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ELM BARK BEETLE BORING AND FEEDING DETERRENTS FROM *PHOMOPSIS OBLONGA*

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Abstract—The two groups of *Phomopsis oblonga* which invade the phloem of stressed elm trees have been investigated for the production *in vitro* of boring/feeding deterrents for elm bark beetles The secondary metabolites of this fungus are compared with those of closely-related *Phomopsis* spp associated with ash and sycamore Active compounds isolated from *P oblonga* include a novel norsesquiterpene γ-lactone, the triglic esters of two novel 5,6-dihydro-5-hydroxy-2-pyrones, nectriapyrone, 4-hydroxyphenylethanol, 5-methylmellein, 2-furoic, orsellinic and 3-nitropropanoic acids and mellein-5-carboxylic acid, portensterol and thymine, from *P oblonga*, were inactive (+)-Mellein and furan-2,5-dicarboxylic acid were obtained as minor metabolites of the ash and sycamore *Phomopsis* strains, which also produced the norsesquiterpene γ-lactone and one of the triglic esters

INTRODUCTION

Phomopsis oblonga (Desm) Trav, a fungus frequently found in in the outer bark of healthy *Ulmus* spp, particularly wych elm (*U glabra*), can invade the phloem of stressed trees, principally those infected by *Ceratocystis ulmi*, the causative agent of Dutch elm disease [1, 2] Bark beetles of *Scolytus* spp, the insect vectors of the disease, reject *P oblonga*-invaded phloem as being unsuitable for breeding and such trees do not become brood trees [3] Although depletion of essential nutrients is believed to contribute to this effect [4], we have investigated *P oblonga* for the production of secondary metabolites with boring/feeding deterrent activity

RESULTS AND DISCUSSION

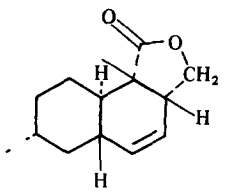
Two morphologically distinct types (groups 1 and 2) of *P oblonga* are found on elm [2, 5] Strains (numbers 118 and 119 respectively) of each group were grown on both malt extract and the natural (elm phloem) medium in both surface and shake culture Only one fermentation of each kind was conducted with the natural medium, which was not readily available On malt, the yields of the more important secondary metabolites appeared to be reproducible

A laboratory bioassay in which adult female *S scolytus* beetles were offered a choice between treated and untreated elm bark was developed It was used to monitor, firstly, organic solvent extracts of culture filtrates for boring/feeding deterrent activity, and secondly, the chromatographic separation of active extracts into their components Extracts containing both neutral and acidic constituents were investigated Neutral constituents were separated by column chromatography followed by preparative TLC Acidic constituents were separated by preparative TLC and by vacuum sublimation In some cases, the active products identified did not account for all of the activity in the original extract Unless solid products were readily obtained, the identification of the constituents of inactive gummy fractions was not vigorously pursued

A number of active novel fungal metabolites were isolated from strain 118 comprising the norsesquiterpene γ-lactone **1** [6] and the triglic esters **2** and **3** of two 5,6-dihydro-5-hydroxy-2-pyrones [7] Because compounds **1** and **3** were also produced by closely-related *Phomopsis* spp associated with ash and sycamore [6, 7], a more detailed examination of the secondary metabolites of these strains (numbers 123 and 124, respectively), grown on malt medium, was undertaken

Additional active, but known, compounds isolated from fermentations with strain 118 were 2-furoic (**4**, R = H), orsellinic (**5**) and 3-nitropropanoic (**6**) acids and 5-methylmellein (**7**, R = Me), inactive compounds obtained were hydroquinone (an artefact) and fumaric and succinic acids Active known compounds isolated from strain 119 were 5-methylmellein, nectriapyrone (**8**), 4-hydroxyphenylethanol (**9**, R = OH), mellein-5-carboxylic acid (**7**, R = CO_2H) and 2-furoic acid, portensterol (**10**) and thymine (**11**), each obtained on only one occasion, were inactive The fermentation conditions responsible for these isolations are summarized in Table 1 Metabolites isolated from strains 123 and 124 are listed in Table 2

The conditions under which compounds **1**–**3** are produced have been discussed elsewhere [6, 7] 5-Methylmellein (**7**, R = Me), a metabolic product of the almond pathogen *Fusicoccum amygdali* [8] and of numerous *Hypoxylon* spp and *Numularia* spp [9], was produced in reasonable yield (2–8 mg/l) by strain 119 under all the conditions investigated, and by strain 118 in shake culture on elm medium (1 mg/l) It was not produced by strain 123 or 124, which, however, both produced, in surface culture (1 mg/l), (+)-mellein (**12**) [10] with the opposite configuration at position 3 5-Methylmellein is



