

Metabolic Products of *Entomophthora virulenta*

By Norman Claydon and John Frederick Grove,* ARC Unit of Invertebrate Chemistry and Physiology, University of Sussex, Falmer, Brighton BN1 9QJ

The new natural products 4'-hydroxymethylazoxybenzene-4-carboxylic acid and azoxybenzene-4,4'-dicarboxylic acid have been obtained from the entomopathogenic fungus *Entomophthora virulenta*.

AMONG entomopathogenic fungi the genus *Entomophthora* (Entomophorales, Entomophoraceae) has not, hitherto, been examined for the production of insecticidal secondary metabolites. We have recently studied two strains of *E. virulenta* and have shown that they produce the same, solvent-extractable, insecticidal, acidic material when grown on a synthetic medium.¹ 4'-Hydroxymethylazoxybenzene-4-carboxylic acid (2; R = CO₂H, R' = CH₂OH) and azoxybenzene-4,4'-dicarboxylic acid (1; R = H) have now been identified as major constituents of this extract and the identification has been confirmed by synthesis. The hydroxy-acid is responsible for the insecticidal activity.¹

The mass spectrum of the crude extract suggested the presence of two compounds of compositions C₁₄H₁₀N₂O₅ and C₁₄H₁₂N₂O₄. They were separated by preparative t.l.c. of the methyl esters and subsequent alkaline

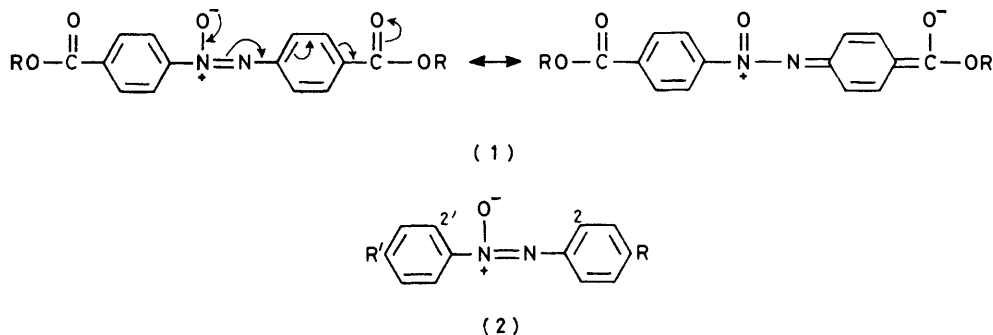
hydrolysis to the parent acids. The less polar of the two esters had the composition C₁₆H₁₄N₂O₅. This, together with the ¹H n.m.r. spectrum, which showed two three-proton singlets close to τ 6, was consistent with the presence of two methoxycarbonyl groups. Signals for the remaining eight protons were present in the aromatic region at τ 1.6—2.0, four in a singlet at τ 1.87, and four in an AA'BB' multiplet, suggesting the presence of two aromatic rings, of which at least one was *p*-disubstituted. This evidence, together with the u.v. spectrum, λ_{max.} 265 and 330 nm (log ε 4.01 and 4.24) suggested the azoxybenzene structure (1; R = Me). The assignment was confirmed by synthesis² of the diacid (1; R = H), by mild reduction of 4-nitrobenzoic acid, followed by methylation with diazomethane.

¹ N. Claydon, *J. Invert. Pathol.*, in the press.

² C. B. Ried and E. G. Pritchett, *J. Org. Chem.*, 1953, **18**, 716.

The more polar of the two esters had the composition $C_{15}H_{14}N_2O_4$. It showed broad absorption in the OH region of the i.r. spectrum and formed a monoacetate. The n.m.r. spectrum showed the presence of only one methoxycarbonyl group, together with a two-proton multiplet at τ 5.25 which moved downfield to τ 4.85 on acetylation, consistent with the presence of a hydroxymethyl substituent and the structure (2; $RR' = CO_2Me, CH_2OH$).

With lithium borohydride, reduction of the ester (1; $R = Me$) was selective, the NO group being unaffected,



and regiospecific, giving only one product of composition $C_{15}H_{14}N_2O_4$. This is presumably the 4'-hydroxymethyl compound (2; $R = CO_2Me, R' = CH_2OH$) since the 4-CO group is deactivated by the conjugative effect [see (1)]. Some of the bis-hydroxymethyl compound (2; $R = R' = CH_2OH$)³ was also formed and some starting material recovered.

