The simple code names adopted refer to the different substituents in the basic ring system, HH referring to the ring methylene, HMe to the ring methyl-methylene, Me to the methyls of the acetyl groups and OEt to the ethoxy groups of the ester side chains. See also Figs. 5 and 6.

One recrystallization from ethanol was sufficient to give a product whose melting point agreed with that given in the literature, but up to three more were required before the colour of the crystals did not change further. There was also a marked increase in the fluorescence, particularly in the last compound (HMe/OEt). The melting points were also raised slightly, particularly the first compound (HH/Me, or DDL). These are given in Table 3, together with the analyses. As found by others (Hantzsch, Knoevenagel, quoted by Schiff & Prosio, 1895) there was a general deficiency in the carbon content. Repeat analyses on HH/Me gave figures for carbon of 66.7, 67.7 and 68.6% (averaged for Table 3), while two using a copper tube for combustion gave 69.4 and 69.7%.

Absorption spectra. It is clear from Figs. 5 and 6 that the compound HH/Me (DDL) is in no way exceptional. The strong-yellow colour is due to the absorption extending sufficiently far into the visible region; only the last compound, HMe/OEt, was finally obtained as completely

colourless crystals, the two intermediate ones showing some absorption in the extreme blue. The optical properties are summarized in Table 2.

SUMMARY

A colour reaction for formaldehyde is described which depends on the synthesis of diacetyldihydro-lutidine from acetylacetone and formaldehyde in the presence of excess of ammonium salt. The conditions are mild enough to allow of its use with living material, and its sensitivity and degree of specificity are comparable to those of other reactions requiring more severe conditions.

The work arose from investigations carried out on behalf of the Public Health Laboratory Service Committee on Formaldehyde Disinfection. I am indebted to Dr A. S. McFarlane and to Dr S. Jacobs, of the National Institute of Medical Research, for the use of a spectrophotometer, and to Dr J. Walker, also of the National Institute, for the micro-analyses.

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Studies in the Biochemistry of Micro-organisms

90. ALTERNARIOL AND ALTERNARIOL MONOMETHYL ETHER, METABOLIC PRODUCTS OF *ALTERNARIA TENUIS*

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Although species of *Alternaria*, in the Dematiaceae family of moulds, are of common occurrence and of world-wide distribution, little work had been published until recently on their biochemical activities. Brian, Curtis, Hemming, Unwin & Wright (1949) reported that several strains of *A. solani* (E. and M.) Jones and Grout and one strain of *A. porri* (Ell.) Sacc. produce, in a variety of liquid media and in fair yields, a metabolic product which they named alternaric acid. This substance, which is not appreciably antibacterial, has a remarkably

specific antifungal activity and is phytotoxic. The same workers and Jefferys (Brian *et al.* 1951) determined the optimum conditions for the production and isolation of alternaric acid and gave a more detailed account of its antifungal properties. They showed that while germination of the spores of some fungi, e.g. *Absidia glauca* and *Myrothecium verrucaria*, is prevented by 1 µg./ml. of alternaric acid, concentrations as high as 100 µg./ml. are required to prevent the germination of the spores of other fungi, e.g. *Botrytis allii*, although, with this fungus,

extension of the germ tubes is markedly retarded at a concentration as low as 0.01 $\mu\text{g.}/\text{ml.}$ Grove (1952) described alternaric acid, $\text{C}_{21}\text{H}_{30}\text{O}_8$, m.p. 138° , as a highly crystalline, colourless, optically inactive, unsaturated dibasic acid, giving a monomethyl ester, m.p. $106\text{--}107^\circ$.

Darpoux, Faivre-Amiot & Roux (1950) recorded the isolation of the following metabolic products from culture filtrates of a strain of *A. solani*: (a) a substance, which they named alternarine, colourless needles, m.p. 230° , which has marked antibacterial activity against a number of Gram + and Gram - plant pathogenic bacteria; (b) an unnamed red pigment crystallizing in clusters of needles, which has no definite melting point but decomposes at about 260° ; (c) a colourless crystalline substance, m.p. 136° , which they believe to be alternaric acid. They also isolated, by ether extraction of the dried mycelium of the same fungus: (d) an unnamed yellow crystalline substance which has no melting point but sublimes completely at 270° and dissolves immediately in alkalis giving an orange-red solution; (e) a red varnish which was not obtained crystalline. No analyses are recorded for any of these substances.

We have found that ethanolic extracts of the dried mycelium of two different strains of *A. tenuis*, which were isolated from tomatoes, give a strong purple colour with ethanolic ferric chloride. The present communication describes the isolation and study of the two substances responsible for this colour reaction. A comprehensive account of *A. tenuis* is given by Neergaard (1945) in a monograph on Danish species of *Alternaria* and *Stemphylium*. He points out that this species, which has an extraordinarily wide distribution and a great range of hosts, is usually found on the dead and weakened parts of plants on which it lives as a saprophyte. Infection experiments have shown, however, that it may appear as a weak parasite on a great many species of plants. Industrially, *A. tenuis* is also of considerable importance since, *inter alia*, it is the cause of serious losses in paper manufacture through its discoloration of wood pulp, and it is also the cause of the destruction of flax fibres because of its ability to decompose both pure pectin and pure cellulose.

The two strains of *A. tenuis*, L.S.H.T.M. catalogue numbers 94 and 430, were grown for a period of 5 weeks at 24° on Czapek-Dox solution. The washed, dried and ground mycelium was extracted with light petroleum to remove fatty materials. It was then redried and re-extracted with ether which removed completely the substances giving the purple ferric reaction. There separated from the ether a colourless crystalline solid, which, on fractionation from ethanol, gave two pure crystalline substances which we propose to name alternariol and alternariol

monomethyl ether since neither of them is identical with alternaric acid or with the metabolic products of *A. solani* described by Darpoux *et al.* (1950).

The yields of the crystalline ether extract obtained presented points of some interest. With *A. tenuis* strain no. 94, when freshly isolated from nature, we obtained 8 g. of crystals from a batch of fifty flasks. On continued cultivation this yield steadily decreased until it had dropped to less than 0.1 g. per fifty flasks after 6 months. With strain no. 430 the first three batches of fifty flasks, after isolation from nature, yielded 8.3, 4.4 and 4.8 g. Sand cultures of this strain were then made and were preserved in evacuated and hermetically sealed tubes. Almost 18 months afterwards a fourth batch of fifty flasks, prepared from these sand cultures, gave the rather surprising yield of 18.0 g. The crystalline ether extracts, on fractionation from ethanol, gave about 10 times as much alternariol methyl ether as of alternariol itself.

Alternariol, $\text{C}_{14}\text{H}_{10}\text{O}_5$, m.p. 350° (decomp.), sublimes without decomposition at 250° in a high vacuum. It contains no methoxyl group. It does not dissolve in aqueous sodium bicarbonate but dissolves readily in cold aqueous sodium carbonate or sodium hydroxide giving a weak yellow-green solution which changes to orange-red on standing overnight. Its ethanolic solution, which gives no precipitate with Brady's reagent, gives an intense purple colour with ethanolic ferric chloride. It slowly reduces Fehling's solution on boiling and, in a parallel test with resorcinol, reduction occurs at approximately the same rate. On boiling a solution of it, in aqueous sodium hydroxide, with an equal volume of chloroform a bright cherry-red colour forms in the aqueous layer. Resorcinol gives an almost identical reaction, but catechol gives no significant colour. It dissolves in cold concentrated sulphuric acid giving a yellow solution with a strong blue-green fluorescence. It shows moderate antibacterial activity completely suppressing the growth of *Staphylococcus aureus* (Oxford H strain) at a concentration of 1:40 000 and that of *Escherichia coli* at a concentration of 1:20 000.

Alternariol methyl ether, $\text{C}_{15}\text{H}_{12}\text{O}_5$, m.p. 267° (decomp.), sublimes without decomposition at $180\text{--}200^\circ$ in a high vacuum. It contains one methoxyl group, one methyl group attached to carbon and two active hydrogen atoms. It shows no optical activity. It does not dissolve in aqueous sodium bicarbonate, but is soluble in hot aqueous sodium carbonate. It readily dissolves in cold dilute aqueous sodium hydroxide giving a yellow-green solution which changes overnight to cherry-red. It gives the same reactions with ferric chloride, Fehling's solution, chloroform and sodium hydroxide and concentrated sulphuric acid as does alternariol. On demethylation, by heating at 140° with con-

centrated hydriodic acid, it gives an almost quantitative yield of alternariol.

