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SESQUITERPENOID METABOLITES FROM *STEREUM COMPLICATUM*

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Key Word Index—*Stereum complicatum:* Basidiomycete; complicatic acid; hirsutic acid *C*; sesquiterpenes; antibiotics; biosynthesis.

Abstract—A new sesquiterpene antibiotic, complicatic acid, isolated from cultures of *Stereum complicatum* (Fr.) Fr. has been shown to be dehydrohirsutic acid *C*. Hirsutic acid *C* was also isolated from the same fungus. [2-^{14}C]-MVA was incorporated into both metabolites and complicatic acid has been shown to be formed from hirsutic acid *C* both *in vivo* and *in vitro*.

INTRODUCTION

IN A PREVIOUS paper[1] we reported that, in submerged fermentations where the pH value was controlled, the American timber decomposing Basidiomycete, *Stereum complicatum* (Fr.) Fr., produced an oily, acidic, antimicrobial metabolite (*ca.* 200 mg/l. after 12 days) to which we ascribed the name complicatic acid (substance *B*). We have since shown[2] that a fermentation in which the pH value was allowed to fall produced, in addition to small amounts of *B*, a second acidic substance, *A* (500 mg/l.). We now report on the identity and biosynthesis of *A* and *B*.

RESULTS AND DISCUSSION

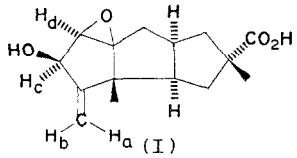
The m.p., $[\alpha]_D$ and spectral data of metabolite *A* and its *p*-bromophenacyl ester were in excellent agreement with literature values for hirsutic acid *C* (I, R=H) and its ester (I,R = $CH_2COC_6H_4$ *p*-Br).[3] Unit cell and intensity data for the *p*-bromophenacyl ester of *A* agreed well with those obtained in the X-ray study of hirsutic acid C[4], but, by minimising radiation damage, a higher resolution was achieved. The resulting structure ($R = 0{\cdot}075$) revealed an intermolecular hydrogen bond (2·87(2)Å) between the 5β-hydroxyl and the epoxide oxygen, which had been ruled out in the earlier study.*

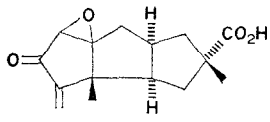
The IR (ν_{max} 1700, 1645 and 900 cm^{-1}) and UV (λ_{max} 234 nm (ϵ4500)) spectra of metabolite *B*, $C_{15}H_{18}O_4$ (high resolution MS), indicated the presence of an exomethylene cyclopentanone (λ_{calc}[5] 230 nm). The NMR spectrum indicated that *A* and *B* were structurally related and showed the loss of H_c. H_a, H_b and H_d appeared as singlets lacking the long-range

* Full details of the refined structure will be published elsewhere.

[1] MANTLE, P. G. and MELLOWS, G. (1972) *J. Gen. Microbiol.* **73**, xxii.

couplings of H_a, H_b to H_c (*ca.* 2–3 Hz) as observed in *A*. Structure II was therefore suggested for *B*. The relationship of *B* to *A* was proved by reduction of *B* with $NaBH_4$ in EtOH which gave *A* and by oxidation of *A* with MnO_2 in $CHCl_3$ which afforded *B*.


(I)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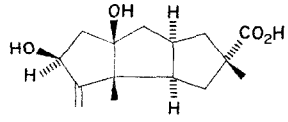
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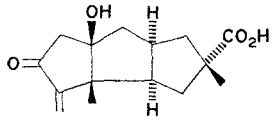
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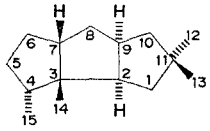
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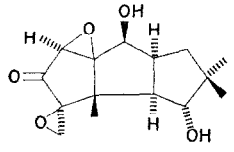
(VI)

Hirsutic acid *C* was first isolated in 1947 from cultures of a filamentous fungus which was thought to be *Stereum hirsutum*.[6] However, subsequent attempts to produce hirsutic acids have been unsuccessful until the present study in which the terpenoid nature of these metabolites has been proved by the efficient incorporation of [2-^{14}C]-mevalonic acid (Table 1).

