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ISOLATION OF ALTERNARIOL AND ALTERNARIOL MONOMETHYL ETHER FROM *ALTERNARIA DAUCI* (KÜHN) GROVES AND SKOLKO

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Abstract—Alternariol and alternariol monomethyl ether have been isolated from the mycelium of *Alternaria dauci*, in proportions which differed considerably from those obtained from *A. tenuis*.[1] The preparation of triacetylalternariol and of alternariol dimethyl ether is described.

INTRODUCTION

SPECIES of *Alternaria* occur widely as parasites on cultivated plants and *Alternaria tenuis* auct. is found on numerous kinds of organic materials in damp situations. The earliest reference to their biochemical activities is Neergaard's[2] observation of the production of crystals in cultures of *A. tenuis*, *A. dauci* (Kühn) Groves and Skolko and *A. anagallidis* Raabe v. *linariae* on malt extract agar medium in which the colourless rosettes were readily visible against the dark background of the mycelium. Alternaric acid, having phytotoxic and antifungal activity, was isolated from *A. solani* (E. and M.) Jones and Grout and *A. porri* (Ell.) Sacc.[3, 4, 5] and its structure established.[6] It is the cause of certain symptoms associated with *A. solani* infections of tomato, etc. The following metabolic products have been isolated[7] from culture filtrates of a strain of *A. solani*: (*a*) alternarine, colourless needles with marked antibacterial activity; (*b*) a red crystalline pigment, which decomposed at about 260° and (*c*) a colourless crystalline substance, m.p. 136°, believed to be alternaric acid. Ether extraction of the mycelium gave two coloured products. Alternariol monomethyl ether and alternariol (I) were isolated from the mycelium of *A. tenuis* by Raistrick *et al.*[1] and the latter was identified by synthesis as 3,4′,5-trihydroxy-6′-methyldibenzo-α-pyrone. The naturally occurring monomethyl ether has its methoxyl group at position 5 or 4′. Alternariol and its methyl ether were the first recorded substituted dibenzo-α-pyrones of fungal origin. One strain of *A. tenuis* gave an ether extract containing alternariol methyl ether and alternariol in the proportions 10:1 and the other strain investigated gave a slightly lower proportion. Thomas[8] studied the chemical degradation of labelled alternariol derived from sodium [1-^{14}C]acetate and demonstrated a biosynthetic mechanism involving the head to tail condensation of acetate units. *A. tenuis* auct. culture filtrates have yielded five metabolic

[1] H. RAISTRICK, C. E. STICKINGS and R. THOMAS, *Biochem. J.* **55**, 421 (1953).
[2] P. NEERGAARD, *Danish Species of* Alternaria *and* Stemphylium. OUP, London (1945).
[3] P. W. BRIAN, P. J. CURTIS, H. G. HEMMING, C. H. UNWIN and J. M. WRIGHT, *Nature* **164**, 534 (1949).
[4] P. W. BRIAN, P. J. CURTIS, H. G. HEMMING, E. G. JEFFERYS, C. H. UNWIN and J. M. WRIGHT, *J. Gen. Microbiol.* **5**, 619 (1951).
[5] J. F. GROVE, *J. Chem. Soc.* 4056 (1952).
[6] J. R. BARTELS-KEITH and J. F. GROVE, *Proc. Chem. Soc.* 398 (1959).
[7] H. DARPOUX, A. FAIVRE-AMIOT and L. ROUX, *Compt. Rend.* **230**, 993 (1950).
[8] R. THOMAS, *Biochem. J.* **78**, 748 (1961).

products, of unknown structure, which are probably related to alternariol;[9] these are: altertenuol ($C_{14}H_{10}O_6$), altenusin ($C_{15}H_{14}O_6$), and three isomeric altenuic acids I, II and III ($C_{15}H_{14}O_8$). Tenuazonic acid ($C_{10}H_{15}O_3N$) was isolated from *A. tenuis*,[9] its structure[10] established and a biosynthetic pathway suggested.[11] The acid has also been found as a metabolite of *Aspergillus* and a member of the *Sphaeropsidales*; it is a growth-inhibitor of human adenocarcinoma-1. NMR data suggest that the metabolite exists in solution as an equilibrium mixture of two tautomeric forms.[11a] The properties of metabolic products of *Alternaria* species have been summarized by Miller.[12]