The following functional derivatives of alternariol and alternariol methyl ether were prepared. (a) Diacetyl alternariol methyl ether, $C_{19}H_{16}O_7$, i.e. $C_{14}H_7O_2 \cdot (OCH_3)(O \cdot CO \cdot CH_3)_2$, by heating alternariol methyl ether with acetic anhydride and anhydrous sodium acetate. The product, m.p. 162–163°, is insoluble in cold aqueous 2*N*-sodium hydroxide, gives no ferric colour in ethanol and gives the same colour with concentrated sulphuric acid as does the parent substance. (b) Alternariol trimethyl ether, $C_{17}H_{14}O_5$, i.e. $C_{14}H_7O_2 \cdot (OCH_3)_3$, by long continued (14 days) methylation with ethereal diazomethane of alternariol methyl ether in dioxan solution or by methylation of alternariol or alternariol methyl ether with dimethyl sulphate and anhydrous potassium carbonate in acetone. The product, m.p. 162.5–164°, gives no colour with ethanolic ferric chloride and is insoluble in cold aqueous 2*N*-sodium hydroxide but dissolves slowly on boiling. (c) Hydrolysis product of alternariol trimethyl ether, $C_{17}H_{16}O_6$, i.e. $C_{14}H_9O_3 \cdot (OCH_3)_3$, by hydrolysis of alternariol trimethyl ether with aqueous *N*-sodium hydroxide in methanol solution followed by careful acidification. The product, m.p. 170–171°, dissolves in aqueous sodium bicarbonate and titrates in the cold as a monobasic acid. It gives no ferric colour in ethanol and does not reduce Fehling's solution. It is readily reconverted into alternariol trimethyl ether by sublimation in a high vacuum at its melting point. (d) Methyl ether methyl ester of (c), $C_{19}H_{22}O_6$, i.e. $C_{14}H_7O \cdot (OCH_3)_5$, by methylation of alternariol methyl ether with dimethyl sulphate and aqueous potassium hydroxide. The ester, m.p. 124–125°, gives no ferric colour in ethanol and is insoluble in cold aqueous sodium hydroxide and only dissolves very slowly on boiling. It gives a purplish precipitate in a hydroxamic acid test, indicative of an ester grouping. (e) Methyl ether of (c), $C_{18}H_{20}O_6$, i.e. $C_{14}H_8O_2 \cdot (OCH_3)_4$, by alkaline hydrolysis in methanol of the ester (d). The free acid forms colourless microtablets, m.p. 181–182°. It titrates in the cold as a monobasic acid and dissolves readily in aqueous sodium bicarbonate. It gives no ferric reaction in ethanol and sublimes unchanged in a high vacuum at 170°.

The general properties just described of the functional derivatives of alternariol and alternariol methyl ether justify the following conclusions as to the nature of the five oxygen atoms present in alternariol and in its methyl ether. Three of the oxygen atoms in alternariol, $C_{14}H_{10}O_5$, are probably phenolic in nature since they can be methylated with diazomethane. The formula for alternariol may therefore be written as $C_{14}H_7O_2 \cdot (OH)_3$. The insolubility of the diacetate (a) $C_{14}H_7O_2 \cdot (O \cdot CO \cdot$

$CH_3)_2 \cdot (OCH_3)$, in cold aqueous alkali demonstrates the absence of a free carboxyl group in alternariol methyl ether and hence, inferentially, in alternariol itself. What then is the function of the two remaining oxygen atoms? We believe that they are present in a lactone ring since this hypothesis offers a simple explanation of the following experimentally established facts.

The alkaline hydrolysis product (c) of alternariol trimethyl ether contains one molecule of water more than its parent substance and is readily soluble in aqueous sodium bicarbonate, whereas the parent substance does not dissolve even in aqueous sodium hydroxide except on heating. These facts are most readily explained by postulating the opening of a lactone ring with the formation of a hydroxycarboxylic acid. Hence the formula $C_{17}H_{16}O_6$ for the hydrolysis product (c) may be expanded to $C_{13}H_7 \cdot (OCH_3)_3 \cdot (OH) \cdot (COOH)$, and the formula $C_{14}H_7O_2 \cdot (OCH_3)_3$ for alternariol trimethyl ether may

be expanded to $C_{13}H_7 \cdot (OCH_3)_3 \cdot \begin{array}{c} O \\ | \\ CO \end{array}$. The correct-

ness of this hypothesis was established by methylating the hydrolysis product (c) when the methyl ether methyl ester (d), $C_{19}H_{22}O_6$, was obtained; this has therefore the expanded formula $C_{13}H_7 \cdot (OCH_3)_4 \cdot (COOCH_3)$. As would be expected, this substance is insoluble in cold aqueous sodium hydroxide but on hydrolysis with hot methanolic sodium hydroxide gives the free carboxylic acid (e), $C_{18}H_{20}O_6$, i.e. $C_{13}H_7 \cdot (OCH_3)_4 \cdot (COOH)$, which titrates sharply as a monobasic acid and is readily soluble in aqueous sodium bicarbonate.

The presence of a $-COOCH_3$ group in the methyl ether methyl ester (d), $C_{19}H_{22}O_6$, was confirmed by heating it with concentrated hydriodic acid and red phosphorus. The substance was demethylated, one molecule of carbon dioxide was evolved and estimated and the decarboxylation product, $C_{13}H_{15}O_4$, was obtained as colourless rectangular platelets, m.p. 246–249°. The same substance was also formed in good yield when alternariol methyl ether was fused with potassium hydroxide at 300°, although, when alternariol methyl ether was heated with concentrated hydriodic acid and red phosphorus no carbon dioxide was evolved and alternariol was isolated in good yield. The decarboxylation product gives a purple-blue ferric reaction in aqueous ethanolic solution. It is insoluble in aqueous sodium bicarbonate but dissolves in aqueous sodium carbonate and in aqueous sodium hydroxide. It contains one methyl group attached to carbon. On methylation with dimethyl sulphate and anhydrous potassium carbonate in dry acetone it forms a tetramethyl ether, $C_{17}H_{20}O_4$, i.e. $C_{13}H_8 \cdot (OCH_3)_4$, as colourless irregular tablets, m.p. 73–74°, which give

no ferric colour and which do not dissolve in aqueous sodium hydroxide even on boiling.

The parent hydrocarbon corresponding to the decarboxylation product $C_{13}H_{12}O_4$ is thus clearly $C_{13}H_{12}$ and, since the decarboxylation product is known to contain a $C-CH_3$ group, the formula for the parent hydrocarbon may be written $C_{13}H_9 \cdot CH_3$. The same conclusion is arrived at from consideration of the formula $C_{13}H_7 \cdot (OCH_3)_4 \cdot (COOCH_3)$ for the methyl ether methyl ester (d).

A plausible structure for the parent hydrocarbon $C_{13}H_9 \cdot CH_3$ was deduced from the following facts. (i) Alternariol methyl ether and the methyl ether methyl ester (d), $C_{13}H_{22}O_6$, resisted all attempts to reduce them catalytically. Further, the methyl ether methyl ester (d) resisted an attempt to ozonolyse it. Hence we may conclude that neither alternariol methyl ether nor the methyl ether methyl ester (d) nor the parent hydrocarbon $C_{13}H_{12}$

$C-CH_3$ group in the parent hydrocarbon $C_{13}H_{12}$ is probably in the ortho position, i.e. 6 or 6', since if it were in any other position a methylfluorene would be expected to arise by zinc-dust distillation.

Accepting this hypothesis, partial structure III may be assigned to the decarboxylation product $C_{13}H_{12}O_4$ and partial structure IV to alternariol trimethyl ether.

Evidence as to the positions occupied by the three methoxyl groups and the lactone grouping in alternariol trimethyl ether was obtained as follows. A solution of the hydrolysis product (c) of alternariol trimethyl ether in cold aqueous N sodium hydroxide was oxidized with potassium permanganate at a temperature not exceeding 35°. The two pure oxidation products isolated were identified as 3:5-dimethoxyphthalic acid (V) and the previously unknown 2-carboxy-3:5-dimethoxyphenylglyoxylic acid (VI). The structure (VI) of the second oxidation product was established as follows. The product, $C_{11}H_{10}O_7$, H_2O , m.p. 173–174°, contains two methoxyl groups and titrates sharply in the cold as a dibasic acid. It is readily soluble in cold aqueous sodium bicarbonate. On oxidizing the product in the cold with alkaline hydrogen peroxide, 3:5-dimethoxyphthalic acid (V), $C_{10}H_{10}O_6$, was formed in good yield. On heating the product with

contains an ethylenic double bond. (ii) A mixture of alternariol methyl ether and electrolytic zinc dust was subjected to distillation in a current of hydrogen. Fluorene ($C_{13}H_{10}$, I) was isolated from the mixture of volatile degradation products obtained and was conclusively identified by analysis and by comparison with an authentic specimen including X-ray powder examination.

The simplest structure for the parent hydrocarbon $C_{13}H_{12}$ which satisfies the following requirements (a) contains a $C-CH_3$ group; (b) contains no ethylenic double bonds; and (c) gives fluorene on zinc-dust distillation, is II.

