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Biosynthesis of tetrahydroanthraquinones in fungi ☆

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Abstract

The tetrahydroanthraquinones (1*S*,3*S*)-austrocortilutein (**4**) and (1*S*,3*S*)-austrocortirubin (**7**) are octaketides formed in the fruit bodies of the fungus *Dermocybe splendida* via the gentiobioside **2** of the dihydroanthracenone (*S*)-torosachrysone (**1**). © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

We reported earlier (Gill et al., 1990) on the isolation and structural elucidation of the antibiotic tetrahydroanthraquinones (1*S*,3*S*)-austrocortilutein (**4**) and (1*S*,3*S*)-austrocortirubin (**7**) from the EtOH extracts of the beautiful red and yellow coloured fruit bodies of the Australasian fungus *Dermocybe splendida* Horak. Later work (Gill et al., 1992a) established that the pigments **4** and **7** occur in the intact toadstools as their respective 8-*O*-β-D-gentiobiosides **5** and **6**. More recently (Gill et al., 2000), we achieved the first total synthesis of the aglycones **4** and **7** in enantiomerically pure form. We report here the results of ^{13}C-labelling experiments that establish that (1*S*,3*S*)-austrocortilutein (**4**) and (1*S*,3*S*)-austrocortirubin (**7**) are octaketides derived biogenetically via the dihydroanthracenone (*S*)-torosachrysone (**1**); the 6-OMe group in **4** and **7** comes from methionine.

2. Results and discussion

We speculated elsewhere (Gill et al., 1990) that the biogenesis of the quinones **4** and **7** involves torosachrysone (**1**), which is a minor metabolite of *D. splendida* and is found in various other Australasian *Dermocybe* toadstools (Gill et al., 1989; Gill, 1996). We have studied the biosynthesis of torosachrysone (**1**) in the fruit bodies of the fungus *Cortinarius* sp. WAT 20880[1] and shown (Gill et al., 1989, 1992b) that it originates, at least formally, from an octaketide that is folded, condensed and decarboxylated as shown in the early steps of Fig. 1. We have also noted previously (Eagle et al., 1993) that the fruit bodies of *Cortinarius* sp. WAT 20880 contain large quantities of the intensely fluorescent, H_2O soluble 8-*O*-β-D-gentiobioside **2** of (*S*)-torosachrysone. We recognised at that time that if a ^{13}C-label could be incorporated efficiently into the gentiobioside **2** then we would have a powerful vehicle for studying the biosynthesis of several other complex fungal metabolites. For some examples, see Gill and Steglich (1987). Accordingly, a solution of [*Me*-^{13}C]methionine in H_2O was injected via syringe into several fruiting bodies of *Cortinarius* sp. WAT 20880 growing in their natural habitat. The fruit bodies were later collected, lyophilised, extracted with MeOH and the extracts were carefully purified by a combination of preparative TLC and GPC to afford

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[1] The code refers to the accession number under which voucher specimens are held in the herbarium of the Royal Botanic Garden, Edinburgh. The species is not described in the botanical literature.