A number of reports, of the identification of *A. dauci* as the cause of leaf blight of carrots, have appeared in the mycological literature. The fungus has been found in the U.S. and in Britain, elsewhere in Europe and in other parts of the world including the tropics. The disease starts in patches on the leaves and spreads causing death of the foliage; the fungus also causes brown lesions on the stems of carrot seedlings, resulting in "damping-off". No information on the biochemical properties of this species has been found except Neergaard's observation.[2]

Cultures of *A. dauci* (formerly known as *A. porri* (Ell.) Neerg. *f. sp. dauci* (Kühn)) were isolated here by Mr. R. B. Maude from carrot seeds and identified as the cause of damping-off of seedlings. Their appearance agreed well with Neergaard's description[2] of this species. The observation of relatively large quantities of crystalline compounds associated with the fungus in culture led to an investigation of their chemical and biological properties.

RESULTS AND DISCUSSION

Two methods were used for isolation of metabolic products from the dried mycelium of *A. dauci*, in which the crystalline components were embedded. In the first, the mycelium, grown in liquid prune medium, was extracted with ether and the fatty components of the extract residue removed by means of boiling benzene. The insoluble fraction, obtained from strain 1, was crude alternariol (8·6 per cent of the dried mycelium). Crystallization from aqueous ethanol gave alternariol (I), $C_{14}H_{10}O_5$, m.p. 350° (decomp.). There was no evidence of production of alternariol monomethyl ether by strain 1. The general properties and colour reactions of our product agreed with those of alternariol from *A. tenuis*[1] and the identity of the product was confirmed by mixed melting point of its trimethyl ether with an authentic specimen, kindly supplied by Dr. C. E. Stickings.

Our product was optically inactive, $[\alpha]_D^{20}$, 0° ± 1 (*c*, 0·8 in ethanol). The following peaks were observed in the u.v. spectrum of an ethanolic solution: 218, 258, 302, and 330 nm; maximum extinction was at 258 nm; ϵ, 38,000. The i.r. spectrum determined on a Nujol mull (by Dr. B. K. Tidd at the Natural Rubber Producers' Research Association, Welwyn Garden City), showed absorption bands at 830, 1610 (aromatic rings), 1660, and 3250, 3550 cm^{-1} (hydroxyl groups).

The second method of extraction of the metabolic products was based on that of Raistrick *et al.*[1] as providing better conditions for isolation of alternariol monomethyl ether. Strain 2 was used because the yields of the required products from strain 1 fell off sharply after about

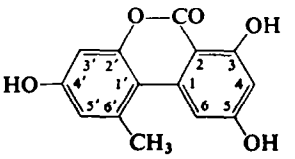
[9] T. ROSETT, R. H. SANKHALA, C. E. STICKINGS, M. E. U. TAYLOR and R. THOMAS, *Biochem. J.* **67**, 390 (1957).
[10] C. E. STICKINGS, *Biochem. J.* **72**, 332 (1959).
[11] C. E. STICKINGS and R. J. TOWNSEND, *Biochem. J.* **78**, 412 (1961).
[11a] E. A. KACZKA, C. O. GITTERMAN, E. L. DULANEY, M. C. SMITH, D. HENDLIN, H. B. WOODRUFF and K. FOLKERS, *Biochem. Biophys. Res. Comm.* **14**, 54 (1964).
[12] M. W. MILLER, *The Pfizer Handbook of Microbial Metabolites*. McGraw-Hill, London (1961).

