Phytochemistry, Vol. 28, No. 3, pp. 775–779, 1989.
Printed in Great Britain.

0031-9422/89 $3.00 + 0.00

5-*EPI*-ARISTOLOCHENE IS A COMMON PRECURSOR OF THE SESQUITERPENOID PHYTOALEXINS CAPSIDIOL AND DEBNEYOL

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(*Received* 21 *June* 1988)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; cell suspension cultures; biosynthesis; sesquiterpenoid phytoalexins; eremophilenes; 5-*epi*-aristolochene; capsidiol; debneyol.

Abstract—5-*epi*-Aristolochene (4-*epi*-eremophila-9,11-diene) has been identified (GC-MS and high-field ^{1}H NMR) as the hydrocarbon (unknown **I**) which is accumulated when the formation of capsidiol and debneyol from [1-$^3H_2$]FPP in cell-free preparations of cells from cellulase-elicited cell-suspension cultures of *Nicotiana tabacum* is inhibited by the omission of NADPH or the exclusion of molecular oxygen from the incubation mixture. Feeding experiments with ^{14}C-labelled 5-*epi*-aristolochene have shown this compound to be a common precursor of the two sesquiterpenoid phytoalexins capsidiol and debneyol which accumulate in the elicited cultures. The evidence suggests that the biosynthesis of capsidiol is regulated, in part, by the activity of the first of the two hydroxylases which catalyse its formation from this hydrocarbon and furthermore, that this 3-hydroxylase is induced during the elicitation process.

INTRODUCTION

In two recent papers, we reported on the successful preparation of a cell-free system from cellulase-elicited cell suspension cultures of *Nicotiana tabacum* L. which was capable of supporting the synthesis of the sesquiterpenoid phytoalexins capsidiol and debneyol in the presence of NADPH and molecular oxygen from either IPP or FPP [1, 2]. The omission of NADPH or replacement of the gas phase with nitrogen, prevented the synthesis of the two phytoalexins and led instead to the accumulation of a hydrocarbon compound (referred to as unknown **I** in refs [1, 2]). Although we were unable to obtain any direct evidence for the involvement of this unknown hydrocarbon in the biosynthesis of the two phytoalexins and lacked any spectroscopic data, the circumstantial evidence obtained from the experiments with and without the cofactors led us to propose that the hydrocarbon was a common precursor of the two phytoalexins. Further work with marker compounds encouraged us to believe that the hydrocarbon was most probably a bicyclic eremophilene compound and not a germacrene. Based on our observations we proposed the unknown to be 5-*epi*-aristolochene (4-*epi*-eremophila-9,11-diene) (**1**) [1, 2].

In this paper, we report the isolation and characterization of unknown **I** and show it to be 5-*epi*-aristolochene. We show also that when radio-labelled 5-*epi*-aristolochene (isolated from cell-free incubations) is fed to elicited tobacco cultures it is incorporated rapidly and efficiently into both phytoalexins. Furthermore, the results of the feeding experiments suggest that the biosynthesis of capsidiol is regulated, in part, by the activity of the first of the two hydroxylases on the pathway from 5-*epi*-aristolochene to capsidiol, and further that this hydroxylase is induced during the elicitation process.

Abbreviations—IPP, isopentenyl pyrophosphate; FPP, *E,E*-farnesyl pyrophosphate;

RESULTS

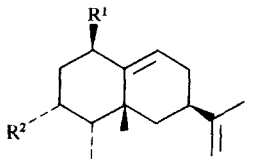
Characterization of unknown I *as* 5-epi-*aristolochene*

Unknown **I**, $C_{15}H_{24}$ ($[M]^+$, *m/z* 204), was obtained as an oil from a large scale incubation of a cell-free preparation of elicited cells with [1-$^3H_2$]FPP and was assigned the hydrocarbon structure **1** (5-*epi*-aristolochene) on the basis of spectroscopic evidence. The ^{1}H NMR spectrum in $CDCl_3$ was obtained at 270.17 MHz and the principal features of this spectrum are given in Table 1 together with comparative data for related sesquiterpenoid compounds. These are hydroxy derivatives of the parent eremophiladiene in which the diene character is either retained (1-deoxycapsidiol **2**, capsidiol **3**) or converted to a monoene (debneyol **4**, 1-hydroxydebneyol **5**). All of these compounds retained the 9,10-double bond. The ^{1}H spectrum of **1** confirmed the absence of CHOH groups but revealed several features highly characteristic of the 4-*epi*-eremophiladiene structure. The presence of the isopropenyl substituent at C-7 is indicated by the chemical shifts of the methyl group Me-13 and the two exocyclic