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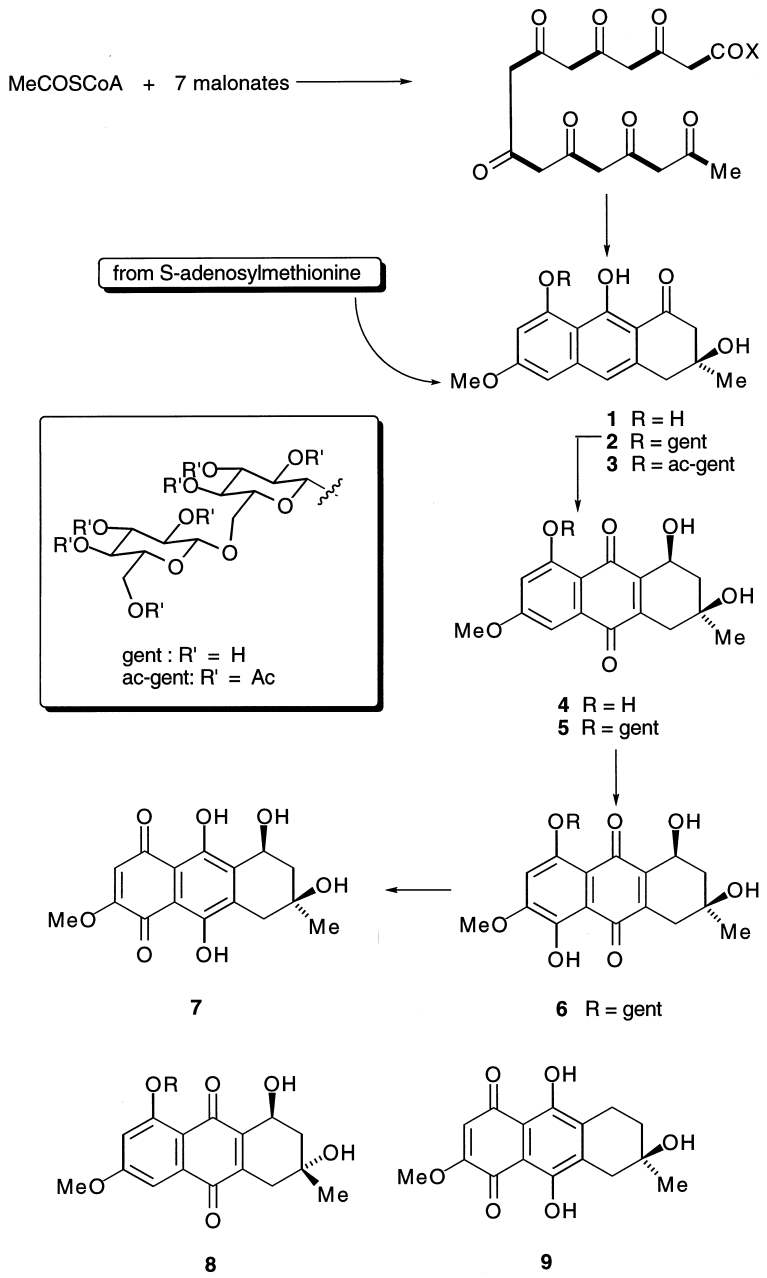

Fig. 1. Biogenesis of tetrahydroanthraquinones in *D. splendida*.

the gentiobioside **2** of (*S*)-torosachrysone (**1**) in a yield of 4.7% of the dry weight of the fungus. The level of enrichment in ^{13}C-content in **2** (10 at.% above natural abundance) was determined by comparison of the 6-OMe resonance (δ 55.55) in the ^{13}C NMR spectrum of both labelled and natural abundance samples of **2** in DMSO-d_6 (Eagle et al., 1993). The enrichment was confirmed and the enantiomeric purity of **2** was determined after acidic hydrolysis of the natural product and isolation of the aglycone **1**. In this case, integration of the ^{13}C–^{1}H satallites (J 144.5 Hz) flanking the 6-OMe resonance at δ 3.88 in the ^{1}H NMR spectrum of **1** corresponded to a ^{13}C content of 11 at.%. The sample of **1** obtained in this way was determined by chiral HPLC analysis to be >99% e.e.

The ^{13}C-labelled gentiobioside **2** in H_2O was, in turn, injected by syringe into fruiting bodies of *D. splendida* in their natural environment. Harvesting, work-up and preparative TLC, as before (Gill et al., 1990), gave the aglycones **4** and **7**. The region of the ^{13}C NMR spectrum of (1*S*,3*S*)-austrocortilutein (**4**) that includes the signal from the 6-OMe group (δ 55.8) is shown both at natural abundance and after feeding the enriched gentiobioside **2** in Fig. 2(a); the corre-

sponding partial spectrum from (1*S*,3*S*)-austrocortirbin (**7**), which includes the 6-OMe signal at δ 56.9, is shown in Fig. 2(b). It follows from these pairs of spectra that the levels of enrichment in the ^{13}C content above natural abundance in the 6-OMe group in the tetrahydroanthraquinones **4** and **7** is 1.5 and 2.1%, respectively.

[*Me*-^{13}C]Methionine is also incorporated efficiently into the 6-OMe group of the tetrahydroanthraquinones **4**, **7**, **8** and **9** by *D. splendida*. In these cases, the high levels of enrichment (13–28 at.% above natural abundance) were again measured best from the corresponding ^{1}H NMR spectrum.

