Natural Acetylenes. Part XXXV.[1] Polyacetylenic Acid and Benzenoid Metabolites from Cultures of the Fungus *Lepista diemii* Singer

By **V. Thaller** * and **J. L. Turner**, The Dyson Perrins Laboratory, Oxford University, Oxford OX1 3QY

Four known polyacetylenes ($NC\cdot[C\equiv C]_2\cdot CH=^{t}CH\cdot CO_2H$, $H[C\equiv C]_3\cdot CH=^{t}CH\cdot CO_2H$, $HO\cdot CH_2\cdot[C\equiv C]_3\cdot CH=^{t}CH\cdot CO_2H$, and $HO_2C\cdot CH=^{t}CH\cdot[C\equiv C]_2\cdot CH=^{t}CH\cdot CO_2H$) as well as four benzene derivatives (*p*-nitroanisole, *p*-nitrobenzaldehyde, 4-chloro-3-methoxybenzaldehyde, and 3-chloro-4-methoxybenzaldehyde) have been isolated from culture fluids. The aromatic compounds have not been reported previously as fungal metabolites.

ANALYSIS of the ether extract of the culture fluid of the Basidiomycete fungus *Lepista diemii* Singer revealed the presence of the four known polyacetylenic acids (I—IV; R = H); two more were present in trace amounts but were not identified. Diatretyne 2 (I; R = H) and diatretyne 3 (III; R = H) were the main metabolites; their concentrations in the culture fluid varied considerably with the conditions of fungal growth (Table). The occurrence of diatretynes in *Clitocybe* and *Lepista* species has been tentatively proposed [2] as support for Singer's classification of the Agaricaceae family of fungi. The biogenesis of the *L. diemii* polyacetylenes is being investigated.

The fungus produced in addition to the polyacetylenic acids a variety of benzenoid metabolites in low concentrations, and four of these [(V)—(VIII)], not previously reported as fungal metabolites, were isolated from the neutral fraction of the extract. This production also varied with growth conditions (Table). *p*-Nitrobenzaldehyde could be a degradation product of chloramphenicol; the latter was, however, not detected by comparative t.l.c. (limit of detection 3 μg per l of culture fluid) in the relevant fractions of the neutral part of the extract.

None of the metabolites were detected during the first 10 days of fungal growth.

EXPERIMENTAL

Instruments used: u.v., Unicam SP 800; i.r., Unicam SP 1000 and Perkin-Elmer 257; n.m.r., Perkin-Elmer R14; mass spectra (direct insertion) Varian-MAT CH7; m.p.s (corr.), Kofler hot-stage apparatus.

Liquid chromatography: silica gel H.B.L. M60 in columns and Merck $HF_{254+366}$ and $PF_{254+366}$ in 0·3 mm (t.l.c.) and 1 mm (p.l.c.) layers, respectively.

Light petroleum refers to the fraction b.p. 40—60°. All evaporations were carried out under reduced pressure.

Growth of Lepista diemii.—The fungus was grown on 3% malt extract; when maximum polyacetylene concentration (estimated by u.v.) was reached (38—48 days in surface cultures and 30—32 days in shaken cultures) the medium was withdrawn and replaced by a 4% glucose solution. In this, maximum polyacetylene concentration occurred within 25—30 days after reflooding in surface cultures and within 15—20 days in shaken cultures. Optimal growth conditions for the aromatic compounds were not investigated.

Isolation of Metabolites.—The metabolite concentrations which were found for the different fungal growth conditions are summarised in the Table. All metabolites (the acids as methyl esters), except the cyano-ester (I; R = Me), were directly compared with authentic specimens, which, when not already available, were synthesised by routes previously described. Only spectral data not published before are quoted. The isolation of metabolites from a surface culture grown on malt extract illustrates the procedure generally used.

[1] Part XXXIV, V. Thaller and J. L. Turner, *J.C.S. Perkin I*, 1972, 552.

[2] M. Anchel, W. B. Silverman, N. Valanju, and C. T. Rogerson, *Mycologia*, 1962, **54**, 249.

The culture medium (90 l; 120 flasks) was continuously extracted with ether for 36 h. The extract was concentrated to 300 ml and separated with saturated sodium hydrogen carbonate solution into an acidic and neutral fraction. Only the former showed polyacetylene absorption in the u.v.

