. wt.) as an orange powder, mp 185–187 °C (CHCl₃–petrol) (Found: M^+, 584.1682. Calc. for $C_{33}H_{28}O_{10}$: M, 584.1682); $[a]_D$ +66.7 (c 0.03, CHCl₃); CD 300 (Δε, 0.0), 283 (−34.4), 273 (0.0), 267 (+22.6), 248 (+10.4), 234 (+22.9), 227 (0.0), 218 (−41.4), 206 (0.0), 202 nm (+2.7); ν_{max} 3436, 1692, 1672 and 1613 cm⁻¹; λ_{max} 209 (4.84), 210sh (4.70), 274 (4.74), 399 (4.06) and 462sh (3.84); λ_{max} (EtOH + OH⁻) 400 (4.01) and 530 nm (3.87); m/z 584 (M^+, 23%) and 566 (100); δ_H Table 1; δ_C Table 2.

The orange zone (R_f 0.20) was filtered through Sephadex LH-20 to afford *(2'S,3S,3'S,P)-2'-hydroxyanhydrophlegmacin-9,10-quinone 8'-O-methyl ether* **3** (83.3 mg, 8.33×10^{-3}% fr. wt.) as an orange powder, mp 191–193 °C (CHCl₃–petrol) (Found: M^+, 600.1630. $C_{33}H_{28}O_{11}$ requires M, 600.1631); $[a]_D$ −51 (c 0.03 in CHCl₃); CD 340 (Δε, 0.0), 330 (+1.8), 320 (+0.7), 311 (+3.0), 302 (0.0), 283 (−33.6), 274 (0.0), 269 (+23.2), 254 (+6.8), 235 (+23.4), 228 (0.0), 218 (−40.3), 206 (0.0), 201 nm (+5.2); ν_{max} 3436, 1672, 1620 and 1613 cm⁻¹; λ_{max} 209 (4.84), 210sh (4.70), 274 (4.71), 399 (4.03) and 464sh nm (3.81); λ_{max} (EtOH + OH⁻) 400 (4.01) and 530 nm (3.87); m/z 600 (M^+, 4%), 582 (100), 566 (38), 553 (50) and 539 (42); δ_H Table 1; δ_C Table 2.

Acetonide 8. Pigment **3** (2 mg) was dissolved in 2,2-dimethoxypropane (1 ml) containing a catalytic amount of toluene-*p*-sulfonic acid and the solution was stirred at room temperature for 16 h. Aqueous sodium hydrogen carbonate was added (1 M, 2 ml) and the mixture was diluted with water (30 ml) and extracted with chloroform (3 × 50 ml). The chloroform extracts were combined, dried and evaporated to dry-

ness. The residue (2.2 mg) was purified by PLC [toluene–ethyl formate–formic acid (50:49:1)], to afford the acetonide **8** (1.7 mg, 70%) as a yellow powder, mp 158–161 °C (CHCl₃–petrol); ν_{max} 3434, 1669, 1625 and 1598 cm⁻¹; λ_{max} 210 (4.82), 213sh (4.68), 270 (4.70), 401 (3.87) and 465sh nm (3.80); δ_H 1.35 (3 H, s, 3'-Me), 1.41 and 1.45 (3 H, each br s, Me₂-C), 2.51 (3 H, s, 3-Me), 2.60 (1 H, dd, J 15.1 and 1.5 Hz, 4'-H$_{eq}$), 2.88 (1 H, d, J 15.1 Hz, 4'-H$_{ax}$), 3.67 (3 H, s, 6-OMe), 3.87 (3 H, s, 6'-OMe), 4.01 (3 H, s, 8'-OMe), 4.31 (1 H, d, J 1.5 Hz, 2'-H$_{eq}$), 6.14 (1 H, d, J 2.4 Hz, 5'-H), 6.48 (1 H, d, J 2.4 Hz, 7'-H), 7.14 (1 H, br s, 2-H), 7.58 (1 H, s, 5-H), 7.72 (1 H, d, J 1.5 Hz, 4-H), 12.06 (1 H, s, 8-OH), 12.40 (1 H, s, 1-OH) and 15.08 (1 H, s, 9'-OH).

Labelling experiments

(i) [*Me*-13C]methionine. Each of four young specimens were injected by using a syringe with an aqueous solution (500 μl) of [*Me*-13C]methionine (0.22 M) on days 1, 4 and 8. The toadstools were harvested on day 13, soaked in ethanol (500 ml) and the phlegmacin **3** (3.7 mg) was isolated by chromatography as before. The levels of incorporation of label into the carbons of the three methoxy groups in **3** (see text) was measured by comparison of the height of the corresponding signals in the natural abundance and in the enhanced 13C NMR spectra after normalisation.

(ii) Sodium [2-13C]acetate. Ten young fruit bodies were each injected with an aqueous solution (150 μl) of sodium [2-13C]-acetate (1.2 M) on days 1, 4 and 6, and finally on day 10. The toadstools were harvested on day 11 and worked up in the usual way to yield the phlegmacin **3** (12.5 mg). Enrichment in 13C content over and above natural abundance was measured by comparing the peak heights of individual signals in the enriched and natural abundance 13C NMR spectra after normalisation. Results are collected in Table 3.

(iii) 6-O-[*Me*-13C]Torosachrysone 8-O-β-D-gentiobioside. Two young fruiting bodies were each injected with an aqueous solution (150 μl) of 6-O-[*Me*-13C]torosachrysone 8-O-β-D-gentiobioside **5** (11 atom% 13C, 1% w/v) on days 1, 4 and 6. The toadstools were harvested on day 10 and worked up in the usual way to yield the phlegmacin **3** (2.9 mg). Enrichment in 13C content over and above natural abundance was measured by comparing the peak heights of individual methoxy signals in the enriched and natural abundance 13C NMR spectra after normalisation. The spectra are shown in Fig. 5.

Reductive cleavage of phlegmacinquinone 2

To the dark red solution of anhydrophlegmacin-9,10-quinone 8'-O-methyl ether **2** (6 mg) in aqueous sodium hydroxide (2 M, 1.5 ml) was added solid sodium dithionite (60 mg). The colour of the solution changed immediately to yellow and after 1 min further portions of sodium dithionite (120 mg in total) were added. After 3 min the pale yellow solution was cooled in ice, neutralised with aqueous hydrochloric acid (10%, *ca.* 1 ml) and the products were extracted into ethyl acetate (3 × 20 ml). The extracts were dried and evaporated and the residue was passed through a column containing Sephadex LH-20 (40 × 2 cm) using methanol–dichloromethane (1:1) as eluent. The first band to elute contained unchanged phlegmacinquinone **2**, the second consisted of a mixture of cleavage products that was further analysed by chiral HPLC [Daicel Chiralpak-AD (10 μm; 0.46 × 25 cm)] with (i) ethanol, and (ii) ethanol–hexane (2:3) as eluent (0.5 ml min⁻¹). Solvent (i) separated physcion **7** (retention time 72 min) from other degradation products (retention times 10–34 min) which were collected together and chromatographed further using solvent (ii). (S)-Torosachrysone 8'-O-methyl ether **6** eluted after a retention time of 15.7 min, its enantiomer at 65.2 min. In solvent (i), **6** and its enantiomer show retention times of 10.8

and 31.6 min, respectively. Chromatograms were calibrated with a sample of 66% ee (*S*)-torosachrysone 8′-*O*-methyl ether **6**,[16] the individual enatiomers of which were separated by chiral HPLC and characterised by comparing their CD spectra with published data.[17]

Acknowledgements

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