Although the i.r. spectrum of the fermentation-derived $C_{15}H_{14}N_2O_4$ ester was identical with that of the hydroxy-ester (2; $R = CO_2Me, R' = CH_2OH$), the n.m.r. spectrum showed the presence of an impurity. The OMe signal at τ 6 integrated correctly for three protons, but appeared as an unsymmetrical doublet, line separation 2 Hz; and four of the aromatic protons gave a multiplet consisting of overlapping AA'XX' patterns in which the two sets of AA' lines corresponded in chemical shift to the AA'BB' pattern of the ester (1; $R = Me$). The synthetic hydroxy-ester showed only a three-proton singlet at τ 6 and signals for four aromatic protons in a deceptively simple AA'XX' pattern. Similar results were obtained with the corresponding acetates.

It is concluded that the hydroxy-ester (2; $R = CO_2Me, R' = CH_2OH$) is the major component of the naturally derived $C_{15}H_{14}N_2O_4$ ester, but is accompanied therein by the isomer (2; $R = CH_2OH, R' = CO_2Me$) from which it is not separated by t.l.c.

Although several naturally occurring aliphatic azoxy-compounds have been described, for example, cycasin and its relatives⁴ and, among microbial products, elaiomycin,⁵ this constitutes the first report of the occurrence of aryl azoxy-compounds in nature.

EXPERIMENTAL

M.p.s were taken on a Kofler hot-stage apparatus and are corrected. I.r. spectra were determined for mulls in

Nujol and u.v. spectra for solutions in methanol. N.m.r. spectra were obtained in deuteriochloroform at 90 MHz with tetramethylsilane as internal standard. Molecular weights were obtained from mass spectra. Mass spectra at high resolution were recorded at 70 eV with a Varian CH5 double-focusing instrument interfaced with a Varian 620L computer. Merck silica gel HF254 was used in t.l.c. In preparative t.l.c. silica layers ($20 \times 20 \times 0.075$ cm) were developed in chloroform-methanol (95:5) and the R_F values quoted are for this solvent system.

Fermentations with *E. virulenta* and the isolation of the crude acidic insecticidal extract are described elsewhere.¹

The extract was an orange yellow amorphous powder, λ_{max} 290br nm, ν_{max} 2 840, 2 660, and 1 690 cm^{-1} , soluble in hot methanol and hot ethyl acetate. The mass spectrum showed two parent ions at m/e 286.0598 (calc. for $C_{14}H_{10}N_2O_5$: 286.0589) and 272.0768 (calc. for $C_{14}H_{12}N_2O_4$: 272.0797).

Identification of the Fermentation Products.—The crude acidic extract (50 mg) in methanol (2 ml) was methylated with ethereal diazomethane. Preparative t.l.c. of the neutral yellow amorphous product gave two major bands, R_F 0.74 and 0.49, which furnished solid products. Minor bands at R_F 0.64, 0.58, 0.36, and 0.15 gave traces of biologically inactive gummy products which were not investigated further.

The product of R_F 0.74 crystallised from methanol in yellow needles (18 mg), m.p. 204–205°, of the ester (1; $R = Me$) (Found: C, 60.4; H, 4.7; N, 9.0%; M , 314.0889. Calc. for $C_{16}H_{14}N_2O_5$: C, 61.1; H, 4.5; N, 8.9%; M , 314.0920), λ_{max} 265 and 330 nm (ϵ 10 320 and 17 520), ν_{max} 1 730, 1 605, and 1 565 cm^{-1} , τ 1.6–1.9 (4 H, m), 1.87 (4 H, s), 6.17 (3 H, OMe), and 6.20 (3 H, OMe), identical with synthetic material (see below).

The product of R_F 0.49 crystallised from methanol in yellow needles (15 mg), m.p. 156°, of a mixture of the hydroxy-esters (2; $R, R' = CO_2Me, CH_2OH$) but predominantly (2; $R = CO_2Me, R' = CH_2OH$) (Found: C, 61.9; H, 5.1; N, 9.4%; M , 286.0949. Calc. for $C_{15}H_{14}N_2O_4$: C, 61.6; H, 5.3; N, 9.3%; M , 286.0953), λ_{max} 233, 265, and 335 nm (ϵ 8 860, 8 580, and 19 240), ν_{max} 3 400–3 100br, 1 730, 1 600, and 1 565 cm^{-1} , τ 1.6–2.6 (5 H, m), 1.89 (4 H, s), 5.25 (2 H, m), and 6.1 (3 H, OMe). The addition of deuterium oxide removed a one-proton signal from the five-proton multiplet.

The acetate, prepared as described below, formed yellow

³ J. B. Shoemith and W. E. Taylor, *J. Chem. Soc.*, 1926, 2832.

⁴ N. V. Riggs, *Chem. and Ind.*, 1956, 926.