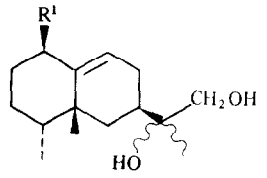
Table 1. ^{1}H NMR data for 5-*epi*-aristolochene and related compounds (270.17 MHz, $CDCl_3$, TMS as internal standard)

H	1	2	3	4	5
9	5.54 *dt* (6.5, 1.8, 1.8)*	5.60 *dd* (6.5, 1.8, 1.8)	5.94 *dd* (6.5, 2.0)	5.49 *dt* (6.4, 1.8, 1.8)	5.83 *dd* (7.3, 1.3)
12	4.68 *m*	4.68 *m*	4.69 *m*	—	—
12′	4.69 *m*	4.71 *m*	4.73 *m*	—	—
13	1.73 *m*	1.74 *m*	1.74 *m*	—	—
14	0.99 *d*	0.92 *d*	0.88 *d*	0.97 *d*	0.95 *d*
15	1.17 *s*	1.18 *s*	1.37 *s*	1.17 *s*	1.37 *s*

* Coupling constants in parenthesis.



1 $R^1 = R^2 = H$
2 $R^1 = H, R^2 = OH$
3 $R^1 = R^2 = OH$



4 $R^1 = H$
5 $R^1 = OH$

olefinic protons H-12, H-12′. Furthermore, the fine structure of the latter peaks (allylic coupling) is essentially identical to that observed in **2** and **3** in keeping with the β-configuration at C-7 shown in **1**. We have established previously [1] that a characteristic of the presence of a C-9,10 double bond in the eremophilane structure is stereospecific coupling between H-9 and H-1_{ax} ($^4J = 1.8$ Hz); coupling between H-9 and H-1_{eq} is not observed. Thus the doublet of triplets observed at δ5.54 in the spectrum of **1** establishes the presence of an endocyclic double bond and its position as Δ^9 (cf data for **2** and **4**), and confirms that the cyclohexene ring has an envelope conformation.

Aristolochene, first isolated in 1970 [3], has *cis* disposed methyl groups Me-14, Me-15 with chemical shifts of δ 0.83 and 0.95 respectively. In contrast 5-*epi*-aristolochene (**1**) has *trans* disposed methyl groups both occuring at lower field, δ0.99 and 1.17 respectively, due to the removal of a mutual shielding effect. The downfield shift is less for Me-14 since it is now in an axial position. With reference values for the shifts of the methyl groups in the parent system it is apparent now that the shifts recorded for the substituted derivatives **2**–**5** are exactly as expected, the effect of axial hydroxylation at C-1 being a shielding of Me-14 and a deshielding of Me-15. Precise chemical shifts could not be established for the remaining endocyclic protons due to severe overlap but those protons alpha to a double bond, H-1_{eq}, H-1_{ax}, H-7, H-8_{eq} and H-8_{ax} appeared as multiplets in the range δ1.8 to 2.3.

The mass spectrum of the new hydrocarbon had an $[M]^+$ ion at *m/z* 204 with a base peak at *m/z* 105 and the series of ions at *m/z* 161, 147 and 133. These are characteristic [4] of a Δ^9 eremophilene structure and are attributable to a fragmentation pattern involving loss of the C-7 substituent to give an ion at *m/z* 161 followed by allylic scission of the saturated ring to give an ion at *m/z* 105.

Thus, the NMR and MS data establish the structure of the hydrocarbon formed in the cell-free system as **1** and confirm that the Δ^9 -eremophilene system has the conformation previously established for its derivatives.

In the present study unknown **II** [1, 2] was not produced in either the large scale cell-free incubation mixtures used to prepare sufficient amounts of 5-*epi*-aristolochene for the characterization studies or in the small scale cell-free incubation mixtures used to prepare ^{14}C-labelled 5-*epi*-aristolochene for the feeding experiments. The reason(s) for this are not known.