The fact that an unsubstituted fluorene is obtained by zinc-dust distillation indicates that the

concentrated hydriodic acid and red phosphorus it was demethylated, one molecule of carbon dioxide was evolved and was estimated and 3:5-dihydroxyphenylacetic acid (VII) was isolated. The identity of the 3:5-dihydroxyphenylacetic acid, m.p. 130–131°, was established by comparison (as acid and as the previously unknown methyl ester) with authentic synthetic specimens.

The isolation of (VI) fixes the structure of ring B in structure IV which may now be expanded to VIII. This structure is supported by the fact that alternariol itself gives an intense purple salicylic ferric colour so that one of the hydroxyl groups in alternariol is almost certainly in the ortho position to the potential carboxyl group.

The structure of the alkaline hydrolysis product (c), $C_{17}H_{18}O_8$, of alternariol trimethyl ether then becomes IX. This compound (IX), but not its methyl ether, gives a positive Gibbs reaction (1927). Hence position 5' in ring A is probably unsubstituted and, if so, the OCH_3 group in ring A must be either in position 3' or 4'.

The general correctness of these deductions and the fact that alternariol trimethyl ether has probably structure X and not the alternative structure with the CH_3O group in position 3' was proved by its synthesis.

Alternariol trimethyl ether

Hurtley (1929) condensed resorcinol with *o*-bromobenzoic acid in an alkaline medium with copper sulphate as catalyst and obtained what has since been shown to be 4'-hydroxydibenzo- α -pyrone (XI, $R' = OH$, $R'' = R''' = H$, $R'''' = >O$).

This reaction has proved to be of general application in the synthesis of substituted dibenzo- α -pyrones. It was extensively studied and used by Adams and his collaborators in their synthesis of cannabinol (XI, $R'''' = (Me)_2$, $R'' = Me$, $R' = OH$, $R' = n-C_5H_{11}$) and substances related to cannabinol. Cannabinol is a constituent of Egyptian and Indian hashish which is obtained from *Cannabis indica*. During the course of this work they synthesized 6'-*n*-amyl-4'-hydroxy-5-methyldibenzo- α -pyrone

by condensing olivetol (1-*n*-amyl-3:5-dihydroxybenzene) with 2-bromo-4-methylbenzoic acid (Adams, Pease, Clark & Baker, 1940*a*; Adams, Baker & Wearn, 1940*b*). The constitution of the pyrone was rigorously proved.

In a number of analogous syntheses orcinol (3:5-dihydroxy-1-methylbenzene) was condensed with different substituted *o*-bromobenzoic acids. Thus Adams *et al.* (1940*a*) obtained 4'-hydroxy-6'-methyldibenzo- α -pyrone from orcinol and *o*-bromobenzoic acid, and 4'-hydroxy-5:6'-dimethyldibenzo- α -pyrone from orcinol and 2-bromo-4-

methylbenzoic acid. Lederer & Polonsky (1948), in a study of the synthesis of 4:4'-dihydroxydibenzo- α -pyrone which Lederer (1942) had isolated from castoreum, the secretion of the scent glands of the beaver (*Castor fiber*), condensed orcinol with 2-bromo-5-methoxybenzoic acid and obtained 4'-hydroxy-4-methoxy-6'-methyldibenzo- α -pyrone. All these findings point to the conclusion that, when substituted *o*-bromobenzoic acids are condensed in the Hurtley reaction with orcinol, condensation takes place at the carbon atom in the orcinol nucleus which is ortho and para to the two hydroxyl groups.

In an analogous manner we used the Hurtley reaction to condense orcinol (XII) with 2-bromo-

4:6-dimethoxybenzoic acid, m.p. 151–152° (XIII), which was synthesized from 2-amino-4:6-dimethoxytoluene (Curd & Robertson, 1933) via the Sandmeyer reaction followed by permanganate oxidation of the methyl group to carboxyl. The resulting condensation product, 4'-hydroxy-3:5-dimethoxy-6'-methyldibenzo- α -pyrone (XIV), m.p. 293–296°, was methylated giving 3:4':5-trimethoxy-6'-methyldibenzo- α -pyrone (X), m.p. 162.5–163°, not depressed on admixture with alternariol trimethyl ether, m.p. 162.5–164°. Both specimens could also be obtained in a form melting at a lower temperature (about 140°), and the yellow solution of each specimen in cold concentrated sulphuric acid showed a strong blue-green fluorescence in ultraviolet light.

It follows, therefore, that alternariol is 3:4':5-trihydroxy-6'-methyldibenzo- α -pyrone (XV).

(XV)

Alternariol.

Accepting structure XV for alternariol the designations of the different methyl derivatives and partial degradation products of alternariol follow. Thus the hydrolysis product of alternariol trimethyl ether becomes 2'-hydroxy-3:4':5-trimethoxy-6'-methyldiphenyl-2-carboxylic acid (c) p. 428, and the decarboxylation product of the methyl ether methyl ester (d) and the potash fusion product of alternariol methyl ether becomes 2':3:4':5-tetrahydroxy-6'-methyldiphenyl.

The exact position of the methoxyl group in the second metabolic product of *A. tenuis*, alternariol monomethyl ether, is uncertain though it is believed to be in either position 4' or 5 for the following reasons. (i) Alternariol monomethyl ether, like alternariol itself, gives an intense purple salicylic ferric colour. Hence we believe that position 3 in alternariol monomethyl ether is occupied by a hydroxyl and not by a methoxyl group. (ii) In the Gibbs test (1927) alternariol gives a strong purple colour in 1 min.; after the same time alternariol monomethyl ether gives a similar but paler purple colour. This is readily understood if the methoxyl group occupies either position 4' or 5 but not if it occupies position 3. Hence we believe that the metabolic product, alternariol monomethyl ether, is 3:(4' or 5)-dihydroxy-(5 or 4')-methoxy-6'-methyldibenzo- α -pyrone. It is of interest here to note that both orcinol and resorcinol give a strong

purple colour in the Gibbs test, though most other phenols, including phenol itself, give a strong pure-blue colour.

Although a number of substituted dibenzo- α -pyrones of both plant and animal origin have been described we believe that alternariol and its monomethyl ether are the first recorded substituted dibenzo- α -pyrones of fungal origin.

EXPERIMENTAL

All melting points are uncorrected except where otherwise stated. Methoxyl estimations and determination of titration equivalents were carried out in this Department, all other estimations by Weiler and Strauss, Oxford.

History of cultures

We are indebted to Mr G. Smith of this Department for the following mycological notes on the cultures used in this investigation.

Both the strains L.S.H.T.M. catalogue numbers 94 and 430 were isolated here by Mrs S. Marcus from tomato. In both cases the first cultures produced spores abundantly, but, whilst no. 430 is still in good condition and sporulating freely on most culture media, no. 94 has deteriorated and produces spores only sparsely, and on some media not at all. However, when first isolated, the two strains were very similar, in colour and texture of colonies, in length of spore chains and in shape and size of spores.

Colonies spread rapidly on all media; on potato agar fairly dense, with a basal felt of dark mycelium overgrown with dirty-white to greyish funiculate hyphae; on Czapek agar similar; on corn-meal agar thin, transparent, very dark dull green to almost black, sporing well; spore chains mostly long, often branched, with up to 10 or 11 spores; spores extremely polymorphous, ovate, conical, cylindrical or irregular, mostly beakless or with very short beak but occasionally with fairly long definite beak, with or without longitudinal septa, rough or smooth, mostly fairly deep olive-brown, number of cross septa 1–9, of longitudinal septa 0–4, size 15–50 $\times$ 8–17 μ .

The most recent taxonomic study of *Alternaria* is by Neergaard (1945). He divides the genus into three groups on the basis of length of spore chains. The species to be considered in the present connexion are the three species in the group Longicatenatae, *A. tenuis*, *A. circinans* and *A. resedae*, and also *A. tenuissima*, in the group Brevicatenatae, which is stated to produce long chains in artificial culture. The spores of *A. circinans* and *A. resedae* are somewhat similar, being comparatively uniform in shape and mostly lacking longitudinal septa. *A. tenuis* is sharply distinguished from these by its markedly polymorphous spores, mostly with longitudinal septa. *A. tenuissima* differs from *A. tenuis* in the somewhat greater size of the spores, in the spores mostly having fairly long beaks, and in the lesser degree of variation in shape and size. There is little doubt that the two isolates nos. 94 and 430 are to be assigned to *A. tenuis* auct. sensu stricto in Neergaard's sense. Neergaard states that this species is a ubiquitous saprophyte and weak polyphagous parasite. Although he does not state categorically that it attacks tomato, one of the isolates on which he based his study, and assigned by him to *A. tenuis* sensu stricto, was obtained from tomato.