⁵ C. L. Stevens, B. T. Gillis, J. C. French, and T. H. Haskell, *J. Amer. Chem. Soc.* 1958, **80**, 6088.

needles, m.p. 124° (from methanol) (Found: M , 328.1051. Calc. for $C_{17}H_{16}N_2O_5$: M , 328.1059), ν_{\max} . ca. 1 745, 1 725, 1 600, and 1 560 cm^{-1} , τ 1.65—2.6 (4 H), 1.90 (4 H, s), 4.85 (2 H, m), 6.10 (3 H, OMe), and 7.89 (3 H, MeCO).

Dimethyl Azoxybenzene-4,4'-dicarboxylate (1; R = Me).—The acid (1; R = H)² [decomp. >400°; λ_{\max} . 263 and 334 nm (ϵ 9 200 and 17 500); 150 mg] was suspended in methanol (6 ml) and treated with an excess of ethereal diazomethane during 5 h. The recovered solid was extracted with chloroform, and the product was purified by preparative t.l.c. and crystallisation from methanol, giving yellow needles (108 mg) of the ester (1; R = Me), m.p. 204° (lit.,² 204—205°). Alkaline hydrolysis² regenerated the acid (1; R = H).

Methyl 4'-Hydroxymethylazoxybenzene-4-carboxylate (2; R = CO₂Me, R' = CH₂OH).—The ester (1; R = Me) (50 mg) in tetrahydrofuran (25 ml) was stirred at room temperature with lithium borohydride (20 mg) and the course of the reduction was followed by t.l.c. Two products, R_F 0.49 and 0.20, were detected, in addition to starting material, R_F 0.74. After 5 days, when the yield of the product R_F 0.49 was optimum, water (20 ml) was added. The solution was concentrated *in vacuo* to small bulk and extracted with chloroform (2 × 5 ml). The recovered solid was separated into its components by preparative t.l.c.

The material of R_F 0.49 crystallised from methanol in yellow needles (11 mg), m.p. 164° of the *hydroxy-ester* (2; R = CO₂Me, R' = CH₂OH) (Found: M , 286.0959. $C_{15}H_{14}N_2O_4$ requires M , 286.0953), ν_{\max} . 3 400—3 100br, 1 730, 1 600, and 1 565 cm^{-1} , τ 1.75 (2 H, d), 1.91 (4 H, s), 2.55 (2 H, d), 5.22 (2 H, s), and 6.10 (3 H, OMe).

The *acetate* (2; R = CO₂Me, R' = CH₂OAc), prepared

with acetic anhydride in pyridine during 2 days at room temperature, formed yellow needles, m.p. 134° (from methanol) (Found: M , 328.1061. $C_{17}H_{16}N_2O_5$ requires M , 328.1059), ν_{\max} . 1 725, 1 600, and 1 560 cm^{-1} , λ_{\max} . 230, 265, and 330 nm (ϵ 7 500, 7 800, and 16 650), τ 1.65—2.6 (4 H), 1.9 (4 H, s), 4.80 (2 H, s), 6.10 (3 H, OMe), and 7.88 (3 H, MeCO).

The material of R_F 0.20 formed yellow needles (6 mg), m.p. 162° (Found: M , 258.0992. Calc. for $C_{14}H_{14}N_2O_3$: M , 258.1004), ν_{\max} . 3 400—3 100br and 1 600 cm^{-1} , λ_{\max} . 233 and 330 nm (ϵ 7 900 and 10 950), of the diol (2; R = R' = CH₂OH) (lit.,³ m.p. 167°).

Starting material (8 mg) was recovered from the band at R_F 0.74. No azobenzene analogues were detected by mass spectrometry in any of the crude products.

4'-Hydroxymethylazoxybenzene-4-carboxylic Acid (2; R = CO₂H, R' = CH₂OH).—The hydroxy-ester (2; R = CO₂Me, R' = CH₂OH) (20 mg) in methanol (10 ml) and 0.2N-potassium hydroxide (0.5 ml) was heated under reflux for 2 h. The cooled solution was acidified to pH 3 with 3N-hydrochloric acid and the precipitate was filtered off. Reprecipitation from methanol afforded the *acid* (2; R = CO₂H, R' = CH₂OH) as a yellow amorphous powder (11 mg), m.p. 251° (decomp.) (Found: M , 272.0802. $C_{14}H_{12}N_2O_4$ requires M , 272.0797), ν_{\max} . 3 400—3 120br, 2 680, 2 520, 1 690, and 1 603 cm^{-1} , λ_{\max} . 232, 265, and 332 nm (ϵ 7 970, 7 260, and 17 070).

We thank Albert and Grete Olney for the microanalyses, Dr. F. A. Mellon for the mass spectra, and Mrs. B. O'Connor for the n.m.r. spectra.

[7/1246 Received, 13th July, 1977]