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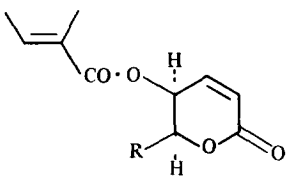
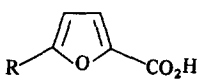


2 R = Me·CHOH·CO·CH^{Z}=CH—
3 R = Me·CHOH·CHOH·CH^{E}=CH—



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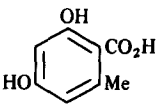

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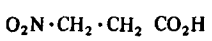

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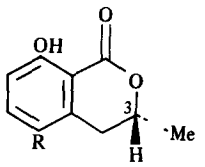

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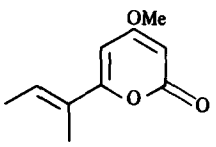

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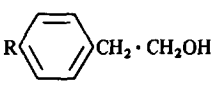

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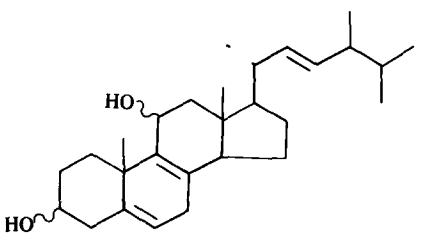

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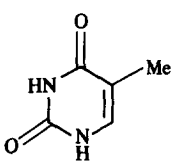

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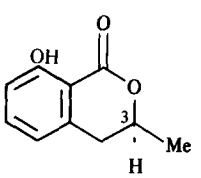

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sometimes accompanied by mellein-5-carboxylic acid (7; R = CO_2H) [9], but in the *P oblonga* fermentations this acid was only isolated from strain 119 in shake culture on malt Nectriapyrone (8), a metabolic product of *Gyrostroma missouriense* [11], was obtained only in low yield (1 mg/l) in shake culture on malt and its characteristic UV absorption makes it unlikely that it would have remained undetected if produced in surface culture or on elm medium The phytotoxic 4-hydroxyphenylethanol (9, R = OH), a metabolic product of the plant pathogens *Ceratocystis fimbriata* and *Gloeosporium lacticolor* [12, 13], was produced in reasonable yield (3 mg/l) in surface culture on malt Under these conditions, the ubiquitous related fungal volatile phenylethanol (9, R = H) was a metabolic product of strain 124 Portensterol (10) has previously only been obtained from the fruiting bodies of *Trichloma portentosum*, *Rhodopaxillus nudus* and *Clitocybe nebularis* [14, 15]

Initially, because of the known presence of 2-furfuraldehyde and 2-furfuryl alcohol in malt extract [16], little importance was attached to the isolation of 2-furoic acid (4, R = H) (2–7 mg/l) when strain 118 was grown on this medium However, these obvious precursors are absent from elm phloem medium and the acid (4, R = H) would therefore appear to be a genuine metabolic product of both groups of *P oblonga* It was not produced by strains 123 and 124, both of which produced 1–3 mg/l of the novel fungal metabolite furan-2,5-dicarboxylic acid (4, R = CO_2H) 2-Furoic acid has previously only been recorded as a metabolite of *Cercospora beticola* [17] though it is presumed to have been produced by *Penicillium chrysogenum* [18] The highly active orsellinic acid (5) and 3-nitropropanoic acid (6) occur commonly as fungal secondary metabolites particularly of *Penicillium* sp and *Aspergillus* sp [19, 20] They were obtained from strain 118 on malt medium in surface (1 mg/l) and shake

Table 1 Isolation conditions and boring/feeding deterrent activity of *P oblonga* metabolites

		Group (strain number)							
		1 (118)				2 (119)			
		Surface culture		Shake culture		Surface culture		Shake culture	
Compound	Activity	Malt†	Elm†	Malt	Elm	Malt	Elm	Malt	Elm
1	+	*	—	—	—	—	—	—	—
2	++	*	—	*	—	—	—	—	—
3	+	*	—	*	—	—	—	—	—
7 (R = Me)	+	—	—	—	*	*	*	*	*
8	+							*	
9 (R = OH)	+					*			
10	−							*	
11	−							*	
4 (R = OH)	+	*	*	*	—	*	*	*	*
5	++	*	—	—	—				
6	++			*	—	—		—	
7 (R = CO_2H)	+					—	—	*	—

* = present, — = absent
†Type of medium

Table 2 Metabolites obtained from *Phomopsis* spp 123 (ash) and 124 (sycamore) on malt