Table 1. *Cultural characteristics of Alternaria tenuis strain no. L.S.H.T.M. 94*

Incubation period (days)	pH of culture filtrate	Residual glucose by polarimeter (%)	Culture filtrate ether extract/flask		Mycelium wt./flask (g.)	Mycelium ether extract/flask	
			Total extract (g.)	Crystalline extract (mg.)		Total extract (g.)	Crystalline extract (mg.)
21	7.3	1.24	—	—	3.19	0.27	69
25	7.3	0.78	0.23	37	3.82	0.33	81
31	7.3	0.56	0.24	40	3.99	0.41	161
39	7.5	0.09	0.28	54	4.11	0.44	160

Cultural conditions

Batches of a hundred 1 l. conical flasks, plugged with cotton wool, were prepared, each containing 350 ml. of Czapek-Dox solution, i.e. glucose, 50.0 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 l. After sterilization, each flask was inoculated with spore suspensions obtained from freshly grown Czapek-agar slopes of the chosen strain of *A. tenuis*, either no. 94 or no. 430, and incubated in the dark at 24°. Growth of the mould was rather slow. The mycelium was a dirty-grey colour and contained patches of dull-olive green and black with a black reverse. It was normally very slimy to the touch. The culture fluid became pale brown in colour towards the end of the incubation period.

In order to determine the optimum time for harvesting, batches of fifty flasks were harvested after 21, 25, 31 and 39 days' incubation. Determinations were made of pH (colorimetrically), residual glucose (polarimetrically) in the culture filtrates, weight of mycelium (dried to constant weight), and of ether extract from the mycelium and from the culture filtrate as described below. The results obtained are shown in Table 1.

Isolation and purification of alternariol and alternariol methyl ether

Batches of fifty flasks were harvested after 35 days. The pale-brown culture fluid was decanted from the mould mycelium which was scraped out and squeezed in butter muslin. The mycelium was then thoroughly washed with water, strained again through muslin and pressed as dry as possible in a tincture press. It was thoroughly dried in a vacuum oven at 45–50° and ground to a fine powder in a coffee mill. The average weight of dry mycelium from a batch of fifty flasks of either strain of *A. tenuis* was 200–210 g.

The dried, ground mycelium was extracted in Soxhlets with light petroleum (b.p. 40–60°) for 8 hr. The resulting light-orange-yellow extract, on removal of the solvent, gave a yellow oil containing a small quantity of a colourless wax-like solid. This extract gave no ferric reaction in ethanolic solution.

The mycelium was now re-dried and was then re-extracted with dry ether for 12–13 hr. when extraction was practically complete. Within 20 min. of commencing the extraction a colourless crystalline solid began to separate. The majority of the product was extracted in the first hour. After the extraction was complete the separated solid was collected by filtration; the orange-pink ethereal filtrate was concentrated and yielded initially a further crop of crystals and finally a red oily liquid.

In the case of *A. tenuis* strain no. 94 the crystals which separated during the extraction process formed 80–90% of the total (8 g.) crystalline extract and consisted of almost pure *alternariol methyl ether* melting only 4° below the m.p. of the pure substance, i.e. 267° (decomp.). The crystals which were obtained on concentration of the ethereal mother liquor consisted of a mixture of *alternariol methyl ether* and *alternariol*, which, when purified, melted at 350° (decomp.).

With *A. tenuis* strain no. 430 the crystals which separated during the extraction process consisted of a mixture of *alternariol methyl ether* and *alternariol*, which on heating softened at approx. 260° and decomposed completely at 330–340°. The combined yields of crystalline solid from the first four batches of fifty flasks of strain no. 430 were 8.3, 4.4, 4.8 and 18.0 g. (see p. 422). The proportion of *alternariol methyl ether* to *alternariol* was approximately 10:1 with strain no. 94 and was slightly less with strain no. 430.

Mixtures of *alternariol methyl ether* and *alternariol* can be readily separated into their components by means of the much higher solubility of the latter compound in ethanol. Thus a hot ethanolic solution of a mixture of the two substances, on cooling, deposits *alternariol methyl ether* in the form of colourless feathery needles. *Alternariol* remains in solution in the ethanol from which it may be separated, by diluting with hot water, in the form of colourless needles which are finally purified by sublimation in a high vacuum at 250°. *Alternariol methyl ether* crystallizes readily from ethanol or dioxan, the latter solvent being more suitable for larger quantities. The approximate solubilities in these solvents at their boiling points and at 0–5° are, in ethanol 0.6 and 0.2%, and in dioxan 5.0 and 1.4% respectively. The product isolated from some batches of mycelium, particularly the early batches of strain no. 94, was accompanied by a pink contaminant which persisted throughout the above purification processes.

General properties of alternariol

Alternariol crystallizes from aqueous ethanol in colourless needles, m.p. 350° with decomposition and with the formation of a small microcrystalline sublimate of unchanged material. It sublimes without decomposition in a high vacuum at 250°. It contains no N, S, Cl or methoxyl. (Found: C, 65.4; H, 3.9. $\text{C}_{14}\text{H}_{10}\text{O}_6$ requires C, 65.1; H, 3.9%.) *Alternariol* is more soluble than its methyl ether in ethanol, methanol, acetone and ether and almost insoluble in benzene or light petroleum. It does not dissolve in water or in aqueous NaHCO_3 , but readily dissolves in cold aqueous Na_2CO_3 or NaOH giving a weak yellow-green solution which changes to an orange-red colour on standing overnight. Its ethanolic solution gives no precipitate on the addition of an

equal volume of Brady's reagent (0.32% 2,4-dinitrophenylhydrazine in aqueous 2N-HCl). It gives the following reactions. (a) Its ethanolic solution gives an intense purple colour with ethanolic FeCl_3 ; (b) on boiling with a mixture of equal volumes of CHCl_3 and aqueous NaOH, a bright cherry-red colour develops in the alkali layer; resorcinol gives an almost identical reaction but catechol gives no significant colour; (c) reduces hot Fehling's solution at about the same rate as resorcinol; (d) dissolves in cold conc. H_2SO_4 to a yellow solution with a strong blue-green fluorescence.

General properties of alternariol methyl ether

Alternariol methyl ether crystallizes from ethanol or dioxan in fine colourless needles, m.p. 267° (decomp.). It sublimes without decomposition in a high vacuum at $180\text{--}200^\circ$. It contains no N, S or Cl but contains one methoxyl group. (Found: C, 66.1, 66.3; H, 4.4, 4.5; OCH_3 , 11.0, 11.4; C-CH_3 , 7.5 = 1.36 C-CH_3 groups; active H atoms (in pyridine), 1.67; mol.wt. (Rast), 294. $\text{C}_{17}\text{H}_{16}\text{O}_5$ requires C, 66.2; H, 4.4; OCH_3 , 11.4; C-CH_3 , 5.5%; mol.wt. 272.) A saturated ethanolic solution (approx. 0.3%) did not exhibit any optical activity when viewed in a 2 dm. tube. It is moderately soluble in dioxan, ethanol and acetone, slightly soluble in ether and almost insoluble in benzene and light petroleum. It does not dissolve in water or in aqueous NaHCO_3 but dissolves in hot aqueous Na_2CO_3 , although on cooling a microcrystalline deposit separates. It readily dissolves in cold dilute aqueous NaOH giving a yellow-green solution which changes overnight to cherry red. It gave the same reactions (a), (b), (c) and (d) as are described above for alternariol.

Demethylation of alternariol methyl ether.

Isolation of alternariol

In a Zeisel estimation alternariol methyl ether (0.0981 g.) was refluxed for 4 hr. at 140° with constant b.p. HI (15 ml.). The CH_3I formed was led over in CO_2 into ethanolic AgNO_3 . (Found: OCH_3 , 11.9%.) On cooling, the HI solution contained a solid which was collected, washed with hot water and dried; 0.0874 g. = 94% of the theoretical; colourless microneedles. The product was purified by sublimation in a high vacuum at 250° giving a colourless sublimate, m.p. 350° (decomp.) not depressed on admixture with alternariol from *A. tenuis*. (Found: C, 65.3; H, 3.9. $\text{C}_{14}\text{H}_{10}\text{O}_5$ requires C, 65.1; H, 3.9%.)

Derivatives of alternariol and alternariol methyl ether

Acetylation of alternariol methyl ether. Alternariol methyl ether was boiled with anhydrous sodium acetate and acetic anhydride for 5 min. and the cooled mixture was diluted with water. Diacetylated alternariol methyl ether after three crystallizations from ethanol formed long colourless needles, m.p. $162\text{--}163^\circ$. (Found: C, 63.9; H, 4.4; OCH_3 , 8.7. $\text{C}_{19}\text{H}_{16}\text{O}_7$, i.e. $\text{C}_{15}\text{H}_{10}\text{O}_3 \cdot (\text{O} \cdot \text{CO} \cdot \text{CH}_3)_2$, requires C, 64.0; H, 4.5; OCH_3 , 8.7%.) It is insoluble in cold aqueous 2N-NaOH. Its ethanolic solution gives no colour with ethanolic FeCl_3 . It gives the same colour with conc. H_2SO_4 as does the parent substance.