a year in laboratory culture and cultures no longer contained obvious crystals or gave a positive reaction with alcoholic ferric chloride. Strain 2 was freshly isolated from carrot seeds. The dried mycelium, grown on liquid prune medium, was extracted with petroleum ether to remove fatty components. After redrying, extraction with ether gave a mixture of alternariol and alternariol monomethyl ether in the proportion of about 3 to 1 (total 11·3 per cent of the mycelium). The former was much more soluble than the latter in cold absolute ethanol and separation was based on this difference in solubility. The mycelium of *A. tenuis* No. 94[1] gave 3·9 per cent of crystalline extract (after 39 days' incubation), in which the proportion of alternariol methyl ether to alternariol was 10:1. The corresponding proportion was slightly less with strain No. 430.

A. dauci strain 1 grew well in malt extract medium but gave smaller quantities of cruder alternariol (and no detectable alternariol monomethyl ether) than when cultured in the prune medium. The product (0·73 per cent of the mycelium) was isolated and characterized as triacetylalternariol.

Our specimen of alternariol monomethyl ether, m.p. 266–267°, analysed for $C_{15}H_{12}O_5$, gave an intense purple colour with alcoholic ferric chloride and had general properties and colour reactions in agreement with those described earlier.[1]

On treatment with acetic anhydride and pyridine, alternariol gave a good yield (96 per cent of theory) of triacetylalternariol, m.p. 163–165°, $C_{20}H_{16}O_8$ ($C_{14}H_7O_5.3CH_3CO$). The i.r. spectrum (Nujol mull) was similar to that of alternariol but the bands at 1660, 3250 and 3550 cm^{-1} were absent in the spectrum of the acetyl derivative and there were new bands indicative of ester groups at 1737 and 1761 cm^{-1}. The nuclear magnetic resonance spectrum of triacetylalternariol was recorded on a Varian HA100 NMR spectrometer in carbon tetrachloride solution. Peak positions were recorded in ppm downfield from tetramethylsilane internal standard. The spectrum showed peaks in regions characteristic of three acetyl groups (2·49, 2·42 and 2·38 ppm), an aromatic C-methyl group (2·87 ppm) and four protons on aromatic rings (8·05 ppm, $J \simeq 2$ c/s; 7·09 ppm, $J \simeq 2$ c/s; 7·04 ppm, $J \simeq 2$ c/s; 6·95 ppm, $J \simeq 2$ c/s). The coupling constants found for the aromatic ring protons are typical[13] of *meta* couplings, and imply the presence of two aromatic rings each bearing a pair of metarelated hydrogen atoms. Furthermore, the weak splitting of the resonance due to the aromatic C-methyl group, as evidenced by its unsymmetrical shape implies[14] the presence of a hydrogen atom in the position *ortho* to it. These observations are completely consistent with the structure already suggested for alternariol (I).



(I) Alternariol

Methylation of alternariol with dimethyl sulphate and potassium carbonate in acetone under the conditions described by Raistrick *et al.*[1] gave alternariol trimethyl ether, m.p. 164–165°, $C_{17}H_{16}O_5$, which was shown by mixed melting point determination to be identical

[13] L. M. JACKMAN, *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*. Pergamon Press, Oxford (1959).
[14] S. STERNHELL, *Rev. Pure Appl. Chem.* **14**, 15 (1964).

with an authentic specimen kindly provided by Dr. C. E. Stickings. Methylation under slightly milder conditions by refluxing on a water bath gave the dimethyl ether as light feathery needles, melting very sharply at 186–186·5°, $C_{16}H_{14}O_5$, which unlike the trimethyl ether gave a strongly positive reaction with alcoholic ferric chloride. The check in the progress of the reaction at the dimethyl stage may be due to the presence of traces of water. The melting points of alternariol and its mono-, di- and trimethyl ethers decreased regularly as the number of methoxyl groups increased. The melting points of our dimethyl ether and of 4′-hydroxy-3,5-dimethoxy-6′-methyldibenzo-α-pyrone (m.p. 293–296°) synthesized by Raistrick *et al.*[1] are widely separated and the two compounds are clearly different. The two methoxyl groups in our derivative must therefore occupy the 4′ and either 3 or 5 positions. The compound gave an intense purple salicylic reaction with ferric chloride suggesting that position 3 is occupied by a hydroxyl group. It is therefore probable that its structure is 3-hydroxy-4′,5-dimethoxy-6′-methyldibenzo-α-pyrone.