TABLE 1. INCORPORATION OF (±)-[2-^{14}C]-MEVALONIC ACID INTO HIRSUTIC ACID *C* AND COMPLICATIC ACID

		Hirsutic acid *C*			Complicatic acid		
Expt. No.	[2-^{14}C]-MVA fed (dpm)	Wt. isolated (mg)	Sp. act.‡ (dpm/mg)	Incorporation§ (%)	Wt isolated (mg)	Sp act (dpm/mg)	Incorporation§ (%)
1*	$6\,12 \times 10^6$	100·4	803·6	2·64	60·6	200	0·40
2†	$4{\cdot}28 \times 10^7$	193	1339	1·26	0 0	—	—

* 1 × 100 ml culture used; harvested on day 16
† 3 × 100 ml cultures used; harvested on day 7.
‡ Sequentially crystallized to constant activity (± 5%).
§ Allowing for the utilization of only the (+) enantiomer of (±) [2-^{14}C]-MVA.

Although there is no direct evidence, it is possible that complicatic acid is identical to hirsutic acid *N* (an uncharacterized antibiotic substance which was shown to be formed from hirsutic acid *C* *in vivo*[6]) and ramealin (a metabolite, also uncharacterized, of *Stereum complicatum* (Fr.) Fr. (= *Stereum rameale* Schw.)).[2,7] In support of this we have shown that [^{14}C]-hirsutic acid *C* (biosynthesized from [2-^{14}C]-MVA) is efficiently transformed *in vivo* into II (Table 2). Furthermore, when washed mycelium of *Stereum complicatum*, harvested

[2] MANTLE, P G. and MELLOWS, G. (1973) *Trans. Brit. Mycol. Soc.* in press.
[3] COMER, F. W., MCCAPRA, F., QURESHI, I. H. and SCOTT, A. I. (1967) *Tetrahedron* **23**, 4761.
[4] COMER, F. W. and TROTTER, J. (1966) *J. Chem. Soc.* **B**, 11.
[5] SCOTT, A I (1964) *Interpretation of the Ultraviolet Spectra of Natural Products*, Pergamon, Oxford.
[6] HEATLEY, N. G., JENNINGS, M. A. and FLOREY, H. W. (1947) *Br J. Exp. Path.* **28**, 35.
[7] FLOREY, H. W., CHAIN, E. B., HEATLEY, N. G., JENNINGS, M. A., SAUNDERS, A. G., ABRAHAM, E. P. and FLOREY, M. E. (1949) *Antibiotics* Vol I, p. 366, Oxford University Press, Oxford.

at the time when II begins to appear in the culture fluid (*ca.* 10 days' growth), was incubated with an aqueous solution of hirsutic acid *C* at 24° during 14 hr the latter was completely converted to II. These observations are in conflict with Lansbury's assumption[8] that hirsutic acid *N* is identical with isohirsutic acid *C* (IV). Since hirsutic acid *N* was shown to be antimicrobial when bioassayed against *Staphylococcus aureus*[6] we have synthesized IV from I(R–H) and examined its antimicrobial activity. I(R=H) was reduced with $LiBH_4$ in THF to give dihydroisohirsutic acid *C*(III), m.p. 176–179°, oxidation of which with MnO_2 in $CHCl_3$ afforded IV as an oil. Isohirsutic acid C showed approximately equivalent antimicrobial activity to complicatic acid,[2] whereas III and I (R=H) were inactive. Hence, this parameter cannot assist in determining whether hirsutic acid *N* is identical with II or IV.

TABLE 2. INCORPORATION OF [^{14}C] LABELLED HIRSUTIC ACID *C* INTO COMPLICATIC ACID

Expt.	[^{14}C] Hirsutic acid *C* fed* Wt. (mg)	Sp. act. (dpm/mg)	Complicated acid isolated (mg)	Sp. act. of hirsutic acid *C*‡ (dpm/mg)	Incorporation (%)
1†	10·2	1317	60·2	46	20·5
2†	10·2	1317	65·1	41	20·2

* The [^{14}C]-hirsutic acid *C* (biosynthesized from (±)-[2-^{14}C]-MVA) was fed as an aq. soln. to one production flask (100 ml) immediately after inoculation.

† The flasks were harvested on day 14.

‡ The complicatic acid isolated was reduced ($NaBH_4$ in EtOH) to hirsutic acid *C* which was crystallized to constant activity.

We are currently isolating other related metabolites from *Stereum complicatum* and to avoid further confusion in the literature over trivial names we propose that the hypothetical parent hydrocarbon V be named hirsutane, numbered as shown. Thus metabolites *A* (hirsutic acid *C*) and *B* (complicatic acid) would be 5β-hydroxy-6β,7β-oxido hirsut-4(15)-en-12-oic acid and 6β,7β-oxido-5-oxohirsut-4(15)en-12-oic acid respectively. The coriolins[9] would also be systematically named from this skeleton. Thus, coriolin(VI),[9a] the parent metabolite of this group, would be 1α,8β-dihydroxy-4β, 15:6β,7β-dioxido-5-oxo-hirsutane.