	Strain number			
	123		124	
Compound	Surface culture	Shake culture	Surface culture	Shake culture
1	*	—	*	*
3	*	—	—	*
9 (R = H)			*	
12	*		*	
4 (R = CO_2H)	*	*		*

* = present, — = absent

(4 mg/l) culture respectively, but under no other conditions

The course of a typical surface fermentation with *P oblonga* strain 118 on malt extract is shown in Table 3 Despite the formation of acidic metabolites, the pH of the culture filtrate stayed roughly constant whilst the optical rotation fell to zero in 28 days. The amount of solvent-extractable product barely doubled between days 7 and 35 and TLC of these extracts suggested that, with the exception of orsellinic acid for which there was no evidence before day 14, metabolites **1–5** were present at least from day 7 onwards The course of a similar fermentation with strain 119 is shown in Table 4 Apart from a sharp rise in pH when the optical rotation of the culture filtrate reached zero, the fermentation took a similar course Because the amounts of 5-methylmellein produced were small, it was not found possible to devise an accurate simple analytical procedure for this compound in fermentation broth based on the characteristic UV absorption λ_{max} 249, 324 nm (log ε 3 84, 3 65) TLC of the neutral extract suggested that 5-methylmellein was present in the broth from at least day 7 onwards and the metabolite was formally identified at day 10, as was 4-hydroxyphenylethanol

Although it contains a number of plant pathogens associated with leafspot and die back, the genus *Phomopsis* has been little investigated for the formation of biologically active secondary metabolites Mammalian toxicity and phytotoxicity associated with *P paspalli* and *P sojae* have been attributed to the presence of cyto-

Table 3 Course of a typical surface fermentation with *P oblonga* strain 118 on malt medium

	Time (days)					
	0	7	14	21	28	35
Optical rotation (°)	+2 205	+1 543	+0 984	+0 381	−0 025	+0 021
pH	5 1	5 3	5 4	5 3	5 4	5 5
Neutral extract (mg/l)	0	29	32		55	54
Acidic extract (mg/l)	0	21	53		69	62

Table 4 Course of a typical surface fermentation with *P oblonga* strain 119 on malt medium

	Time (days)					
	0	7	14	21	28	35
Optical rotation (°)	+2 205	+2 051	+1 462	+0 734	+0 113	−0 039
pH	5 1	4 7	5 0	4 8	4 7	5 7

chalasins, notably cytochalasin H and deacetylcytochalasin H and their close relatives [21, 22] The tetrahydroanthraquinone altersolanol A and a number of related anthraquinone pigments have been identified from *P juniperovora* [23] Phomopsin A, a toxic halogen-containing cyclic hexapeptide, has been isolated from *P leptostromiformis* [24], and *P viticola* is a source of methanethiol [25] None of these compounds were detected in the present work

Although the *Phomopsis* strains from ash and sycamore resembled *P oblonga* group 1 in producing compounds **1** and **3**, the resemblance ended there (compare Tables 1 and 2) From this investigation there was no evidence for the formation in high yield of a single, highly active, boring/feeding deterrent by *P oblonga* Rather, both *P oblonga* groups were capable of producing small amounts of a range of secondary metabolites with moderate deterrent activity The pattern of secondary metabolites produced by the two groups was very different Of the active compounds isolated, only 5-methylmellein and 2-furoic acid were produced on both media and by both groups there was no evidence for the formation in elm medium of the chemically more interesting metabolites **1**, **2**, **3** and **6**

EXPERIMENTAL

Mps were taken on a Kofler hot-stage apparatus and are corr IR spectra were determined on Nujol mulls and UV spectra and optical rotations were measured in MeOH NMR spectra, at 90 MHz, were obtained in $CDCl_3$ with TMS as internal standard M_rs were taken from high-resolution mass spectra In analytical TLC, Merck silica gel F_{254} was used with $CHCl_3$–MeOH (19 1) (neutral material) or di-isopropyl ether–HCO_2H–H_2O (90 7 3) (acidic material) Merck silica gels 7739 and 7734 were used in prep TLC (0 1 cm layer) and in CC, respectively Petrol had bp 60–80°