Incorporation of ^{14}C-*labelled* 5-epi-*aristolochene into capsidiol and debneyol*

Biogenetically ^{14}C-labelled 5-*epi*-aristolochene was incorporated very rapidly and very efficiently into both capsidiol and debneyol by cellulase elicited cultures but only significantly into debneyol in unelicited cultures (Table 2). Furthermore, the ratio of [^{14}C]debneyol biosynthesized from the substrate relative to that of [^{14}C]capsidiol increased with time after elicitation as expected from previous findings [2] (see Table 2 and discussion).

DISCUSSION

It is notoriously difficult to establish that a compound is a true *in planta* intermediate in the later stages of the biosynthesis of a secondary metabolite due to the low

Table 2. Incorporation of radioactivity from ^{14}C-labelled 5-*epi*-aristolochene (**1**) into capsidiol (**3**) and debneyol (**4**) by unelicited and cellulase-elicited cultures of tobacco

Time after elicitation (hr)	Incorporation of radioactivity into choloroform-soluble lipids (% dose)				Ratio of capsidiol to debneyol
	Total	Capsidiol	Debneyol	Unknowns	
Unelicited	42.7	5.0	25.0	10.6*	1:5.0
3	64.0	40.0	19.7	1.0†	1:0.5
15	60.2	23.4	32.3	1.9†	1:1.4
35	54.2	11.3	40.6	0.0	1:3.6

The 5-*epi*-[^{14}C]aristolochene (370 pmol, 138,000 dpm) was added at the times indicated and the cultures harvested after 1 hr.

* R_f 0.33 i.e. just above debneyol.

† R_f 0.60 i.e. same as substrate.

substrate specificities of some of the enzymes concerned and the occurrence of aberrant reactions. Indeed, "it is much easier to show that a compound can serve as a precursor of a cell constituent than to determine whether it is a normal, obligatory intermediate in the biosynthesis of that constituent" [5]. In the case of 5-*epi*-aristolochene (**1**), however, the evidence is overwhelmingly in favour of it being a true *in planta* intermediate in the biosynthesis of capsidiol (**3**) and debneyol (**4**). Thus: it represents the first committed intermediate on the pathway leading from FPP to capsidiol and debneyol, and by analogy with other pathways of secondary metabolism, it is to be expected that its formation from FPP would be catalysed by enzymes showing a high substrate specificity; it is produced in good yield from FPP in cell-free systems prepared from elicited cells, and when the incubations are performed in the presence of NADPH and molecular oxygen the amount accumulated is reduced in proportion by an amount equivalent to the amount of capsidiol and debneyol synthesised [1, 2]; and it is very rapidly and very efficiently incorporated into capsidiol and debneyol when fed to elicited cultures (Table 2).

The biosynthesis of 5-*epi*-aristolochene from FPP can be envisaged to involve just two enzymes: An FPP-carbocyclase (FPP : germacrene A cyclase) which catalyses the conversion of FPP into the 10-membered ring system of (+)-germacrene A and a germacrene cyclase (germacrene A : 5-*epi*-aristolochene cyclase) which catalyses the conversion of germacrene A directly into the bicyclic eremophilene 5-*epi*-aristolochene. Based on the methyl and hydride migrations known to occur during the biosynthesis of capsidiol and debneyol [6–8], it seems plausible that the reaction mechanism of the germacrene cyclase would proceed in a concerted fashion involving a forward cyclization reaction to form a eudesmane carbocation followed by a backward rearrangement involving two Wagner–Meerwein shifts. However, it has not escaped our attention that such a mechanism would lead to the formation of a compound in which the two methyl groups (Me-14, Me-15) are *cis* to each other and not *trans* as found in 5-*epi*-aristolochene and its derivatives.

The present results support our previous assertion that the measurement of the incorporation of radioactivity from [1-$^3H_2$] FPP into capsidiol, debneyol, unknown **I** (5-*epi*-aristolochene) and possibly unknown **II** in cell-free systems, provides an indirect measure of FPP : germacrene A cyclase (FPP carbocyclase) activity [2]. This being the case, it is clear that the biosynthesis of capsidiol/debneyol and other related sesquiterpenoids involves the induction of FPP : germacrene A cyclase and presumably the putative germacrene A : 5-*epi*-aristolochene cyclase.