EXPERIMENTAL

NMR spectra were recorded in $CDCl_3$ on a Varian HA-100 spectrometer using TMS as internal standard. Mass spectra were taken on an AEI MS9 spectrometer. M.ps were determined on a Kofler block and are uncorrected. Merck Kieselgel GF_{254} nach Stahl was used for TLC and PLC. Radiocounting was carried out on a Beckman LS-200B liquid scintillation counter, in 10 ml scintillant (containing butyl PBD (6 g) and naphthalene (50 g) in toluene (11)) and ethyl cellosolve (3 ml).

Production and isolation of I *and* II. The fermentation process for the large scale (up to 300 l.) production of I and II and the extraction and purification of the metabolites, have been described elsewhere.[2]

Biosynthesis experiments. Mycelium from 6- to 10-day-old malt agar slants was used to inoculate seed flasks each containing Medium S[2] (100 ml). After 6–8 days growth in shaken culture, the excess supernatant

[8] LANSBURY, P. T., WANG, N. Y. and RHODES, J. E. (1972) *Tetrahedron Letters* 2053; LANSBURY, P. T. (1972) *Acc. Chem. Res.* **5**, 311.

[9] (a) TAKAHASHI, S., NAGANAWA, H., IINUMA, T., TAKITA, T., MAEDA, K. and UMEZAWA, H. (1971) *Tetrahedron Letters* 1955; (b) TAKAHASHI, S., IINUMA, H., TAKIDA, T., MAEDA, K. and UMEZAWA, H. (1969) *ibid.* 4663; (c) (1970) *ibid.* 1637.

fluid was decanted off and the mycelium blended for 1 min (Omnimix homogenizer). The total homogenate from one seed flask was used to inoculate two production flasks each containing Medium P² (100 ml). Aq. solns. of radioactive substrates were sterilized by filtration through a Sartorius membrane filter (2 μ) before being added to the relevant number of production flasks, immediately after inoculation. The flasks were shaken at 24° for the period indicated (Tables 1 and 2). At the end of the experiment the mycelium was separated by filtration through muslin and washed with dist. H_2O. The combined culture fluid and washings was extracted with $CHCl_3$ at pH 8 and the $CHCl_3$ extract discarded. The aq. layer was extracted at pH 3 with $CHCl_3$ and the latter washed with dist. H_2O, dried and concentrated *in vacuo*. The acid extract was eluted on PLC with $CHCl_3$–isoPrOH (9:1). Band R_f 0·5–0·6, UV absorbing, afforded II and band R_f 0·3–0·4 gave I (R=H), when stripped with $CHCl_3$–isoPrOH (1:1).

Metabolite A (*Hirsutic acid C*, (*I,R=H*)) m.p. 178·5–180° (needles from aq. EtOH), $[\alpha]_D$ + 116° (*c* 1·2, $CHCl_3$) (cf. hirsutic acid *C*[3] m.p. 180°, $[\alpha]_D$ + 116°) (Found: C, 68·0; H, 7·6. Calc. for $C_{15}H_{20}O_4$ *c*, 68·1; H, 7·6%) *p*-Bromo-phenacyl ester (I,R = ·CH_2CO C_6H_4-*p*Br) m.p. 131–132·5° (needles from EtOH) $[\alpha]_D$ + 99° (*c* 1·0, $CHCl_3$) (*cf p*-bromo phenacyl hirsutate[3] m.p. 129–130°, $[\alpha]_D$ + 97° Spectral data for metabolite *A* and its ester were in excellent agreement with lit. values for hirsutic acid *C* and its ester †

Metabolite B (*Complicatic acid*). Isolated as an oil, $[\alpha]_D$ − 79° (*c* 1·1, $CHCl_3$) MS showed M^+ 262.1208 ($C_{15}H_{18}O_4$ requires: 262·1205) with major fragments at *m/e* 205, 159, 85 and 83 ν_{max} ($CHCl_3$) 3600–2400 br, 1730s (CO_2H), 1700s, 1640 m (C=C–C=O), 1470 m, 1410 m, 1310 m, 950 m, 915 w, 900 w cm^{-1}. λ_{max} (EtOH) 234 nm (ϵ4500) τ 8·86, 3 Hs; τ 8·80 1 Ht (*J* 6 Hz); τ 8 64 3 Hs, τ 8 46 1 Hm; τ 8 04, 2 Hm, τ 7·95–7·10, 4 H, τ 6·65, 1 Hs; τ 4·78, 1 Hs; τ 4 01, 1H s and τ 0·0–1 2 1 Hs.