In vitro *bioassay for determent of boring and feeding by* S scolytus The base of a glass Petri dish (9 cm diameter) was coated with a layer (4 mm) of paraffin wax and the wax was covered with a filter paper circle embedded at the edge by the application of heat The test compound or extract (3 0 or 0 3 mg) in EtOH (0 5 ml) was applied to both sides (and ends) of a sliver of bark (2 × 1 × 0 2 cm) cut from a twig of English elm (*U procera*) and the solvent was removed in a stream of warm air A control bark sliver was treated similarly with EtOH The filter paper was moistened with water and the treated and control bark slivers were pinned to the wax approximately 2 cm from the perimeter of the dish and 5 cm apart Five newly-emerged adult female *S scolytus* beetles [26] were introduced, and the dish was closed, placed in an incubator at 28°, and examined daily for 2 days Each assay was carried out in duplicate The amount of boring/feeding activity associated with each bark sliver was assessed on an arbitrary scale of 0–3 Agreement between replicates was usually good After 48 hr, the control had normally achieved a score of 2 (10–50% chewed) or 3 (> 50% chewed) whilst the treated sliver was frequently untouched (score 0) or only slightly chewed (score 1) In this situation the test material was considered to be active in determent of boring and feeding at the concn used Activity was then assigned according to the following scale −, inactive at 3 0 mg, ±, doubtful activity at 3 0 mg, +, active at 3 0 mg, + +, active at 0 3 mg Towards the end of the investigation, 2-furoic acid (+) was sometimes included as a standard

Media Elm medium was most easily prepared in the month of June The outer bark was stripped from a section (30 × 45 cm diameter) of a freshly cut log of English elm (*U procera*), and the phloem (*ca* 2 cm thick) was prized away, broken up, cooled in liquid N_2, and powdered in a steel mortar Batches (125 g) of the powder were shaken with H_2O (1 l) for 5 min and then autoclaved for 20 min at 120° The solid was filtered off and the filtrate was dispensed into flasks or Roux bottles, which were then autoclaved in the usual way

Malt medium contained 2% 'Oxoid' malt extract

Fermentations (a) *Surface culture* Roux bottles (usually batches of 50) containing the medium (200 ml) were inoculated with a mycelial suspension (1 ml) of the *Phomopsis* strain prepared from a 5-day shake culture on the same medium The bottles were incubated at 25° in artificial light At intervals (usually 1 week), a bottle was selected at random and harvested and the pH and optical rotation of the culture filtrate were measured (the latter analysis was not possible with the dark-coloured elm medium) The culture filtrate was extracted with EtOAc, first at the natural pH, usually 5 0–6 0, and then, after the addition of HCl, at pH 2 5–3 0 The neutral and acidic extracts were then examined for specific UV absorption and by TLC The bottles remaining were harvested soon after the optical rotation had come close to zero (30–40 days)

(b) *Shake culture* Conical flasks (250 ml) containing the medium (100 ml) were inoculated as described above and shaken at 140 rpm on a rotary shaking machine Essentially the same conditions for incubation and harvesting (14–20 days) were employed

Separation of extracts into their constituents by prep TLC The air-dried plate was examined in UV light With acidic constituents, the silica from each band was extracted with warm H_2O and filtered off The aq filtrate was adjusted (if necessary) to pH 3 with HCl, extracted with EtOAc and the solute recovered Neutral constituents were extracted from silica with $CHCl_3$ or $CHCl_3$–MeOH (49 1), depending on the R_f being > or < 0 2, respectively

Isolation of secondary metabolites (A) *Strain* 118 (a) *Surface culture* (i) *Malt medium* In the fermentation described elsewhere [6], extraction of the culture filtrate (11 1 l) gave neutral (508 mg, activity + +) and acidic (293 mg, activity +) material CC of the neutral material giving compounds **1**–**3** has already been described [6, 7]

Prep TLC of the acidic material (six 40 × 20 cm plates) gave three bands visible in UV light at R_f 0 71, 0 57 and 0 22

Extraction of the R_f 0 71 band furnished prisms (7 mg), mp 170° dec (from EtOAc), UV λ_{max} 260, 300 nm, identified as orsellinic acid (**5**), activity + + (Found M_r 168 0422 Calc for $C_8H_8O_4$ M_r 168 0422) by comparison of the IR spectrum (ν_{max} 3520, 3440, 1640, 1615 cm^{-1}) with an authentic specimen supplied by Prof R Thomas (lit [19] mp 176° dec) The R_f 0 57 band furnished prisms (18 mg), mp 123–125° (from EtOAc) λ_{max} 252 nm, identified as 2-furoic acid (**4**, R = H), activity + (Found M_r 112 0151 Calc for $C_5H_4O_3$ M_r 112 0160) by the IR spectrum ν_{max} 3140, 2650, 2560, 1675, 1580, 755 cm^{-1} (lit [18] mp 129–130°) From the R_f 0 22 material, by sublimation at 100–130°/10^{-1} mm and crystallization of the sublimate from EtOAc there was obtained a solid, mp 170°, UV λ_{max} 227, 296 nm, identified as hydroquinone, activity −, R_f 0 78 (Found M_r 110 0370 Calc for $C_6H_6O_2$ M_r 110 0368), by the mass spectrum The R_f indicates that this compound is an artefact Trituration with EtOAc of the acidic material (450 mg) from a replicate fermentation yielded succinic acid (20 mg), activity −, identified by the IR spectrum.