The chemical and spectroscopic properties of the metabolic products from *A. dauci* and their derivatives are consistent with the formulae already established.[1]

Antibacterial Activity of Alternariol

Alternariol (0·008 g) was dissolved in absolute ethanol (3 ml) and the solution diluted to 100 ml with nutrient glycerol medium (≡80 ppm alternariol). Serial dilutions were prepared to give alternariol concentrations of 40, 20, 10, and 5 ppm. Batches of tubes were inoculated with young cultures of *Staphylococcus aureus* N.C.T.C. strain No. 6571; *Corynebacterium betae* N.V.R.S. isolate No. 1064a and *Escherichia coli* N.C.T.C. strain No. 86, respectively. Growth was recorded after 6 days at 25° (pH 7·0) as shown in Table 1. It was concluded that alternariol has fairly powerful activity against Gram +ve bacteria but only moderate activity against the Gram −ve bacterium tested. Raistrick *et al.*[1] found that alternariol completely inhibited the growth of *S. aureus* at 25 ppm and of *E. coli* at 50 ppm in glucose broth culture.

TABLE 1. ACTIVITY OF ALTERNARIOL AGAINST GRAM +VE AND −VE BACTERIA

	Alternariol concentration (ppm)						
	80	40	20	10	5	0	0+ethanol
S. aureus	−	−	−	−	−	+++	++++
C. betae	−	−	−	++	++	+++	+++
E. coli	+	++	++	+++	+++	+++	+++

Concentrations of alternariol in the range 5–80 ppm were incorporated into Czapek-Dox yeast agar for examination of its effect on *Aspergillus niger*, *Fusarium oxysporum lycopersici*, *Penicillium digitatum*, *Rhizoctonia solani* and *Trichothecium roseum*. There was no detectable effect on the growth of these fungi after 6 days at 25°. This lack of antifungal activity may be due to the insolubility of alternariol in aqueous media at the pH in question (pH 3·7); microscopic crystals of the metabolic product separated out in the cultures initially containing 40 and 80 ppm.

The phytotoxic properties of alternariol were tested on carrot plants. Alternariol (18·6 mg) was dissolved in ethanol (2 ml), Teepol (0·1 ml) was added and the mixture poured into water (248 ml) with vigorous shaking. The slightly opalescent solution (alternariol, 74 ppm),

which remained stable and free from gross suspended matter, was (1) sprayed on to young carrot seedlings and (2) applied to the roots of seedlings cut off at the crown (6 plants/50 ml solution). After 7 days at 20° there were no observable changes as compared with control plants and it was concluded that alternariol had no phytotoxic effect under these conditions.

EXPERIMENTAL

Melting points are uncorrected. Microanalyses were carried out by Drs. Weiler and Strauss, Oxford.

Culture Media

(i) *Prune medium.* Prunes (200 g) were steeped in boiling water (500 ml) for 1 hr and, after cooling, the extract was strained off through cotton wool, diluted to 1 l. and sterilized by autoclaving. The extract (50 ml) was diluted with water to 500 ml, dispensed into suitable culture vessels and sterilized by autoclaving. The medium had initial pH 6·2 and its initial reducing sugar concentration was 0·5–0·7 g/100 ml as apparent glucose as determined by a combination of the methods of Munson and Walker[15] and Bertrand[16] after removal of proteins and inversion by the method described by Freeman and Morrison.[17]

(ii) *Malt extract medium.* Concentrated malt extract ("Wandermalt"*) (20 g) was dissolved in water and the solution diluted to 1 l. The medium was sterilized by autoclaving; it had initial pH 5·95 and reducing sugar concentration, 1·1 g/100 ml as glucose.