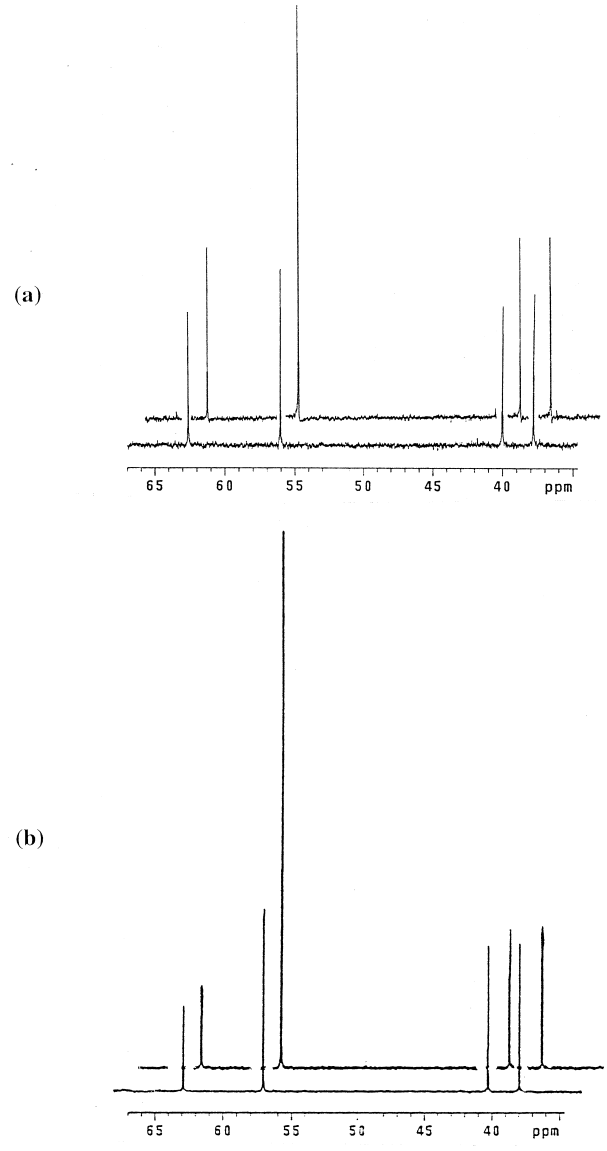

Fig. 2. Partial ^{13}C NMR spectrum (100 MHz, $CHCl_3$) of (a) (1*S*,3*S*)-austrocortilutein (**4**) at natural abundance (lower trace) and enriched by incorporation of the gentiobioside **2** (upper trace), and (b) (1*S*,3*S*)-austrocortirubin (**7**) at natural abundance (lower trace) and enriched by incorporation of the gentiobioside **2** (upper trace).

The results discussed above are fully supportive of the biosynthesis of tetrahydroanthraquinones in *D. splendida* by the route summarised in Fig. 1. The perceived involvement of torosachrysone (**1**) in the biogenesis of more complex fungal metabolites has been recognised for some time (Gill and Steglich, 1987) but it is only recently that experimental evidence has been obtained which firmly establishes that as a fact (Elsworth et al., 1999a, 1999b; Müller, 1995). This important pathway is here extended to include tetrahydroanthraquinones and, with the ready availability of the pivotal ^{13}C-labelled intermediate **2**, it should be possible to further extend our knowledge of the origins of several other classes of natural products.

3. Experimental

3.1. General

NMR spectra were recorded with a JEOL JNM-GX-400 spectrometer (^{1}H at 399.65 MHz and ^{13}C at 100.4 MHz) for solutions in $CDCl_3$ unless stated otherwise. Pigments **4**, **7**, **8** and **9** were identified spectroscopically by direct comparison with data obtained from authentic materials (Gill et al., 1990).

Preparative TLC was performed on Merck Kieselgel 60 GF_{254} (20 g spread on a 20 × 20 cm glass plate). Visualisation was under UV light (254 or 366 nm). Chromatography employed toluene/HCO_2Et/HCO_2H (50:49:1) as eluent unless stated otherwise. Gel permeation chromatography (GPC) employed a column (40 × 3.5 cm) of Sephadex LH-20 suspended in and eluted with MeOH.

[*Me*-^{13}C]Methionine (99.6 at.% ^{13}C) was used as purchased from Sigma–Aldrich. $CDCl_3$ (Cambridge Isotope Laboratories) was washed with H_2O, dried (K_2CO_3), distilled, and stored in the dark prior to use.

HPLC was performed on an ISCO model 2350 with an ISCO UA-6 UV–Vis detector, an optical flow cell operating at 280 nm, and a Spectra-Physics SP 270 integrator. A Chiralpak AD column (Daicel Chemical Industries, Tokyo, 0.46 × 25 cm; 10 μm particle size) was used with EtOH (0.5 ml min^{-1}) as eluent.

3.2. Fungal material

Experiments were carried out on fungi growing in mixed native forest in the Kinglake National Park, Victoria (*Cortinarius* sp. WAT 20880) and in the Murrindindi Nature Park, Victoria (*D. splendida*) during June and July 1996–1998. Lyophilised specimens are lodged in the herbarium of the Royal Botanic Garden, Edinburgh under the accession numbers WAT 20880 and WAT 18086. *D. splendida* was identified by Dr R. Watling (E) and Prof. E. Horak (ETH, Zurich).