Methylation of alternariol methyl ether and alternariol.

With dimethyl sulphate and K_2CO_3 in acetone. Alternariol methyl ether (1 g.), anhydrous K_2CO_3 (4 g.), dimethyl

sulphate (4 ml.) and dry acetone (100 ml.) were heated under reflux for 7 hr. The solution, which no longer gave a purple ferric colour, was filtered and the residue washed with fresh acetone. The pale-yellow filtrate, on concentration, gave clusters of colourless feathery needles (0.95 g.) which on recrystallization from acetone gave alternariol trimethyl ether as colourless feathery needles, m.p. $162.5\text{--}164^\circ$ (corr.), resetting and then remelting at $162.5\text{--}163.5^\circ$. (Found: C, 67.9; H, 5.2; OCH_3 , 30.5, 30.7. $\text{C}_{17}\text{H}_{16}\text{O}_5$ requires C, 68.1; H, 5.4; 3OCH_3 , 31.0%.) It is insoluble in cold aqueous 2N-NaOH but dissolves slowly on boiling. It gives no colour with ethanolic FeCl_3 . It gives the same colour as the parent substance with conc. H_2SO_4 . On boiling with a mixture of equal volumes of CHCl_3 and aqueous NaOH, the red colour given under these conditions by alternariol and by its methyl ether is no longer produced. Further, resorcinol dimethyl ether yielded on similar treatment only a light-orange colour in striking contrast to the intense red colour given by resorcinol itself.

Alternariol similarly treated gave an identical product.

With diazomethane. An ethereal solution of diazomethane was added to a solution of alternariol methyl ether (0.10 g.) in dioxan (10 ml.). Methylation took place very slowly, and it was necessary to continue the reaction for 14 days with periodic addition of fresh diazomethane to ensure full methylation. On removal of the solvents there remained a mobile gum (0.09 g.) which crystallized on the addition of ether. Recrystallization from acetone gave colourless feathery needles, m.p. $162.5\text{--}164^\circ$, not depressed on admixture with the trimethyl ether as prepared above.

Alkaline hydrolysis of alternariol trimethyl ether

Isolation of 2'-hydroxy-3:4:5-trimethoxy-6'-methyl-2-phenyl-2-carboxylic acid. A solution of alternariol trimethyl ether (0.2006 g., m.p. $162.5\text{--}164^\circ$) in methanol (40 ml.) and N-NaOH (2.00 ml.) was refluxed for 4.5 hr. in an atmosphere of O_2 - and CO_2 -free N_2 . After cooling, the colourless solution was titrated against N-HCl using phenolphthalein as indicator. (Found, equivalent, corrected for blank: 299. $\text{C}_{17}\text{H}_{16}\text{O}_5$ titrating as a monobasic acid requires 300.) The neutralized solution was concentrated to about 0.25 vol., cooled, acidified to Congo red with N-HCl, and the colourless crystalline solid which separated was collected, washed with water and dried. The product (0.195 g.) consisted of heavy colourless tablets, m.p. $170\text{--}171^\circ$. (Found: C, 63.3; H, 5.8; equiv. by titration, 316. $\text{C}_{17}\text{H}_{18}\text{O}_6$ requires C, 64.1; H, 5.7%; equiv. titrating as a monobasic acid, 318.) The product is insoluble in water but dissolves in aqueous NaHCO_3 . With conc. H_2SO_4 it gives a weak-yellow solution with a slight green fluorescence. Both the colour and the fluorescence are much less intense than those given by alternariol trimethyl ether but both become more intense on heating. It does not reduce Fehling's solution and its ethanolic solution gives no colour with ethanolic FeCl_3 . The product could not be purified by recrystallization since all such attempts yielded a product of slightly lower melting point which was not completely soluble in cold aqueous 2N-NaOH. The m.p. $170\text{--}171^\circ$ was depressed on admixture with alternariol trimethyl ether, m.p. $162.5\text{--}164^\circ$. The acid was readily reconverted into alternariol trimethyl ether by sublimation at its melting point in a high vacuum and crystallization of the colourless transparent sublimate from acetone.

Methylation of alternariol methyl ether with dimethyl sulphate and aqueous KOH

Isolation of the methyl ester of 2':3:4':5-tetramethoxy-6'-methyldiphenyl-2-carboxylic acid. A solution of alternariol methyl ether (0.50 g.) in methanol (10 ml.) was refluxed and dimethyl sulphate (10 ml.) and 20% aqueous KOH (40 ml.) were added dropwise over a period of 20 min., the KOH being added at such a rate as to keep the mixture alkaline; the mixture was then refluxed for 2.75 hr. The opalescent yellow solution, pH approx. 8.0, on cooling deposited a sticky, pale-brown, gum which was collected, washed with water and dried. The gum (0.31 g.), which readily crystallized on trituration with methanol, on recrystallization from methanol, yielded the ester as colourless heavy prisms, m.p. 124–125°. (Found: C, 66.1; H, 6.3; OCH₃, 44.3. C₁₉H₂₂O₆ requires C, 65.9; H, 6.4; 5OCH₃, 44.8%.) The ester is insoluble in cold aqueous NaOH and only dissolves very slowly on boiling. It gives no ferric colour in ethanolic solution. In conc. H₂SO₄ it gives a weak yellow colour with a slight green fluorescence, both colours being far less intense than those given by alternariol methyl ether. In a hydroxamic acid test it gives a purplish precipitate indicative of an ester grouping (Feigl, 1939).

The methylation solution filtrate from the above crude ester was acidified to Congo red. The resulting gummy deposit was collected, washed with water and dried (89 mg.). It was crystallized from aqueous methanol giving flat, colourless microtablets (wt. 56 mg.), m.p. 179°, not depressed on admixture with the acid C₁₈H₂₀O₆ obtained by alkaline hydrolysis of the ester C₁₉H₂₂O₆ (see following paragraph).

Dilutions (× 1000)	1:20	1:40	1:80	1:160	1:320	Control
<i>Staph. aureus</i>	—	—	+	++	++	++
<i>Esch. coli</i>	—	++	++	++	++	++

Alkaline hydrolysis of the ester C₁₉H₂₂O₆

Isolation of 2':3:4':5-tetramethoxy-6'-methyldiphenyl-2-carboxylic acid. A mixture of the ester (0.20 g.), aqueous 2N-NaOH (20 ml.) and methanol (2 ml.) was refluxed for 3 hr. A slight precipitate was separated by filtration and the cooled filtrate was acidified to Congo red dropwise with conc. HCl, when a colourless solid (0.15 g.) separated. This substance crystallized from aqueous methanol giving the hydrolysis product as colourless microtablets, m.p. 181–182°. (Found: C, 64.8; H, 6.2; OCH₃, 37.7; equiv. by titration, 329. C₁₈H₂₀O₆ requires C, 65.1; H, 6.1; 4OCH₃, 37.4%; equiv. titrating as a monobasic acid, 332.) The hydrolysis product sublimes unchanged in a high vacuum at 170°. It is insoluble in water but readily dissolves in aqueous NaHCO₃. It does not give a ferric reaction in ethanolic solution and conc. H₂SO₄ produces only a pale-yellow solution with a slight green fluorescence but this colour becomes more intense on heating the solution.

Colour reactions with 2:6-dichloroquinone chloroimide (Gibbs, 1927)

The reaction was carried out as follows. About 1 mg. of the substance under test was dissolved in 1 ml. of sodium borate buffer, pH 9.2, by heating if necessary. To each solution, after cooling, was added one drop of a freshly prepared aqueous suspension of 2:6-dichloroquinone chloro-

imide. The following colour reactions were obtained: natural alternariol, immediate strong purple reaching maximum after 1 min.; natural alternariol monomethyl ether, immediate paler purple; synthetic alternariol dimethyl ether (see p. 432) and natural alternariol trimethyl ether (in acetone solution), negative even after 2 hr.; alkaline hydrolysis product, C₁₇H₁₈O₆, of alternariol trimethyl ether, i.e. 2'-hydroxy-3:4':5'-trimethoxy-6'-methyldiphenyl-2-carboxylic acid (see p. 428), immediate deep blue reaching maximum after 1 min.; 2'-methyl ether, C₁₈H₂₀O₆, of the above, i.e. 2':3:4':5'-tetramethoxy-6'-methyldiphenyl-2-carboxylic acid (see previous para.), negative; orcinol or resorcinol, deep purple; phenol, deep blue.