Our findings disprove previous proposals [9, 10] which had postulated that oxyfunctionalization occured prior to, and (in some schemes) was a prerequisite for, the formation of an eremophilene ring system from germacrene A. They also suggest that cyclization of germacrene A prior to oxyfunctionalization will be a characteristic of the other pathways of cyclic sesquiterpenoid biosynthesis which give rise to the vetispirane lubimin, the lubimin-derived *nor*-eudesmane rishitin and the *seco*-eudesmane phytuberin. Indeed, we have recently shown that a ^{14}C-labelled hydrocarbon with the TLC properties expected of vetispira-1(10),11-diene is accumulated in cell-free preparations of elicited potato tubers in which ^{14}C-lubimin synthesis from [1-^{14}C]IPP [11] or [1-$^3H_2$]FPP has been inhibited by the omission of NADPH or the exclusion of oxygen (DRT/IMW unpublished results).

At the present time neither the nature nor the sequence of the reactions involved in the conversion of 5-*epi*-aristolochene into either capsidiol or debneyol is known. However, it seems likely that both hydroxyl groups in capsidiol and at least one of the two hydroxyl groups in debneyol will be introduced by way of NADPH- and O_2-dependent hydroxylation reactions catalysed by mixed function monooxygenases (hydroxylases) [2]. Whilst it is possible that the biosynthesis of capsidiol from 5-*epi*-aristolochene involves a short metabolic grid there are several pieces of evidence which suggest that a single pathway is involved. Of the two possible monohydroxy derivatives of 5-*epi*-aristolochene only one, 3-hydroxy-5-*epi*-aristolochene (1-deoxycapsidiol) (**2**), has been isolated from natural sources [12]. When 9 μmol of this compound (chemically synthesized from capsidiol) was added to unelicited cultures of tobacco *ca* 1 μmol was converted into capsidiol in one hr (IMW unpublished work). This result suggests that the 1-hydroxylase is already present in unelicited cultures. In contrast to this, 5-*epi* [^{14}C]aristolochene is only very poorly incorporated into capsidiol in unelicited cultures (see Table 2). This evidence is consistent with specific induction of the 3-hydroxylase during the elicitation process and its catalysis of the first committed step in the biosynthesis of capsidiol from 5-*epi*-aristolochene. The data from the feeding experiments indicate that both of the enzyme activities needed for the biosynthesis of debneyol from 5-*epi*-aristolochene are present in unelicited cells (Table 2). These activities, however, may represent low basal levels since, unlike the feeding experiment with unlabelled 1-deoxycapsidiol just described, only 370 pmol of 5-*epi*-[^{14}C]aristolochene was fed to the unelicited culture. Our analytical and cell-free studies establish that the hydroxylases involved in the formation of capsidiol and debneyol from 5-*epi*-aristolochene show a high degree of substrate specificity.

The intermediacy of 5-*epi*-aristolochene in the biosynthesis of both capsidiol and debneyol poses some interesting questions with regard to the factors which determine the amounts of the phytoalexins accumulated by elicited cultures. Without invoking some theory on compartmentalization, the simplest scenario is that 5-*epi*-aristolochene is competed for directly by the enzymes which catalyse the first step on each specific pathway leading to either capsidiol or debneyol. In our recent study with crude cell-free preparations from elicited tobacco cultures we found the apparent K_m values for the overall synthesis of 5-*epi*-aristolochene, capsidiol and debneyol from FPP to be 10, 2 and 12 μM respectively [2]. These results suggest that the low overall K_m of the cell-free system synthesizing capsidiol is imposed by enzymes catalysing reactions subsequent to the formation of 5-*epi*-aristolochene (possibly the 3-hydroxylase).

Previous work in our laboratory [13] had indicated that the concentration of FPP is limiting for the biosynthesis of the early post-FPP intermediates of sterol biosynthesis in unelicited cultures of potato. In elicited tobacco cultures the activity of squalene synthetase falls rapidly to very low levels [2, J. Chappell, personal communication]. This response appears to represent a specific mechanism to channel available FPP away from sterol synthesis and into sesquiterpenoid phytoalexin formation. The level of microsomal HMG-CoA reductase (the putative key regulatory enzyme of phytosterol bio-

synthesis [14]) activity increases transiently in elicited tobacco cultures [15]. However, the measured activities of this enzyme were too low to account for the levels of capsidiol synthesized by the cultures. Although the *in vitro* levels are probably lower than the true *in vivo* levels, this result and the data with potato cell cultures [13] indicates that FPP concentrations are limiting for sesquiterpenoid phytoalexin biosynthesis in elicited cultures. At low FPP, and therefore low 5-*epi*-aristolochene concentrations, the higher affinity of the enzyme(s) synthesizing capsidiol will maintain the flux of carbon into that pathway. However, even at substrate limiting concentrations of FPP, the high V_{max} value for the overall synthesis of debneyol from FPP compared to that of capsidiol means that roughly equal amounts of both phytoalexins are produced [2]. The failure of the debneyol to accumulate in elicited cultures at the same rate as capsidiol is due to the very active catabolism of debneyol, but not capsidiol [2]. The inhibition of capsidiol catabolism after elicitation represents yet another control mechanism in the tier of events which regulate the accumulation of this compound.