(ii) *Elm medium* Extraction of the culture filtrate (3 5 l, pH 7 5) after 42 days yielded neutral (90 mg, +) and acidic (105 mg, −) material CC (6 g silica, 12 × 1 2 cm) of the neutral material and elution with C_6H_6 (100 ml), and C_6H_6–MeOH (100 1, 150 ml, 50 1, 100 ml) yielded a series of inactive fractions (total 31 mg) C_6H_6–MeOH (20 1, 50 ml) then eluted a resin (6 mg, +), UV λ_{max} 227 nm, which proved intractable It did not contain the dihydropyrone **3**, as judged by the mass spectrum Compounds **1** and **2** were absent from the earlier fractions on spectroscopic evidence Prep TLC (three 40 × 20 cm plates) of the acidic material showed only one band, R_f 0 57, in UV light Extraction furnished 2-furoic acid (7 mg), identified as described above

(b) *Shake culture* (i) *Malt medium* Extraction of the culture filtrate (3 5 l, pH 5 6) after 14 days gave neutral (483 mg, +) and acidic (465 mg, +) material CC of the neutral material gave active fractions from which the dihydropyrones **2** and **3** were obtained [7] Prep TLC (two 40 × 20 cm plates) of a portion (115 mg) of the acidic material led to the isolation of 2-furoic acid (6 mg), as described above The remainder, when triturated with EtOAc, yielded a solid (11 mg), mp 62°, R_f 0 53 Recrystallization from C_6H_6 gave hexagonal plates, mp 68–69° (Found C, 30 2, H, 4 5, N, 11 2 Calc for $C_3H_5O_4N$ C, 30 3, H, 4 2, N, 11 7%), identified as 3-nitropropanoic acid (**6**) (lit [20] mp 67–68°) by the NMR [δ_H 3 00 (2H, *t*), 4 60 (2H, *t*), 8 1 (1H, *br*, OH)] and IR spectra [ν_{max} OH br 1700, 1560 cm^{-1}] Sublimation at 80°/10^{-2} mm of the residue recovered from soln in EtOAc gave more (4 mg) of the acid **6**

(ii) *Elm medium* After 15 days, extraction of the culture filtrate (4 9 l) gave neutral (148 mg, ±) and acidic (117 mg, +) material The neutral material (141 mg) in C_6H_6 (2 ml) was applied to a column of silica gel (6 g, 12 × 1 2 cm) made up in C_6H_6 After inactive gummy fractions (total 44 mg) had been eluted with C_6H_6 (50 ml), C_6H_6–MeOH (100 1, 100 ml) eluted a gum (7 mg) which crystallized from petrol in prisms, mp 121–122° (+), identified as 5-methylmellein (**7**, R = Me) by its spectroscopic properties (see below) Further elution of the column with C_6H_6–MeOH (50 1, 100 ml and 20 1, 50 ml) gave inactive gums (46 mg)

Prep TLC of a portion (74 g) of the acidic material gave three bands, R_f 0 70, 0 56 and 0 34 The R_f 0 70 material (5 mg) on trituration with EtOAc yielded a powder, mp 285° dec, identified as fumaric acid (−) by the IR spectrum The R_f 0 56 material (5 mg) was inactive The R_f 0 34 material (8 mg, +) crystallized from EtOAc in prisms, mp 234–237° dec of an acid (Found M_r 224 0683 Calc for $C_{11}H_{12}O_5$ M_r 224 0685), IR ν_{max} 3200–2500 br, 1680, 1607, 1535 cm^{-1}, UV λ_{max} 234, 342 nm (log ε 4 39, 4 02), which could not be identified

The remainder (37 mg) of the acidic material was heated in a vacuum-sublimation apparatus at 80–130°/10^{-2} mm, but no sublimate of 3-nitropropanoic acid was obtained