Antibacterial activity of alternariol
(by Mrs S. MARCUS)

Alternariol is insoluble in water but a dilute solution was obtained in phosphate buffer pH 8.0. A 1:10 000 solution of alternariol in phosphate buffer pH 8.0 (4.5 ml.) was mixed with an equal volume of sterile glucose broth. Half of the mixture (4.5 ml.) was transferred to a second tube containing glucose broth (4.5 ml.) and so on, giving a series of solutions of concentrations of alternariol of 1:20 000, 1:40 000, 1:80 000, 1:160 000 and 1:320 000. One batch of such tubes was inoculated with a 24 hr. culture of the Oxford H strain of *Staphylococcus aureus* and the other batch with a similar culture of *Escherichia coli* N.C.T.C. strain no. 86. The tubes were incubated for 24 hr. at 37° with the following results (—, no growth; +, growth; ++, growth as in control).

A series of controls using only phosphate buffer, pH 8.0, showed no growth inhibition.

Oxidation of alternariol trimethyl ether with alkaline KMnO₄. Isolation of 3:5-dimethoxyphthalic acid and 2-carboxy-3:5-dimethoxyphenylglyoxylic acid

Alternariol trimethyl ether (2 g.) was refluxed with N-NaOH (80 ml.) in N₂ for 0.5 hr. The resulting colourless solution was cooled, and a solution of KMnO₄ (7 g.) in water (100 ml.) was added with stirring and cooling. The temperature of the reaction solution rose to a maximum of 35°. After 24 hr. at room temperature, the heavy MnO₂ precipitate was separated by filtration and washed with water. The alkaline filtrate and washings were combined and adjusted to pH 7 with 2N-H₂SO₄. The resulting solution was evaporated *in vacuo* at 35° until approximately 20 ml. of distillate had been collected, the vapours being passed through Brady's reagent in which, however, no precipitate was formed. The neutral, partially evaporated, oxidation solution was acidified to Congo red and extracted with ether (4 × 1 vol.). On removal of the solvent there remained a yellow gum (0.69 g.) which, on trituration with dry ether, crystallized in very pale-yellow rectangular plates. Recrystallization from hot water gave oxidation product I (0.48 g.) which on further recrystallization melted at 184° (decomp.).

The ether-extracted oxidation solution was adjusted to pH 7.0 with aqueous NaOH and evaporated *in vacuo* at 35–40° to 30 ml. The evaporated solution was re-acidified and extracted with ether (5 × 1 vol.). Removal of the solvent

gave a pale-yellow gum (0.39 g.) containing colourless needles. The oxidation solution was now extracted continuously with ether for three successive 10 hr. periods yielding extracts weighing 0.35, 0.07 and 0.04 g., respectively. The gum (0.39 g.) obtained above by hand extraction with ether and the first of the continuous ether extracts (0.35 g.) which contained some gum but was largely crystalline were washed with dry ether to remove the gum. The combined crystals were purified by crystallization from water giving oxidation product II as heavy colourless needles, m.p. 173–174° (decomp.).

General properties of oxidation product I. Identification as 3:5-dimethoxyphthalic acid (V). Oxidation product I crystallizes from water in cream-coloured rectangular prisms, which on heating melt at 184° to a colourless liquid which on cooling resets to colourless needles, m.p. 148°. When heated to 100° in a high vacuum, oxidation product I loses water of crystallization (7.34%) without change in melting point. This loss in weight, if due to the removal of 1 mol. water, corresponds to the formation of a compound of mol. wt. 228. (Found, on above dried material: C, 53.3; H, 4.4; OCH₃, 27.0; equiv. by titration, 109. Calc. for C₁₀H₁₀O₆: C, 53.1; H, 4.4; 2OCH₃, 27.4%; mol. wt. 226; equiv. titrating as a dibasic acid, 113.) The acid gives a wine-red colour on heating alone with conc. H₂SO₄. On heating the acid with resorcinol and conc. H₂SO₄, followed by dilution with water and addition of excess NaOH, there is obtained a yellow solution with an intense green fluorescence. Both these reactions are given by 3:5-dimethoxyphthalic acid.

The unusual behaviour on heating of 3:5-dimethoxyphthalic acid, which has been noted in detail by Oxford & Raistrick (1933, p. 650), renders it unsuitable for diagnostic purposes. For this reason the anhydride and dimethyl ester were prepared for the purpose of comparison.

The acid was heated at 190° in an oil bath for 5 min., by which time all effervescence had ceased. On cooling, the melt set to a mass of colourless needles which were purified either by crystallization from *o*-xylene or by sublimation in a high vacuum giving long colourless needles, m.p. 149–150°, not depressing the m.p. 148° of an authentic synthetic specimen of 3:5-dimethoxyphthalic anhydride.

The acid (21 mg.) was treated with an excess of an ethereal solution of diazomethane. The acid gradually dissolved and N₂ was evolved. After removal of the solvent the colourless alkali-insoluble product (22 mg.) was purified by crystallization from aqueous ethanol giving dimethyl 3:5-dimethoxyphthalate as fine colourless needles, m.p. 92°, not depressed on admixture with authentic synthetic dimethyl 3:5-dimethoxyphthalate, m.p. 91–92°. (Found: C, 56.9; H, 5.6; OCH₃, 48.4. Calc. for C₁₂H₁₄O₆: C, 56.7; H, 5.6; 4OCH₃, 48.8%.)

General properties of oxidation product II. Identification as 2-carboxy-3:5-dimethoxyphenylglyoxylic acid (VI). Oxidation product II crystallizes from water as colourless needles, m.p. 173–174° (decomp.). On further heating the melt effervesces and changes to a deep-red liquid which partially resets at about 240° and does not remelt on raising the temperature to 260°. When heated to 100° in a high vacuum 1 mol. water of crystallization is evolved, the crystals become opaque but no change in melting point results. (Found: loss in weight at 100°, 5.7. C₁₁H₁₂O₈·H₂O requires 6.2%. On above dried material: C, 48.6; H, 4.4; OCH₃, 23.7, 23.7; equiv. by titration, 130. C₁₁H₁₂O₈ requires C, 48.5; H, 4.4; 2OCH₃, 22.8%; equiv. titrating as a dibasic acid, 136.) The acid is soluble in NaHCO₃ and also in dilute

aqueous NaOH to give a pale-yellow solution from which it crystallizes unchanged on acidification. No precipitate is formed on the addition of Brady's reagent to an aqueous solution of the acid. It dissolves in cold conc. H₂SO₄ to a pale-yellow solution which on heating becomes an intense wine-red colour. On shaking the solid acid with a mixture of conc. H₂SO₄ and benzene containing a trace of thiophene the acid layer develops a yellow colour which changes through orange to a strong reddish brown in 15 min. and during 2 days to a deep-green colour. No colour develops in the benzene layer. The corresponding colour sequence described for 2-carboxy-4:5-dimethoxyphenylglyoxylic acid by Kuroda & Perkin (1923) is very similar except that the benzene layer developed a green colour.

The acid was identified as 2-carboxy-3:5-dimethoxyphenylglyoxylic acid as follows. (a) A solution of the hydrated acid (90 mg.), neutralized with 0.1 N-NaOH to phenolphthalein, was cooled in ice-water, and ice-cold 10 vol. H₂O₂ (0.5 ml.) was added. The oxidation solution was held at 0° for 0.5 hr., and then at room temperature for 3 hr. The solution (pH 6.9) was acidified with N-HCl (0.5 ml.) when almost colourless, rectangular microplates separated. The crystals (31 mg.) were filtered, washed with water and dried. The filtrate and washings were exhaustively extracted with ether yielding 44 mg. of a mixture of colourless needles and gum. The first crop of crystals (31 mg.) on heating melted at 182–183° with effervescence and on cooling reset to a mass of colourless needles which remelted at 147°. A mixed melt of the crystals with authentic 3:5-dimethoxyphthalic acid also remelted at 147°. The remainder of this crop was methylated with diazomethane as described above. The neutral methylation product, crystallized from *o*-xylene, melted at 93°, undepressed on admixture with authentic synthetic dimethyl 3:5-dimethoxyphthalate, m.p. 92°. (b) A mixture of anhydrous oxidation product II (0.1092 g.), red P (0.25 g.) and constant b.p. HI (4 ml.) was refluxed at 140° in a stream of O₂ and CO₂-free N₂. The effluent gases were passed through a trap containing an aqueous suspension of red P and were then passed through bubblers containing 0.2 N-Ba(OH)₂. CO₂ equivalent to 99.6% of 1 mol. prop. was evolved in 3 hr. In order to ensure complete reduction of the product, the mixture was refluxed for a total of 8 hr. with little further evolution of CO₂. The hot solution was then filtered through a sintered-glass funnel and the residue was washed with hot water. The cooled filtrate and washings were extracted with ether (4 × 1 vol.). The combined ether extracts were shaken with water (5 ml.) to remove HI and then with conc. aqueous Na₂S₂O₃ (5 ml.) to remove I₂. On removal of the solvent there remained an almost colourless gum (25 mg.) which set to a mass of radiating needles on the addition of a small volume of dry ether. The ether-extracted solution was re-extracted with ethyl acetate (4 × 1 vol.) giving a further crop of gummy needles (41 mg.). A suitable solvent could not be found from which to crystallize the small quantity of product available. It was purified by repeated sublimation in a high vacuum at 120°. 3:5-Dihydroxyphenylacetic acid (VII) was thus obtained as fine colourless needles, m.p. 130–131° not depressing the m.p. of an authentic synthetic specimen, m.p. 128–130°. An aqueous solution gave a moderate violet ferric reaction.