The cell-free results [2] and the results of the feeding experiments (Table 2) with 5-*epi*-aristolochene have shown that the rate of synthesis of capsidiol starts to decrease *ca* 12 hr after elicitation whilst the synthesis of 5-*epi*-aristolochene and debneyol continues for (at least) a further 36 hr. This strongly suggests that the activity of the specific enzyme(s) (e.g. the 3-hydroxylase) required for the biosynthesis of capsidiol decreases during this period, with the result that the flux of carbon into the debneyol pathway would be maintained.

The results of the short-term feeding experiments with 5-*epi*-[^{14}C] aristolochene confirms and extends previous suggestions about the regulation of phytoalexin biosynthesis in elicited tobacco cells [2, 15]. Thus, control of sesquiterpenoid phytoalexin synthesis is exerted at a primary level by the inhibition of squalene synthetase and concomitant induction of both HMG-CoA reductase and FPP: germacrene A cyclase, and at a secondary level by the activities of specific hydroxylases.

Now that we have successfully established the identity of the hydrocarbon formed in the cell-free system as 5-*epi*-aristolochene and have confirmed it to be a common, committed precursor of both capsidiol and debneyol, we intend to work on the fractionation of the cell-free system. It is possible that such an approach will give FPP-carbocyclase-containing fractions which synthesize (+)-germacrene A but not 5-*epi*-aristolochene. Furthermore, admixture of these fractions with others may then allow the detection of the germacrene cyclase.

EXPERIMENTAL

Radiochemicals and chemicals. [1-^{14}C]IPP (56 μCi/μmol) was purchased from Amersham International plc, [1-$^{3}H_2$]FPP (0.79 μCi/μmol) was prepared by mixing [1-$^{3}H_2$]FPP (40 μCi/μmol) from a previous synthesis in our laboratory [2] with unlabelled FPP synthesized from *E,E*-farnesol (Aldrich) by the method of ref. [16]. All other chemicals were Analar or of the best grade available. Et_2O and petrol (40–60°) were dried over Na/Pb alloy and redistilled from reduced Fe prior to use.

Biological methods. The growth, maintenance and elicitation of the *Nicotiana tabacum* var. White Burley cell suspension cultures has been described previously [1, 2].