MnO_2 *oxidation of metabolite A*. Metabolite *A* (100 mg) in $CHCl_3$ (2 ml) was stirred during 20 hr with MnO_2 (500 mg). Filtration through celite and removal of solvent *in vacuo* gave a TLC pure, oily product (89 mg) identical by TLC, MS, NMR, IR and UV with metabolite *B* (complicatic acid).

$NaBH_4$ *reduction of metabolite* B. To metabolite *B*(200 mg) in EtOH (15 ml) was added $NaBH_4$ (200 mg) during 30 min. After a further 1 hr, the reaction mixture was poured into H_2O and extracted into $CHCl_3$ at pH 3. Removal of solvent gave a white solid (191 mg) which crystallized as needles (123 mg), m.p. 179–180°, $[\alpha]_D$ + 116° (c 1·0, $CHCl_3$) identical with metabolite *A* (m. m.p., IR, MS and NMR).

$LiBH_4$ *reduction of hirsutic acid* C. A solution of hirsutic acid (600 mg) in dry THF (30 ml) and $LiBH_4$ (250 mg) was heated under reflux during 2·5 hr. (extended reaction time led to reduced yields of III). The complex was decomposed with sat. NH_4Cl soln and the product extracted into isobutyl methyl ketone. The product was eluted on PLC with $CHCl_3$–IPA (9:1) Band R_f 0·4–5·0 was stripped with IPA–$CHCl_3$ (1:1) to give dihydroisohirsutic acid, III, (330 mg) as needles m.p. 176–179° (aq. EtOH), $[\alpha]_D$ − 84° (*c* 1·0, EtOH). (Found: C, 67·5; H, 8·1 $C_{15}H_{22}O_4$ requires: C, 67·6; H, 8·3%). ν_{max} (KBr) 3400s, 3600–2500 br. 1705 s, 1660 w, 1210 s, 1110 s, 910 m, 880 w and 860 w cm^{-1}. NMR (DMSO) τ 9·05, 3 Hs, τ 8·79, 3 Hs; τ 5·82, 1 Ht (*J* 8 Hz), H_c; τ 5·23, 1 H br.s ($J_{w\frac{1}{2}}$ 4·8 Hz), H_b; τ 5·06, 1 H br s ($J_{w\frac{1}{2}}$ 4 Hz), Ha Hirsutic acid C (237 mg) was similarly recovered from band R_F 0·3–0·4.

MnO_2 *oxidation of dihydroisohirsutic acid* A soln of III (140 mg) in $CHCl_3$ (10 ml) was stirred with MnO_2 (1 g) during 24 hr. After usual work up, TLC pure isohirsutic acid C (IV 133 mg) was isolated as an oil MS, M^+ 264·1360 (15%) Calc. for $C_{15}H_{20}O_4$ 264·1362 with major fragments at *m/e* 246 (15%), 222 (12%), 218 (18%), 201 (30%), 200 (20%) and 124 (83%). λ_{max} (EtOH) 232 nm (ϵ4500). ν_{max} ($CHCl_3$) 3600 m, 3580–2500 br, 1725s, 1700s, 1640m, 1475m, 1415m and 950m cm^{-1}.

DL-[2-^{14}C] *Mevalonic Acid feeding experiments* [2-^{14}C] MVA 135 μCi/mg was fed in the amounts indicated and the results are summarized in Table 1.

[^{14}C]-*Hirsutic Acid C feeding experiments* [^{14}C] Hirsutic acid *C*, biosynthesized from [2-^{14}C] MVA, was fed in two parallel experiments during 14 days The complicatic acid isolated was purified by PLC, converted to hirsutic acid *C* by $NaBH_4$ reduction and crystallized to constant activity. The results are summarized in Table 2.

Incubation of hirsutic acid C *with washed mycelium*. The mycelium from one 10-day-old shake flask was thoroughly washed with dist. H_2O and incubated overnight with an aq. soln of hirsutic acid *C* (30 mg in 100 ml). The $CHCl_3$ extract of the aq fraction contained TLC pure complicatic acid.

Bioassay. This was performed using the plate assay method using *Staphylococcus aureus* NCTC 6571 as the test organism

Acknowledgements—We thank Professor A. I Scott for an authentic specimen of hirsutic acid *C* and the S.R.C. for a research studentship (to T.C F)

† Since this work was completed we have received an authentic sample of hirsutic acid *C*, kindly sent by Prof. A. I. Scott, which was identical (TLC, m.m.p.) with metabolite *A*.