(B) *Strain* 119 (a) *Surface culture* (i) *Malt medium.* Extraction of the culture filtrate (6 0 l, pH 4 4) after 38 days gave neutral (305 mg, +) and acidic (348 mg, +) material The neutral material was absorbed onto silica gel (2 g) from EtOAc and placed on top of a column of silica gel (18 g, 24 × 1 5 cm) made up in C_6H_6 After gums (54 mg) had been eluted with C_6H_6 (150 ml), C_6H_6–MeOH (200 1, 100 ml and 100 1, 100 ml) eluted a solid (21 mg) which crystallized from petrol in prisms, mp 126°, R_f 0 65 of 5-methylmellein (**7**, R = Me) (Found M_r 192 0784 Calc for $C_{11}H_{12}O_3$ M_r 192 0786), IR ν_{max} 1660, 1610 cm^{-1}, UV λ_{max} 222, 248, 326 nm, $[\alpha]_D^{20}$ −111° (*c* 0 02515) (lit [8] mp 126–127°, $[\alpha]_D^{21}$ −115°), ^1H NMR δ_H 1 54 (*d*, *J* = 6 4 Hz, 3-Me), 2 20 (*s*, 5-Me), 2 77 (*dd*, *J* = 10 7, 16 6 Hz, 4α-H), 2 97 (*dd*, *J* = 4 1, 16 6 Hz, 4β-H), 4 70 (*ddq*, *J* = 6 4, 10 7, 4 1 Hz, 3-H), 6 80, 7 28 (AB, *J* = 8 5 Hz, 6- and 7-H)

After gums (10 mg) had been eluted with C_6H_6–MeOH (100 1, 150 ml), C_6H_6–MeOH (50 1, 200 ml) eluted a brown oil (56 mg, +) This, on prep TLC, was split into five fractions, R_f 0 75 (3 mg), 0 70 (4 mg), 0 67 (4 mg), 0 60 (2 mg) and 0 56 (6 mg), none of which yielded a pure component Further elution of the column with C_6H_6–MeOH (20 1, 150 ml) furnished a gum (84 mg, +), split by prep TLC into three fractions, R_f 0 43 (6 mg, +), 0 21 (12 mg, +) and 0 15 (19 mg, +) The first two fractions were intractable but the last, UV λ_{max} 225, 280, ~ 286 nm, contained 4-hydroxyphenylethanol (**9**, R = OH) (see below) on spectroscopic evidence

An aliquot (175 mg) of the acidic material was subjected to prep TLC giving five fractions, R_f 0 70 (5 mg) 0 52 (11 mg), 0 43 (9 mg), 0 29 (9 mg) and 0 23 (5 mg) All were inactive apart from the R_f 0 70 (+) material, which contained some 2-furoic acid (TLC, MS) The major component of this fraction, an oil, R_f 0 64, was not identified

On one occasion, part (6 bottles) of a replicate fermentation was harvested (0 75 l, pH 5 0) after 10 days, giving neutral (36 mg, +) and acidic (46 mg, −, discarded) material Prep TLC of the neutral material gave eight fractions, R_f 0 69 (3 mg), 0 62 (3 mg), 0 55 (1 mg), 0 47 (3 mg), 0 31 (4 mg), 0 22 (2 mg), 0 17 (2 mg) and 0 13 (3 mg, +) The R_f 0 62 fraction solidified on trituration with petrol and was identified as 5-methylmellein by the IR spectrum The R_f 0 13 fraction, UV λ_{max} 225, 280, ~ 286 nm, did not crystallize but was identified as 4-hydroxyphenylethanol (**9**, R = OH), R_f 0 13, λ_{max} 226, 280, ~ 286 nm, by the NMR [δ_H 1 6 (2H, *br s*, 20H), 2 80 (2H, *t*, C<u>H</u>$_2$Ar), 3 83 (2H; *t*, C<u>H</u>$_2$OH), 6 76 and 7 05 (4H, AA'BB', *J* = 8 6 Hz)] and mass spectra [*m/z* 138 (26), 107 (100), 77 (15)]

(ii) *Elm medium* After 39 days, extraction of the culture filtrate (9 2 l, pH 8 0) gave neutral (395 mg, + +) and acidic (665 mg, + +) material In CC of the neutral material, after oils (9 mg) had been eluted with C_6H_6 (50 ml), C_6H_6 (100 ml) and C_6H_6–MeOH (200 1, 50 ml) eluted a solid (74 mg) which gave 5-methylmellein (39 mg), mp 125°, on recrystallization from petrol After an intermediate fraction (6 mg), C_6H_6–MeOH (100 1, 50 ml and 20 1, 100 ml) eluted gums, 55 mg, + and 62 mg, +, respectively Preparative TLC of a portion (45 mg) of the former gave three bands which only furnished intractable gums, R_f 0 65 (9 mg), 0 56 (5 mg) and 0 45 (4 mg) The latter, R_f 0 15, was also intractable

Prep TLC (four 40 × 20 cm plates) of an aliquot (333 mg) of the acidic material gave four bands all of which yielded active (+) gums, R_f 0 64 (8 mg), 0 48 (9 mg), 0 40 (7 mg) and 0 26 (11 mg) 2-Furoic acid was obtained from the R_f 0 64 gum but the remaining gums proved intractable