Isolation of Alternariol and Alternariol Monomethyl Ether

Method 1. Prune medium (250 ml) was dispensed into cylindrical mineral-water bottles (28 cm high × 7·5 cm dia., capacity 750 ml) and each inoculated with two 4-mm diameter agar discs from plate cultures of *A. dauci* strain 1. The bottles were supported at an angle of about 20° and incubated in darkness at 22–24°. Crystals were observed in the cultures after about 40 days' incubation as starlike clusters of colourless needles. The cultures were harvested after 75 days' incubation when the dark grey mycelial growth was moderately luxuriant, partially floating and partially submerged. The colour of the medium gradually changed from pale yellowish brown to deep red-brown during incubation. The final pH of the culture filtrate was 7·2 and its apparent glucose content 0·03 g/100 ml. The mycelium was filtered off, washed well with water, pressed as dry as possible on a Buchner funnel and dried *in vacuo* over P_2O_5. Yield from forty-eight bottles, 11·7 g. The mycelium was ground to a fine powder and continuously extracted with ether (Soxhlet) for 8 hr. Evaporation of the extract gave a cream-coloured solid (2·10 g; 18·0% of the mycelium), which was refluxed with benzene (100 ml) for 2 hr and filtered. The residue, which was an almost colourless solid (1·00 g) (8·6% of dry mycelium), was dissolved in boiling absolute ethanol (100 ml), cooled and an equal volume of water added. Alternariol (0·824 g) slowly crystallized as colourless microscopic needles, m.p. 350°, with decomposition. (The absolute alcoholic solution gave only a trace of crystalline deposit after 16 hr at 0° showing the absence of alternariol methyl ether) (Found: C, 64·9; H, 4·0; OMe, 0. Calc. for $C_{14}H_{10}O_5$: C, 65·1; H, 3·9%.) An ethanolic solution of alternariol gave an intense purple colour with ethanolic $FeCl_3$. The general properties and colour reactions of the product agreed with those described by Raistrick *et al.*[1]

* A. Wander Ltd., London, S.W.7.

[15] L. S. Munson and P. H. Walker, *J. Am. Chem. Soc.* **28**, 663 (1906).
[16] G. Bertrand, *Bull. Soc. Chim. Paris* **35**, 1285 (1906).
[17] G. G. Freeman and R. I. Morrison, *Analyst* **71**, 511 (1946).

Alternariol and alternariol methyl ether gave negative reactions in a spot test for resorcinol with pyrocatechol.[18]

The benzene extract, referred to above, on evaporation gave a pale yellow fatty residue (1·092 g) containing traces of a crystalline solid.

The fungus (strain 1) grew much more rapidly and luxuriantly on the malt extract medium but gave smaller quantities of alternariol. After 26 days' incubation, under the above conditions, forty-eight mineral-water bottle cultures gave 46·10 g of dried mycelium. On extraction with ether, 9·22 g of product crystallized after concentration of the extract and was accompanied by a brown oily liquid. The residue (0·453 g) after extraction with boiling benzene was coloured and obviously less pure than the corresponding fraction from the prune medium. The presence of alternariol was established by acetylation, as described below, which gave 0·498 g of acetyl derivative (m.p. 164–165°) which did not depress the melting point of triacetylalternariol. The yield of alternariol was approximately 0·7% of the mycelium as compared with 8·6% from the prune medium.