Acid fraction. The concentrated fraction (*ca.* 1·1 g) was esterified with 5% sulphuric acid in methanol and the resulting esters were chromatographed on a silica gel column (100 g) with stepwise elution (10% ether–light petroleum to 50% methanol–ether), and fifteen 100 ml fractions were collected. Fractions 1, 8, and 11—15 were discarded (no typical absorption in the u.v. and i.r. spectra).

Fractions 2—4 (eluted with 10—20% ether–light petroleum) were concentrated and the residue (550 mg) was separated by p.l.c. (5% ether–light petroleum; 2 runs) into three bands (A, B, and C). The material from the least polar band (A) was rechromatographed and yielded on crystallisation (from light petroleum) needles of methyl non-*trans*-2-ene-4,6,8-triynoate (II; R = Me) (13·5 mg), which decomposed above 60° (lit.,[3,4] decomp. 20° and above 80°, respectively), *m/e* 158 (M^+, 90%), 130 (24), 127 (50), 99 (83), 98 (100), and 87 (49), τ (CCl_4) 7·5 (s, C≡CH), 6·02 (s, $CO_2{\cdot}CH_3$), 3·2 (d, *J* 16 Hz, *trans*-C≡C·CH=C*H*·CO_2Me), and 3·01 (d, *J* 16 Hz, *trans*-C≡C·C*H*=CH·CO_2Me). The material from band B was crystallised (from tetrachloromethane–hexane) and yielded methyl 7-cyanohept-*trans*-2-ene-4,6-diynoate (I; R = Me) (432 mg), m.p. 102—103·5° (lit.,[5] 102—103·5°), *m/e* 159 (M^+, 68%), 128 (100), 100 (60), 99 (36), and 88 (10), τ (CCl_4) 6·2 (s, $CO_2{\cdot}CH_3$), 3·46 (d, *J* 16 Hz, *trans*-C≡C·CH=C*H*·CO_2Me), and 3·21 (d, *J* 16 Hz, *trans*-C≡C·C*H*=CH·CO_2Me); u.v. and i.r. spectra were identical with those recorded.[5] The material from the most polar band (C) was added to fractions 5—7.

Fractions 5—7 (eluted with 20—50% ether–light petroleum) were concentrated and the residue (110 mg) was separated by p.l.c. (10% ether–light petroleum; 3 runs) into four bands [(i)—(iv)] of which the top two [(i) and (ii)] were due to additional small amounts of the esters (I; R = Me) and (II; R = Me). Bands (iii) and (iv) ran very closely: the material from the less polar band (iii) crystallised (from ether) to yield needles of dimethyl deca-*trans*-2,*trans*-8-diene-4,6-diynoate (IV; R = Me) (12 mg), m.p. and mixed m.p. 105—107° (lit.,[6,7] 103—106° and 103—104°, respectively), *m/e* 218 (M^+, 100%), 203 (51), 187 (50), 147 (78), 99 (44), 98 (44), and 74 (41), τ (CCl_4) 6·25 (s, $CO_2{\cdot}CH_3$), 3·95 (d, *J* 15·9 Hz, *trans*-C≡C·CH=C*H*·CO_2Me), and 3·67 (d, *J* 15·9 Hz, *trans*-C≡C·C*H*=CH·CO_2Me). The mother liquors from the crystallisation of the diester (IV; R = Me) were combined with the extract from band (iv) and further separated by t.l.c. (3% ether–benzene; 2 runs) into the diester (IV; R = Me) and two more polar polyacetylenes (<1 mg each) with $\lambda_{max.}$ (Et_2O) 328·5, 307, 288, and 271·5, and 307·5, 291, and 274infl nm, respectively.

The combined fractions 9 and 10 (eluted with ether) were concentrated and the residue yielded on crystallisation (from ether–light petroleum and dichloromethane–hexane) needles of methyl 10-hydroxydec-*trans*-2-ene-4,6,8-triynoate (III; R = Me) (152 mg), m.p. and mixed m.p. 115—116° (decomp.) (lit.,[8] decomp. 116°), *m/e* 188 (M^+, 100%), 173 (20), 157 (20), 145 (57), 117 (32), 99 (20), 89 (35), and 75 (46), τ ($CDCl_3$) 7·81br (OH), 6·29 (s, $CO_2{\cdot}CH_3$), 5·58 (s, $CH_2{\cdot}OH$), 3·57 (d, *J* 16 Hz, *trans*-C≡C·CH=C*H*·CO_2Me), and 3·16 (d, *J* 16 Hz, *trans*-C*H*=CH·CO_2Me).