(b) *Shake culture* (i) *Malt medium.* After 14 days, extraction of the culture filtrate (6 51, pH 4 8) gave neutral (435 mg, ±)

and acidic (378 mg, +) material The neutral material (425 mg) in C_6H_6 (5 ml) was chromatographed on a column of silica gel (12 g, 25 × 1 2 cm) made up in C_6H_6 After an inactive oil (10 mg) had been eluted with C_6H_6 (50 ml), C_6H_6 (100 ml) followed by C_6H_6–MeOH (200 1, 100 ml) eluted a solid (73 mg) which gave 5-methylmellein (51 mg), mp 125°, on crystallization from petrol Further elution of the column with C_6H_6–MeOH (100 1, 200 ml) gave a yellow oil (92 mg), which was subjected to prep TLC (two 40 × 20 cm plates) Semi-solid material, R_f 0 65 (16 mg, +), was sublimed at 80°/10^{-2} mm and then crystallized from petrol in needles, mp 100–102°, UV λ_{max} 228, 332 nm (log ε4 61, 4 16) (Found M_r 194 0942 Calc for $C_{11}H_{14}O_3$ M_r 194 0943), identified as nectriapyrone (**8**) (lit [11] mp 100–102°) by comparison of the IR spectrum (ν_{max} 1710 sh, 1690, 1650 w, 1622, 1557, 1170, 800, 742 cm^{-1}) with a spectrum (provided by Dr M S R Nair) of authentic material

A gum, R_f 0 35 (12 mg, +), UV λ_{max} 221, 250, 320 nm, ν_{max} (film) 3280, 1675, 1610 cm^{-1}, MS *m/z* 219, 191, 105, was not identified Continued elution of the column with C_6H_6–MeOH (50 1, 100 ml and 20 1, 200 ml) gave an oil (144 mg), which separated into two inactive gums, R_f 0 28 (20 mg) and R_f 0 19 (26 mg), on prep TLC The R_f 0 28 gum had λ_{max} 300 nm (Found M_r 212 1060 Calc for $C_{11}H_{16}O_4$ M_r 212 1048) On one occasion, the oil crystallized from EtOAc in prisms (8 mg), mp 240° (−), R_f 0 08 (Found M_r 412 3309 Calc for $C_{28}H_{44}O_2$ M_r 412 3341), IR ν_{max} 3330, 1658, 1620, 1160, 1045, 1020, 962, 935, 855, 800, 720 cm^{-1}, ^1H NMR δ_H 0 60 (*s*, 3H), 0 84 (*d*, 6H, *J* = 6 8 Hz), 0 95 (*s*, 3H), 1 63 (*d*, 6H, *J* = 6 8 Hz), 3 57 (*m*, 1H), 4 00 (*m*, 1H), 5 25 (*m*, 3H), MS *m/z* (rel int) 412 (12), 394 (23), 379 (14), 376 (43), 361 (12), 287 (3), 269 (18), 251 (85), 69 (100) Identified as portensterol (**10**) (lit [14, 15] mp 248–250°) by comparison of the IR spectrum with one (provided by Dr I Morelli) of authentic material It formed a diacetate, mp 155–160° (Found *m/z* 436 [M − 60]$^+$ $C_{32}H_{48}O_4$ requires M_r 496) (lit [14] mp 160°)

On another occasion, the $C_{28}H_{44}O_2$ compound was absent and thymine (−) mp > 300° (Found M_r 126 0435 Calc for $C_5H_6N_2O_2$ M_r 126 0429) R_f 0 08 was obtained from this fraction and identified by the IR spectrum (ν_{max} 3200, 1735, 1670 cm^{-1})

Prep TLC of an aliquot (180 mg) of the acidic material gave three fractions, R_f 0 72 (10 mg, −), 0 60 (5 mg, +) and 0 35 (35 mg, +). the R_f 0 60 fraction crystallized from EtOAc–petrol in small prisms, mp 245°, $[\alpha]_D^{20}$ −55° (*c* 0 0246) (Found M_r 222 0539 Calc for $C_{11}H_{10}O_5$ M_r 222 0528) of mellein-5-carboxylic acid (**7**, R = CO_2H), UV λ_{max} 226, 252, 314 nm, IR ν_{max} 3170, 1695, 1660, 1580 cm^{-1} (lit [9] mp 247–249°), identified by comparison of the IR spectrum with that of an authentic specimen provided by Dr R L Edwards The R_f 0 35 fraction crystallized from EtOAc in small prisms (2 mg), mp 200° dec, which were not identified