Method 2. Prune medium (50 ml) was dispensed into rectangular medicine bottles (15 × 7 × 4 cm external dimensions, capacity 280 ml) and inoculated with *A. dauci*, strain 2. The bottles were incubated, standing on edge, at 22–24° for 68 days with cotton wool plugs and screw cap closures. There was a fairly heavy growth of dark, practically wholly submerged mycelium with obvious starlike clusters of colourless needles. The initial and final apparent glucose concentrations of the culture filtrate were 0·7 and 0·24 g/100 ml. The dried mycelium (5·8 g) was extracted successively with light petrol (b.p. 40–60°) and with ether. The light petrol extract was a pale yellow oil (0·427 g) containing a few colourless crystals. After redrying, the mycelium was extracted with ether. Crystals appeared in the receiver after 20 min of ether extraction. Evaporation of the solvent gave slightly pink crystals (0·65 g, 11·3% of mycelium), which consisted of alternariol and alternariol monomethyl ether in the proportion of about 3 to 1. The two products were separated on the basis of their different solubilities in ethanol. The mixture was dissolved in boiling ethanol (25 ml), cooled to 0° and after 4 days the crystalline deposit of alternariol methyl ether (0·137 g) was collected, m.p. 266–267° (Found: C, 65·6; H, 4·3; OCH_3, 10·6. Calc. for $C_{15}H_{12}O_5$: C, 66·2; H, 4·4; 1 OCH_3, 11·4%). The general properties and colour reactions of the monomethyl ether were as described previously.[1]

The ethanolic filtrate and washings were warmed to 70° and hot water (50 ml) added to give an immediate opalescence and deposition of alternariol (0·413 g), after cooling to 0°, identical with the specimen isolated by method 1.

Triacetylalternariol

Alternariol (54 mg) in acetic anhydride (1 ml) and pyridine (1 ml) gave *triacetylalternariol* (77 mg), recrystallized from methanol as colourless fibrous needles, m.p. 163–165° (Found: C, 62·4; H, 4·3; CH_3CO, 40·0. $C_{20}H_{16}O_8$ required: C, 62·5; H, 4·2; $3CH_3CO$, 33·6%). It gave negative $FeCl_3$ and Gibbs[19] reactions and no colour on boiling with chloroform and N NaOH.

Alternariol Trimethyl Ether

Alternariol trimethyl ether was prepared as described by Raistrick *et al.*[1] Alternariol (0·66 g) gave 0·54 g of product, m.p. 164–165°, and the melting point was not depressed on

[18] F. FEIGL, *Spot Tests in Organic Analysis*, p. 417. Elsevier, London (1960).
[19] H. D. GIBBS, *J. Biol. Chem.* 72, 649 (1927).

admixture with an authentic specimen of the ether, kindly provided by Dr. C. E. Stickings (Found: C, 68·2; H, 5·6; OCH_3, 35·3; mol.wt. (Rast), 257. Calc. for $C_{17}H_{16}O_5$: C, 68·1; H, 5·4; 3 OCH_3, 31·0%; mol.wt. 300).

Alternariol Dimethyl Ether

The *dimethyl ether* was prepared by a similar method to the trimethyl ether except that the reaction mixture was refluxed on a water bath (9 hr) instead of heating directly on an electric hot plate. The reaction mixture gave a +ve reaction with alcoholic $FeCl_3$. It was filtered hot and on cooling deposited clusters of feathery colourless needles (0·257 g from 0·51 g of alternariol). The crude product was recrystallized from acetone, m.p. 186–186·5°. The dimethyl ether was only slightly soluble in cold ethanol but the solution gave a purple reaction with alcoholic $FeCl_3$; the reaction was more intense when the ether was dissolved in acetone. There was no reaction with Gibbs reagent even after 2 hr. The dimethyl ether dissolved in cold conc. H_2SO_4 giving a characteristically more intense fluorescence in visible light than the parent compound (Found: C, 67·1; H, 5·2; OCH_3, 20·4; mol.wt. (Rast), 258 $C_{16}H_{14}O_5$ required: C, 67·1; H, 4·9; 2 OCH_3, 21·7%; mol.wt. 286).

Acknowledgements—I wish to thank my colleague, Dr. W. G. Keyworth, for drawing attention to crystal production in cultures of *A. dauci* and for carrying out the mycological work; and Dr. B. K. Tidd (The Natural Rubber Producers' Research Association) for the i.r. and NMR spectra.