Neutral fraction. The concentrated fraction (brown oil; 1·2 g) was chromatographed on a silica gel column (60 g) with stepwise elution (5% ether–light petroleum to 10% methanol–ether) and seventeen 100 ml fractions were collected. Fractions 1, 2, and 10—17 were discarded (no aromatic absorption in the u.v.).

Fractions 3 and 4 (eluted with 5—10% ether in light petroleum) were combined and concentrated, and the residue was separated by p.l.c. (5% ether–light petroleum) into two bands. The less polar band (R_F *ca.* 0·5) yielded on extraction and crystallisation (ether–light petroleum) *p*-nitroanisole (V) (11·6 mg), m.p. and mixed m.p. 51·5—52·5°. The extract from the more polar band (R_F *ca.* 0·4) was combined with fractions 5—7.

Fractions 5—7 (eluted with 10—20% ether in light petroleum) were combined and concentrated, and the residue (98 mg) was separated by p.l.c. (5% ether–light petroleum; 4 runs) into three major bands [(a)—(c)]. The least polar band (a) (R_F *ca.* 0·6) yielded more *p*-nitroanisole (1 mg). The extract (22 mg) from the middle band (b)

Concentration (mg per l of culture fluid) of metabolites produced by cultures of *Lepista diemii* Singer under various growth conditions

	Metabolite	Surface culture		Shaken culture		Ref.
		Malt extract	Glucose reflood	Malt extract	Glucose reflood	
(I)	NC·[C≡C]$_2$·C^{t}H=CH·CO_2R	4·8	4·2	0·7	0·05	*a, b*
(II)	H[C≡C]$_3$·C^{t}H=CH·CO_2R	0·15	0·15	0·15	0·15	*c*
(III)	HO·CH_2·[C≡C]$_3$·C^{t}H=CH·CO_2R	1·7	3	6·7	8	*d, e*
(IV)	RO_2C·C^{t}H=CH·[C≡C]$_2$·C^{t}H=CH·CO_2R	0·0—0·1	0·1	0·05	0·05	*f*
(V)	*p*-O_2N·C_6H_4·OMe	0·13	Traces	Traces	Traces	
(VI)	*p*-O_2N·C_6H_4·CHO	0·04	Traces	None	None	
(VII)	4-Cl,3-MeO·C_6H_3·CHO	0·12	0·25	0·25	0·25	
(VIII)	3-Cl,4-MeO·C_6H_3·CHO	0·05	0·2	Traces	Traces	

[a] M. Anchel, *Arch. Biochem. Biophys.*, 1958, **78**, 100. [b] N. G. Heatley and J. S. Stephenson, *Nature*, 1957, **179**, 1078. [c] Ref. 3. [d] M. Anchel, *Arch. Biochem. Biophys.*, 1959, **85**, 569. [e] Ref. 8. [f] Ref. 6.

[3] R. C. Cambie, J. N. Gardner, E. R. H. Jones, G. Lowe, and G. Read, *J. Chem. Soc.*, 1963, 2056.

[4] F. Bohlmann, W. Sucrow, and I. Queck, *Chem. Ber.*, 1964, **97**, 2586.

[5] P. J. Ashworth, E. R. H. Jones, G. H. Mansfield, K. Schlögl, J. M. Thompson, and M. C. Whiting, *J. Chem. Soc.*, 1958, 950.

[6] E. R. H. Jones, P. R. Leeming, and W. A. Remers, *J. Chem. Soc.*, 1960, 2257.

[7] Sir Ian Heilbron, E. R. H. Jones, and F. Sondheimer, *J. Chem. Soc.*, 1947, 1586.

[8] J. N. Gardner, E. R. H. Jones, P. R. Leeming, and J. S. Stephenson, *J. Chem. Soc.*, 1960, 691.