The above 3:5-dihydroxyphenylacetic acid (0.10 g.; m.p. 130–131°) was treated in the usual way with an ethereal solution of diazomethane; the product was crystallized thrice from benzene giving methyl 3:5-dihydroxyphenylacetate as colourless rectangular plates, m.p. 112° not

depressed on admixture with an authentic synthetic specimen of the same m.p. The ester is soluble in cold water, and its aqueous solution gives a stable bluish purple ferric reaction which differs slightly from that given by the free acid. The ester gives a strong brownish red colour in a hydroxamic acid test whereas the parent free acid gave none.

Synthesis of 3:5-dihydroxyphenylacetic acid and its methyl ester. Synthetic triethyl orcinoltricarboxylate (Jordan, 1899) (0.2368 g.; m.p. 96–98°) was refluxed with red P (0.25 g.) and constant b.p. HI (4 ml.). The CO₂ evolved was estimated as described under section (b) p. 430. CO₂ (100.4% of 2 mol. equiv.) was evolved in 0.75 hr. but the heating was continued for a total of 1.5 hr. The reaction solution was filtered and extracted first with ether and then with ethyl acetate as described in section (b). The ether extract (77 mg.) consisted of colourless needles of 3:5-dihydroxyphenylacetic acid, m.p. 127–128°, raised to 128–130° by sublimation in a high vacuum at 120° and not depressed on admixture with the corresponding product, m.p. 130–131°, from oxidation product II. The ethyl acetate extract was a partly crystalline gum (40 mg.). von Pechmann & Wolman (1898) recorded that 3:5-dihydroxyphenylacetic acid, when isolated from aqueous solution, melts at 54° but that drying raises the m.p. to 127–128°. The crystalline form and the general properties, including the violet ferric reaction, of the synthetic acid were identical with those of the acid obtained from oxidation product II.

Synthetic 3:5-dihydroxyphenylacetic acid (0.26 g.), dissolved in ethanol (1 ml.), was treated with a 10% excess (for 1 mol.) of an ethereal solution of diazomethane standardized against benzoic acid. On removal of the solvent after 30 min. the yellow oily residue crystallized and was sublimed in a high vacuum at 100–105°. The colourless sublimate of *methyl 3:5-dihydroxyphenylacetate* (0.20 g.), rosettes of needles, melted at 112–113° not depressed on admixture with the methyl ester, m.p. 112°, described above. (Found: C, 59.1; H, 5.5; OCH₃, 16.9. C₉H₁₀O₄ requires C, 59.3; H, 5.5; 1 OCH₃, 17.0%.)

Zinc-dust distillation of alternariol methyl ether.

Isolation of fluorene

An intimate mixture of alternariol methyl ether (1 g.) and electrolytic Zn dust (20 g.) was subjected to distillation in a combustion tube at a dull red heat in a slow current of H₂, the volatile degradation products passing through an adjacent column of heated electrolytic Zn dust (20 g.). An orange-red oil condensed in the cooler part of the tube and deposited a quantity of colourless needles. The mixture was collected in ether and shaken with aqueous NaOH, acidification of which failed to reveal any phenolic component. On removal of the ether there remained an orange-red crystalline oil (0.12 g.) with a cresol-like smell, which was distilled in steam. A red gum (0.01 g.) remained and a mixture of colourless solid and oil separated from the steam distillate. Extraction with ether and removal of the solvent yielded a mixture (0.11 g.) of colourless oil and needles. It was dissolved in a small volume of ethanol, the solution showing a light blue-purple fluorescence in daylight; on cooling, short needles (40 mg.), m.p. 99°, separated. Further crystallization from ethanol yielded colourless needles (10 mg.), m.p. 106–107° raised to 107–108° by sublimation in a high vacuum at 50–55°. (Found: C, 93.8, 93.5; H, 6.3, 6.0. Calc. for C₁₃H₁₀: C, 94.0; H, 6.0%.) A mixture of the crystals, m.p. 107–108°, with authentic

fluorene, m.p. 113–114°, melted at 110–111°. Also the two specimens had the following properties in common. Both specimens possessed a slight cresol-like smell; ethanolic solutions showed the same weak blue-purple fluorescence in daylight; on heating with conc. H₂SO₄ both gave an intensely blue solution though the Zn-dust distillation product had a somewhat greenish tint probably due to traces of the accompanying colourless oil which itself gave a strong yellow-brown colour on heating with conc. H₂SO₄; both rapidly sublimed in a high vacuum at 55°, the sublimate having identical crystalline forms (spiky needles).

We are indebted to Mr R. Bones, Department of Chemistry, University College, London, for conclusive proof that the zinc-dust distillation product is identical with synthetic fluorene. Mr Bones prepared comparative X-ray powder photographs of the Zn-dust product and of synthetic fluorene using sectors to screen off half the film at a time. The two specimens gave identical diffraction lines, the actual values agreeing with those previously quoted for fluorene by Hofer & Peebles (1951), who claim that a characteristic powder photograph is given by fluorene even when not completely pure.

Degree of unsaturation

Catalytic hydrogenation. A solution of alternariol methyl ether (0.544 g.) in dioxan (35 ml.) was shaken for 1.5 hr. in an atmosphere of H₂ at 22° and 746 mm. with a palladium-norite catalyst. No significant absorption of H₂ was observed and unchanged alternariol methyl ether (0.52 g.) was recovered.

The ester C₁₉H₂₂O₆ was shaken with H₂ and a Pd-norite catalyst in methanol, and in a separate experiment with Adams's PtO₂ catalyst. There was no significant uptake of H₂ and the starting material was recovered unchanged in each case.

Ozonolysis of the ester C₁₉H₂₂O₆. Ozonized O₂ was passed for 2 hr. through a solution of the ester C₁₉H₂₂O₆ (0.29 g.) in CHCl₃ (10 ml.) at room temperature. No CO₂ was evolved. The CHCl₃ solution, which had developed a pale-yellow colour, was shaken with an equal volume of water at 60°. The CHCl₃ layer was shown to contain no NaHCO₃-extractable material. On removal of the solvent there remained a colourless, mobile gum (0.26 g.) which solidified on trituration with methanol, and, on crystallization from the same solvent, yielded unchanged starting material (0.15 g.), m.p. 124°.

A portion of the aqueous layer gave no precipitate with Brady's reagent and evaporation of the remainder to dryness gave no residue.

KOH fusion of alternariol methyl ether.

Isolation of 2':3:4':5'-tetrahydroxy-6'-methylidiphenyl

Alternariol methyl ether (2 g.), KOH (10 g.) and water (4 ml.) were heated slowly in a nickel crucible to 180° with stirring. A brown frothing liquid was formed. KOH (10 g.) was added, the temperature was raised to 300° in 10–15 min. when the melt had become quiescent. The dark-brown mixture was cooled, dissolved in water (70 ml.) and slowly acidified with conc. HCl (25 ml.). The cooled acid liquid was extracted with ether (5 × 1 vol.). Removal of the solvent yielded a brown gum (1.50 g.) which slowly crystallized in almost colourless needles. The *product* was purified by crystallization from water and sublimation in a high

vacuum at 200° and was obtained as colourless rectangular platelets, m.p. 247–250°. (Found: C, 66.7; H, 5.4; OCH₃, nil; C-CH₃, 7.3% = 1.12 C-CH₃ groups. C₁₃H₁₂O₄ requires C, 67.2; H, 5.2; C-CH₃, 6.5%.) The product is insoluble in aqueous NaHCO₃ but is soluble in aqueous Na₂CO₃ and in NaOH. Its aqueous solution gives a purple-blue ferric reaction, but an ethanolic solution gives no colour with ethanolic FeCl₃. With conc. H₂SO₄ it gives only a pale-yellow solution with a green fluorescence which is only evident in ultraviolet light. On boiling its solution in aqueous NaOH with CHCl₃ a cherry-red colour forms in the alkali layer. Unlike alternariol (see p. 429) it shows no antibacterial activity against *Staph. aureus* and *Esch. coli* at the highest concentration tested, i.e. 1:2000.