(ii) *Elm medium.* Extraction of the culture filtrate (8 3 l, pH 7 8) after 18 days gave neutral (410 mg, +) and acidic (286 mg, +) material The neutral material was absorbed onto silica gel (3 g) from EtOAc and added to the top of a column of silica gel (12 g, 25 × 1 2 cm) made up in C_6H_6 Elution of a yellow band with C_6H_6 (100 ml) followed by C_6H_6–MeOH (200 1, 100 ml) gave a solid (91 mg), which furnished 5-methylmellein, mp 122–123° (31 mg), on recrystallization from petrol After elution of an inactive oil (16 mg) with C_6H_6–MeOH (100 1, 150 ml), C_6H_6–MeOH (50 1, 200 ml) eluted a yellow band giving an oil (71 mg). A portion (42 mg) of this was subjected to prep TLC giving three fractions, R_f 0 59 (7 mg, +) 0 45 (10 mg, −) and 0 38 (4 mg, −) The R_f 0 59 fraction possibly contained nectriapyrone on the basis of the UV absorption, λ_{max} 325 nm Continued elution of the column with C_6H_6–MeOH (20 1, 200 ml) gave an intractable inactive gum (65 mg), R_f 0 15

Prep TLC (three 40 × 20 cm plates) of a portion (240 mg) of the acidic material yielded two fractions, R_f 0 57 (4 mg, +) identified as 2-furoic acid, and R_f 0 31 (9 mg, +), a gum, which was not identified

(C) *Strain* 123 (a) *Surface culture* Extraction of the culture fluid (6 0 l, pH 7 0) after 25 days (optical rotation + 0 055) gave neutral (2 312 g) and acidic (1 070 g) material The isolation of compounds **1** and **3** from the neutral material by CC has been described elsewhere [6, 7] Before elution of the lactone **1**, C_6H_6–MeOH (200 1, 100 ml) eluted an oil (26 mg), which crystallized from petrol in prisms, mp 52°, $[\alpha]_D^{20}$ +90° (*c* 0 03), UV λ_{max} 217, 248, 316 nm, identified as (+)-mellein (**12**) by comparison of the IR spectrum (ν_{max} 1660, 1620, 1580 cm^{-1}) with that of an authentic specimen (lit [10] mp 56–57°, $[\alpha]_D^{21}$ +97°)

Trituration of the acidic material with EtOAc gave a solid (15 mg), mp > 280°, λ_{max} 264 nm (Found M_r 156 Calc for $C_6H_4O_5$ M_r 156), identified as furan-2,5-dicarboxylic acid (**4**, R = CO_2H) by comparison of the IR spectrum (ν_{max} 3300–2500 br, 1690, 1575 cm^{-1}) with that of a specimen prepared by the dehydration of mucic acid [27]

(b) *Shake culture* Extraction of the culture filtrate (4 8 l, pH 6 0) after 21 days gave neutral (770 mg) and acidic (207 mg) material This fermentation did not yield identifiable products other than furan-2,5-dicarboxylic acid (4 mg) from the acid fraction

(D) *Strain* 124 (a) *Surface culture* Extraction of the culture fluid (7 2 l, pH 5 1) after 31 days (optical rotation − 0 040) gave neutral (300 mg) and acidic (741 mg) material The isolation of the lactone **1** from the neutral material by CC has been described [6] Before elution of the lactone (**1**), C_6H_6–MeOH (200 1, 50 ml) eluted an oil (18 mg), which crystallized from petrol in prisms, mp 55°, of (+)-mellein, identified as described above After elution of the lactone (**1**), C_6H_6–MeOH (100 1, 50 ml) eluted a brown oil (75 mg) consisting of at least five components (TLC) One of these (17 mg), R_f 0 45, UV λ_{max} 220, 262 nm, was shown to be phenylethanol (**9**; R = H) by the IR (ν_{max} 3350, 1602, 1045, 745, 700 cm^{-1}) and NMR (δ_H 1 65, OH, 2 90, *t*, $C\underline{H}_2Ar$, 3 85, *t*, $C\underline{H}_2OH$, 7 3, *s*, 5H) spectra

Crystallization of the acidic material from EtOAc yielded succinic acid (105 mg), identified by the IR spectrum

(b) *Shake culture* Extraction of the culture filtrate (4 1 l, pH 6 7) after 21 days gave neutral (506 mg) and acidic (138 mg) material Compounds **1** and **3** were isolated from the neutral material [6, 7] but no other metabolites were identified Crystallization of the acidic material from EtOAc furnished small prisms (2 mg), mp > 250°, of furan-2,5-dicarboxylic acid

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