(R_F *ca.* 0·5) was rechromatographed (t.l.c.; 5% ether–light petroleum; 6 runs). The extract (12 mg) from the major band (b)i (R_F *ca.* 0·6) was crystallised (light petroleum) and yielded blades (11 mg) of 4-chloro-3-methoxybenzaldehyde (VII), m.p. 52°, mixed m.p. 51—52° (lit.,[9] 52—53·5°), M^+ 172 and 170, $\lambda_{max.}$ (EtOH) 298 (ε 5700), 288·5sh (8300), 270 (14,200), and 224 nm (17,000), τ (CCl_4) 6·01 (s, O·CH_3), 2·4—2·8 (m, aromatic H), and 0·09 (s, CHO). The aldehyde (VII) was converted with sodium borohydride into 4-chloro-3-methoxybenzyl alcohol, M^+ 174 and 172, τ (CCl_4) 6·07 (s, O·CH_3), 5·37 (s, CH_2·OH), 3·17 (dd, *J* 8 and 1·5 Hz, aromatic 5-H), 3·04 (d, *J* 1·5 Hz, aromatic 2-H), and 2·68 (d, *J* 8 Hz, aromatic 6-H). The mother liquors from the aldehyde (VII) crystallisation and a close-running more polar band (b)ii from the t.l.c. purification contained another aromatic compound, possibly a positional isomer (i.r.), which was not identified. The extract (40 mg) from the most polar band (c) (R_F *ca.* 0·3) was rechromatographed (p.l.c.; 5% ether–light petroleum; continuous elution for 6 h). Of the three bands thus obtained only the middle band (c)ii contained aromatic substances, and crystallisation of this from ether yielded needles of *p*-nitrobenzaldehyde (VI) (3·6 mg), m.p. and mixed m.p. 106°. Small amounts of an unidentified compound which showed aromatic methoxy-protons (n.m.r.) were present in the mother liquors of the *p*-nitrobenzaldehyde crystallisation.

Fractions 8 and 9 (eluted with 50% ether–light petroleum) were combined and concentrated, and the residue (70 mg) was separated by p.l.c. (20% ether–light petroleum; 4 runs) into four bands. The least polar and the two most polar bands contained complex mixtures of small amounts of aromatic compounds and were not investigated. The material from the remaining band (R_F *ca.* 0·5) was rechromatographed (t.l.c.; 5% ether–light petroleum; continuous elution for 10 h) and yielded on crystallisation (from light petroleum) 3-chloro-4-methoxybenzaldehyde (VIII) (4·5 mg), m.p. and mixed m.p. 57—58°; the spectra were identical with those of the synthetic specimen.

Synthesis of 3-*Chloro*-4-*methoxybenzaldehyde* (VIII).—Chlorination of anisaldehyde [10] yielded 3-chloro-4-methoxybenzaldehyde, m.p. 57·5—58° (from light petroleum) (lit.,[10,11] 62·5—63° and 53°, respectively) (Found: C, 56·25; H, 4·1; Cl, 21·0. Calc. for $C_8H_7ClO_2$: C, 56·3; H, 4·15; Cl, 20·8%), M^+ 172 and 170, $\lambda_{max.}$ (EtOH) 309·5 (ε 3150), 262 (9000), and 222·5 nm (15,000), τ (CCl_4) 6·02 (s, O·CH_3), 3·0 (d, *J* 8 Hz, aromatic 5 H), 2·28 (dd, *J* 8 and 2 Hz, aromatic 6-H), 2·13 (d, *J* 2 Hz, aromatic 2-H), and 0·13 (s, CHO). The aldehyde (VIII) was also prepared from *p*-hydroxybenzaldehyde by chlorination [12] and subsequent methylation; this sample also melted at 57·5—58°.

We thank the S.R.C. for a research studentship (to J. L. T.) and Mr J. W. Keeping for the mycological work.

[2/687 *Received*, 23*rd March*, 1972]

[9] H. E. Faith, M. E. Bahler, H. J. Florestano, *J. Amer. Chem. Soc.*, 1955, **77**, 543.
[10] P. Pfeiffer and B. Segall, *Annalen*, 1928, **460**, 123.
[11] L. Gattermann, *Annalen*, 1907, **357**, 348.
[12] C. A. Buechler, R. L. Brown, H. M. Holbert, J. G. Fulmer, and G. W. Parker, *J. Org. Chem.*, 1941, **6**, 902.