The product (1 g.) was methylated with dimethylsulphate (5 ml.), K₂CO₃ (5 g.) and dry acetone (100 ml.). After refluxing under N₂ for 5 hr. the mixture was diluted with an equal volume of water and boiled for 10 min. The resulting brown oil which crystallized on cooling was collected, washed with water and dried, 1.2 g., m.p. 68–70°. The methylated product, 2':3':4':5'-tetramethoxy-6'-methylidiphenyl, purified by crystallization from methanol or light petroleum (b.p. 60–80°), formed irregular tablets, m.p. 73–74°, which were insoluble in aqueous 2N-NaOH even on boiling and gave no ferric reaction in ethanol. (Found: C, 70.5; H, 7.1; OCH₃, 42.7, 43.1. C₁₇H₂₀O₄ requires C, 70.8; H, 7.0; 4 OCH₃, 43.0%.) Attempts to oxidize the methylated product with KMnO₄ led only to uncrystallizable gums.

Action of HI and red phosphorus

The method followed was that described on p. 430 for the preparation of 3:5-dihydroxyphenylacetic acid.

(a) *On alternariol methyl ether. Isolation of alternariol.* A mixture of alternariol methyl ether (0.1863 g.), red P (0.25 g.) and constant b.p. HI (4 ml.) was refluxed for 3 hr. No CO₂ was evolved. After filtering the cooled reaction mixture, there remained on the filter a mixture of red P and a colourless solid. This was dried, extracted with boiling ethanol and filtered. On removal of the solvent from the filtrate there remained colourless needles (0.179 g.), m.p. 347–349° (decomp.) not depressed on admixture with alternariol.

(b) *On the ester C₁₉H₂₂O₆. Isolation of 2':3':4':5'-tetrahydroxy-6'-methylidiphenyl.* A mixture of the ester C₁₉H₂₂O₆ (see p. 429) (0.1101 g.), red P (0.25 g.) and HI (4 ml.) was used. CO₂ began to be evolved at a bath temperature of 130°, decarboxylation was complete in 0.75 hr. and the reaction was continued for 2 hr. to ensure complete demethylation. CO₂ (0.995 mol. prop.) was evolved. The reaction mixture was filtered. Crystals (65 mg.), m.p. 246–249° (decomp.), separated from the cooled filtrate. They were sublimed in a high vacuum at 200° giving a colourless crystalline sublimate, m.p. 246–249° (decomp.) not depressed on admixture with the KOH fusion product C₁₃H₁₂O₄, m.p. 247–250° (decomp.). The two compounds had also identical properties (see p. 431).

Syntheses

2-Bromo-4:6-dimethoxytoluene. CuSO₄, 5H₂O (1.2 g.), copper bronze (0.4 g.), NaBr (3.1 g.), conc. H₂SO₄ (0.33 ml.) and water (20 ml.) were boiled under reflux for 30 min. Meanwhile, hydrated 2-amino-4:6-dimethoxytoluene sulphate (1.0 g.), prepared according to Curd & Robertson (1933), was stirred in conc. H₂SO₄ (0.25 ml.) and water (10 ml.), cooled in an ice bath and diazotized with NaNO₂

(0.35 g.). The Cu₂Br₂ suspension was placed in a steam-distillation apparatus, and when distillation had commenced the diazonium suspension was added and washed in with ice-cold water. Most of the volatile material distilled rapidly, and when a total of 250 ml. had been distilled the distillate was made alkaline with 2N-NaOH (25 ml.) and extracted with ether. The residue after removing the ether (0.22 g.) was used directly for the next stage; it did not solidify on standing at 0° overnight.

2-Bromo-4:6-dimethoxybenzoic acid. The above crude 2-bromo-4:6-dimethoxytoluene (0.22 g.) was refluxed with KMnO₄ (0.34 g.) and 0.33N-NaOH (20 ml.) until all the pink colour had gone (2 hr.); at the end of this period much unreacted material remained as oily drops on the surface. The mixture was cooled and treated with sodium bisulphite and 2N-HCl to decolorize, and finally acidified to Congo red. The solution was then extracted with ether (3 × 1 vol.); from the ethereal solution the acidic products were removed with 0.5N-NaHCO₃ (2 × 5 ml.). After washing with ether the bicarbonate layer was cleared of ether on the water pump, then acidified, when heavy prisms slowly formed. After standing at 0° overnight these were filtered, washed and dried (23 mg., m.p. 143–148°).

The unoxidized material was re-oxidized with the same amount of KMnO₄ and gave a further quantity of acid (33 mg.), m.p. 146–150°. More material (less pure) was obtained on extracting the filtrates with ether. Repeated recrystallization from water gave pure 2-bromo-4:6-dimethoxybenzoic acid as colourless prisms, m.p. 151–152°. (Found: C, 41.2; H, 3.6; Br, 31.0; OCH₃, 23.5. C₉H₆O₄Br requires C, 41.4; H, 3.5; Br, 30.6; 2 OCH₃, 23.8%.)

4'-Hydroxy-3:5-dimethoxy-6'-methylidibenzo-α-pyrone. 2-Bromo-4:6-dimethoxybenzoic acid (68 mg.), orcinol (68 mg.) and N-NaOH (0.52 ml.) were heated on the steam bath and to the solution was added 0.7% CuSO₄, 5H₂O (0.52 ml.). A slight flocculent precipitate was produced at once, and during 4 hr. heating on the steam bath no further solid separated. When 2N-HCl (0.52 ml.) was added immediate precipitation of a crystalline solid occurred; after heating for a further 20 min. the mixture was filtered hot. The precipitate was washed with water, then a little 1% NaHCO₃ and finally with water again. The crude material (36 mg.) was crystallized from ethanol to give pure 4'-hydroxy-3:5-dimethoxy-6'-methylidibenzo-α-pyrone as nearly colourless needles, m.p. 293–296°. (Found: C, 67.1; H, 4.9; OCH₃, 21.6. C₁₆H₁₄O₅ requires C, 67.1; H, 4.9; 2 OCH₃, 21.7%.) The compound dissolved in conc. H₂SO₄ shows a strong greenish fluorescence in ultraviolet light. It is insoluble in aqueous NaHCO₃ and can be crystallized from acetone or acetic acid as well as ethanol. It gives a negative Gibbs test.

3:4':5'-Trimethoxy-6'-methylidibenzo-α-pyrone. 4'-Hydroxy-3:5-dimethoxy-6'-methylidibenzo-α-pyrone (22 mg.) was methylated with dimethyl sulphate (0.1 ml.) and anhydrous K₂CO₃ (0.1 g.) in boiling dry acetone (10 ml.) for 6 hr. The acetone was removed and water (10 ml.) added. Excess dimethyl sulphate was decomposed and the solid was collected and dried. Crystallization from aqueous acetone, then ethanol, yielded colourless needles, m.p. initially 139–140° (incomplete) but resetting on further heating and melting finally quite sharply at 161–162° (a sample of the pure trimethyl ether of alternariol, m.p. 162.5–164° (corr.), was recrystallized from ethanol and showed the same 'double melting point' behaviour). The synthetic material was dissolved in warm acetone, cooled and allowed to evaporate spontaneously at room temperature after seeding

with the natural compound. The colourless needles melted at 162–163° with no softening at 140°. After recrystallization from ethanol (seeding with the higher melting material) a constant m.p. of 162.5–163° (corr.) was obtained. A mixture with alternariol trimethyl ether, m.p. 162.5–164° (corr.), also melted at 162.5–163°; in each case the melt resolidified on removal from the bath and on replacing at 155° partly remelted, then recrystallized slowly; the synthetic compound then remelted at 162–162.5°, the mixture at 162–163°. 3:4':5-Trimethoxy-6'-methylidibenzo- α -pyrone therefore exists in two forms, one of m.p. about 140° which is readily transformed above its melting point to that of m.p. 162–163°. (Found: C, 67.8; H, 5.5; OCH₃, 31.0. C₁₇H₁₆O₆ requires C, 68.0; H, 5.4; 3OCH₃, 31.0%). The substance exhibits a blue-green fluorescence in conc. H₂SO₄ identical with that given by the natural compound.

SUMMARY

1. Two hitherto undescribed mould metabolic products, alternariol and its monomethyl ether, have been isolated from the dried mycelium of laboratory cultures of two different strains of *Alternaria tenuis* grown on Czapek-Dox solution.

2. Alternariol, C₁₄H₁₀O₆, forms colourless needles, m.p. 350° (decomp.), which show moderate antibacterial activity against *Staphylococcus aureus* and

Escherichia coli. Its monomethyl ether, C₁₅H₁₂O₆, forms colourless needles, m.p. 267°.

3. A number of derivatives and breakdown products of alternariol and of its methyl ether are described.

4. Alternariol trimethyl ether, colourless needles, m.p. 162.5–163° (corr.), has been synthesized by condensing orcinol and 2-bromo-4:6-dimethoxybenzoic acid and methylating the condensation product.

5. Alternariol has been shown to be 3:4':5-trihydroxy-6'-methylidibenzo- α -pyrone.

The naturally occurring monomethyl ether has its methoxyl group at position 5 or 4'.

6. Alternariol and its methyl ether are the first recorded substituted dibenzo- α -pyrones of fungal origin.

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