Isolation and characterization of 5-epi-*aristolochene.* The cells from ten 7-day-old cultures (ea 120 ml; late log phase) which had been elicited with cellulase (Ex. *Trichoderma viride*, Sigma) for 12 hr were harvested by filtration of the cultures through Miracloth and whilst still in the filter, washed with 3 l of dist. H_2O. The washed cells (410 g) were mixed with 200 ml ice-cold 0.1 M Pi buffer (pH 7.5)–0.5 M sucrose–2 mM EDTA–2 mM 2-mercaptoethanol containing 40 g insoluble PVP (Sigma) and homogenized in a Waring Blendor (2 × 30 sec). The homogenate, after filtration through two layers of Miracloth, was centrifuged at 4500 *g* for 10 min at 0°. The 4500 *g* supernatant (cell-free preparation) was stirred into an equal vol (370 ml) of ice-cold half-strength homogenization buffer and $MgCl_2$ and [1-$^{3}H_2$]FPP (0.79 μCi/μmol) added to a final concn of 10 and 25 mM respectively. The incubation mixture was dispensed into five 250 ml conical flasks which were incubated in a shaking water bath for 1 hr at 30°. The reaction was stopped by the addition of 4 vol. $CHCl_3$–MeOH (1:2) and, after leaving the mixture to stand for 0.5 hr, the $CHCl_3$-phase was recovered by a suitably scaled up version of our standard method [12]. In all subsequent procedures appropriate precautions were taken to reduce the loss of the volatile product. The $CHCl_3$ phase (*ca* 950 ml) was dried by passage through a 4 cm bed of dry Na_2SO_4 contained in a sintered glass funnel and the volume of the extract reduced to a small vol. *in vacuo* at 30°. The extract was then transferred to a glass centrifuge test tube with $CHCl_3$ and the solvent carefully removed under a stream of N_2. The residue was dissolved in 10–20 ml of boiling petrol (40–60°) and the solution maintained at −10° overnight. The ppt. produced by this procedure was removed by centrifugation (10 min at 4500 *g*), then resuspended in ice-cold petrol and removed again in the same way. (Removal of petrol-insoluble material at this stage was crucial for TLC purification.) The supernatants were combined and the solvent removed under a stream of N_2. The extract was then subjected to prep TLC on 0.5 mm rhodamine 6G-impregnated silica gel G plates (predeveloped with Et_2O) with petrol (40–60°) as developing solvent. The product was detected by radio-TLC (R_f 0.60), eluted from the gel with Et_2O, taken to dryness under a stream of N_2 and subjected to a second prep. TLC step using spectroscopically pure cyclohexane as developing solvent and the product again located by radio-TLC (R_f 0.50). The area of the gel containing all of the radioactivity was coincident with a pink band visible on the plate under UV_{254} light which after elution with Et_2O and careful removal of the solvent under N_2 gave a transparent oil. The yield of 5-*epi*-aristolochene calculated from its radioactive content was 1.1 mg (5.4 μmol). Radio-GC (conditions as described, except column temp. 110° [11]) gave a single mass peak (R_t 1.7 min) containing 85% of the injected radioactivity. GC-EIMS (Finnegan 1020, 25 m SE 54 capillary column, 20 psi He, 70 eV, 130° isothermal) also gave a single mass peak with an R_t of 8.7 min. *M/z* (rel. int.): 204 $[M]^+$ (7.8), 189 $[M-Me]^+$ (5.4), 175 (1.0), 161 $[M-Me(CH)CH_2+H]^+$ (21.4), 147 (10.0), 133 (17.1), 121 (18.6), 105 (100), 91 (35.4), 79 (22.7), 67 (11.4), 55 (18.3) and 41 (30.2). ^{1}H NMR: see Table 1 and text.

Feeding experiments. Extracts containing 5-*epi*-[2,6,8,-$^{14}C_3$]Aristolochene (^{14}C-**1**) were freshly prepared by incubation of cell-free preparations (prepared by method B) with [1-^{14}C]IPP (56 μCi/μmol) as described in [2]. Radio-TLC analysis showed that at least 95% of the radioactivity in the crude $CHCl_3$ extracts was associated with **1** and these were used without further purification. ^{14}C-**1** (138 000 dpm; 168 nCi/nmol) was added in Me_2CO (100 μl) directly to elicited or unelicited cultures at the appropriate times (see Table 2). Cultures were harvested 1 hr after the addition of the label by filtration through Miracloth and rinsed with 100 ml H_2O. The biomass and

filtrates were then analysed by the procedures described below for the presence of radioactive phytoalexins.

Extraction of media. The filtrates were extracted (x2) with an equal vol. of Et_2O. The Et_2O extracts were combined, taken to dryness *in vacuo*, transferred to a small vial and dissolved in 100 μl $CHCl_3$. 10 μl was assayed for radioactivity and 20 μl was TLC'd (as described above) with EtOAc–cyclohexane (1:1) as developing solvent. The ^{14}C-labelled products were detected by radio-TLC. The areas of the gel containing the labelled products were scraped off and assayed for radioactivity by liquid scintillation spectrometry [2]. The ^{14}C-labelled capsidiol and debneyol were characterized by the methods described previously [2].

Extraction of cells. Freshly harvested cells were homogenized in $CHCl_3$–MeOH (1:2; 5 ml/g fr. wt of cells) with an Ultra-Turrax homogeniser and the homogenate left to stand for 30 min. After centrifuging the homogenate at 8000 *g* for 15 min, the supernatant was dild with an equal vol. of H_2O and the mixture centrifuged at 5000 *g* for 15 min. After removal of the aq. layer by means of a pasteur pipette attached via a trap to a vacuum line, the $CHCl_3$ phase was evapd to dryness *in vacuo* and the extract analysed as above.

Acknowledgements—The authors would like to thank Mrs M. Huffee for her expert technical assistance with this project, Mr A. D. Roberts for obtaining the GC-MS data and the AFRC for financial support.

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