3.3. Feeding experiments and extraction and isolation of ^{13}C-labelled metabolites

3.3.1. Cortinarius sp. WAT 20880

Each of the seven young specimens of *Cortinarius* sp. WAT 20880 were injected by using a syringe with a solution (300 μl) of [*Me*-^{13}C]methionine (5% w/v) in H_2O on days 1, 3 and 5. The toadstools (108 g) were harvested on day 10 and immediately lyophilised. The lyophilised fruit bodies (6.8 g) were soaked in MeOH (3 × 200 ml) and the combined extracts were evaporated to dryness. The residue was partitioned between EtOAc (200 ml) and H_2O (100 ml) and the blue fluorescent H_2O phase was lyophilised. The yellow–brown residue was purified by preparative TLC (MeOH) and GPC to afford [*Me*-^{13}C]-(*S*)-torosachrysone 8-*O*-β-D-gentiobioside (**2**) (10 at.% ^{13}C above natural abundance) as a mustard yellow powder (317 mg; 0.29% fr. wt 4.7% dry wt).

3.3.2. Acidic hydrolysis of 2

The gentiobioside **2** (20 mg, 33 mmol) was dissolved in H_2O (50 ml) and carefully acidified with concentrated HCl until the blue fluorescence at 365 nm was extinguished (pH 2). The resulting suspension was extracted with EtOAc (2 × 50 ml) and the extracts were combined, dried (Na_2SO_4) and evaporated. The residue was purified by preparative TLC to afford [*Me*-^{13}C]-(*S*)-torosachrysone (**1**) (10 at.% above natural abundance) as a lime green powder (4.5 mg, 71%).

3.3.3. D. splendida

*3.3.3.1. [Me-^{13}C]-(S)-*Torosachrysone *8-O-*β-D-gentiobioside *(2)*. Two young fruiting bodies of *D. splendida* were each injected with a solution (150 μl) of [*Me*-^{13}C]-(*S*)-torosachrysone 8-*O*-β-D-gentiobioside (**2**) (11 at.% ^{13}C, 1% w/v) in H_2O on days 1 and 4. One sporophore was picked on day 6 while the second was injected with a further aliquot (150 μl) of labelled **2** and was harvested on day 10. The toadstools were soaked separately overnight in EtOH (50 ml) and the deep red extracts were concentrated and purified by preparative TLC and GPC to afford (1*S*,3*S*)-austrocortilutein (**4**) (3.2 mg) and (1*S*,3*S*)-austrocortirubin (**7**) (7.5 mg). Enrichment in ^{13}C content in **4** and **7** over and above natural abundance was measured by comparing the peak heights of individual 6-OMe signals in the enriched and natural abundance ^{13}C NMR spectra after normalisation. The spectra are shown in Fig. 2.

3.3.3.2. [Me-^{13}C]Methionine. Each of the six young specimens were injected by using a syringe with a solution (500 μl) of [*Me*-^{13}C]methionine (0.22 M) in H_2O on days 1, 5, 7 and 10. The toadstools (97 g) were harvested on day 14 and soaked in EtOH (500 ml) overnight. The solvent was evaporated and the residue was partitioned between EtOAc (2 × 100 ml) and H_2O (100 ml). The organic phase was dried (Na_2SO_4) and evaporated and the pigments were purified by preparative TLC and GPC to afford (1*S*,3*S*)-austrocortilutein (**4**) (13 mg; 12.7 at.% ^{13}C above natural abundance), (1*S*,3*S*)-austrocortirubin (**7**) (67 mg; 28.3 at.% ^{13}C), (1*S*,3*R*)-austrocortilutein (**8**) (1.4 mg; 13 at.% ^{13}C) and (*R*)-1-deoxyaustrocortirubin (**9**) (0.6 mg; 13 at.% ^{13}C). Enrichment in ^{13}C content in **4**, **7**, **8** and **9** was measured by integration of the ^{13}C–^{1}H satallites flanking the 6-*O*-[*Me*-^{12}C] signal [δ 3.91 (*J* 145.4 Hz) in the spectrum of **4**, δ 3.95 (*J* 146.9 Hz) in **7**, δ 3.91 (*J* 145.5 Hz) in **8** and δ 3.93 (*J* 146.4 Hz) in **9**] in the ^{1}H NMR spectra.

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