Studies in the Biosynthesis of Fungal Metabolites

4. ALTERNARIOL MONOMETHYL ETHER AND ITS RELATION TO OTHER PHENOLIC METABOLITES OF *ALTERNARIA TENUIS**

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(*Received* 9 *November* 1960)

The isolation of alternariol and its monomethyl ether from the mycelium of *Alternaria tenuis* Auct., after growth on Czapek–Dox solution, was described by Raistrick, Stickings & Thomas (1953). Chemical degradation and synthetic studies established the structure (I) of alternariol, $C_{14}H_{10}O_5$, the biosynthesis of which was subsequently shown to involve the head-to-tail condensation of acetate units (Thomas, 1961*a*).

The position of the methoxyl group in alternariol monomethyl ether was not determined, but of the three possible alternatives (II, III or IV), shown in Fig. 1, the structure (II) was thought to be inconsistent with the intense purple ethanolic ferric reaction, which is normally associated with substituted salicylic acids. Further, the positive purple colour reaction obtained in borate buffer, pH 9·2, on addition of 2:6-dichloroquinonechloroimide, provided further evidence (Gibbs, 1927) for the presence of a phenolic hydroxyl group with a free *para* position, which is absent in structure (II). Additional evidence for the existence of a free hydroxyl *ortho* to the lactone carbonyl is now apparent from consideration of the infrared spectra of alternariol, its monomethyl ether and the trimethyl ether $C_{17}H_{16}O_5$, which is devoid of free hydroxyl substituents. Thus the carbonyl stretching frequencies of alternariol, 1665 cm.$^{-1}$ (in Nujol), 1683 cm.$^{-1}$ (in chloroform) and alternariol monomethyl ether, 1650, 1657 cm.$^{-1}$ (in Nujol), 1672 cm.$^{-1}$ (in chloroform) are appreciably lower than that of alternariol trimethyl ether, 1725 cm.$^{-1}$ (in Nujol). In view of the proximity of the peaks in Nujol and chloroform, the lower frequency of alternariol relative to the trimethyl ether may be correlated with the known structure (I), as resulting from intramolecular hydrogen bonding between the *o*-hydroxyl group and the carbonyl of an unstrained δ-lactone ring (Bellamy, 1958; Duncanson, Grove & Zealley, 1953). The presence of similarly low carbonyl frequencies, in both alternariol and its monomethyl ether, is in accord with the existence in both compounds of an *o*-hydroxyaroyl structure.

Of the two remaining alternatives (III and IV), it was tentatively suggested on the grounds of a possible relationship with the other metabolites of *A. tenuis* that (III) was the more likely (Thomas, 1951), and evidence confirming this structure for alternariol monomethyl ether is presented below.

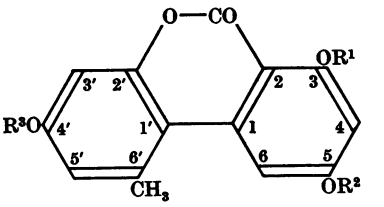

Fig. 1. Alternariol (I) and its monomethyl ethers:

(I) $R^1 = R^2 = R^3 = H$.
(II) $R^1 = CH_3$; $R^2 = H$; $R^3 = H$.
(III) $R^1 = H$; $R^2 = CH_3$; $R^3 = H$.
(IV) $R^1 = H$; $R^2 = H$; $R^3 = CH_3$.

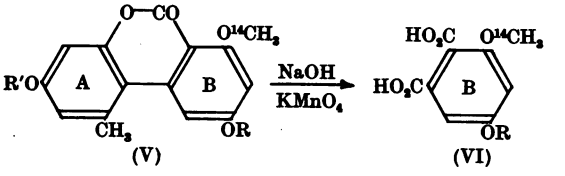

Fig. 2. Oxidation of alternariol monomethyl ether after dimethylation with $^{14}CH_3I$. The table shows the expected ratio of activities of alternariol trimethyl ether (V) and its oxidation product (VI) based on the alternative structures (III) and (IV) for the monomethyl ether.

Alternariol monomethyl ether	Alternariol trimethyl ether (V)		Ratio sp. activities, $\frac{VI}{V} \times 100$ (%)
	R	R′	
(IV)	$^{14}CH_3$	CH_3	100
(III)	CH_3	$^{14}CH_3$	50

* Part 3: Thomas (1961*b*).

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In the course of the determination of the structure of alternariol (Raistrick *et al.* 1953), it was observed that alternariol trimethyl ether, on oxidation with aqueous alkaline permanganate solution, gave rise to a mixture of 3:5-dimethoxyphthalic acid and 4:6-dimethoxyphthalonic acid, in combined yields of about 70%. It was therefore decided to ethylate alternariol monomethyl ether and carry out an analogous oxidation of the diethyl ether, $C_{19}H_{20}O_5$, to the corresponding dialkoxyphthalic acid, the nature of which would unequivocally establish the position of the original methoxyl group. However, repeated oxidation attempts under carefully controlled conditions, known to be satisfactory for the oxidation of alternariol trimethyl ether, failed to produce more than a very low yield of a non-crystalline extract.

In order to overcome this difficulty, an alternative approach was considered which involved the methylation of alternariol monomethyl ether with [^{14}C]methyl iodide. It was anticipated that this would give rise to the trimethyl ether containing two $O^{14}CH_3$ groups (V), oxidation of which would yield 3:5-dimethoxyphthalic acid (VI) containing either one or two $O^{14}CH_3$ groups (i.e. either 50% or 100% of the original activity), thus distinguishing between the alternative structures (III) and (IV) respectively (Fig. 2).

Accordingly, alternariol monomethyl ether, which was shown to be uncontaminated with alternariol, was methylated with methyl iodide in dry acetone solution in the presence of anhydrous potassium carbonate. Despite the use of conditions known to be suitable for complete methylation with dimethyl sulphate, methyl iodide only gave rise to a partially methylated product, even on prolonged refluxing with a tenfold excess of the freshly distilled liquid. Attempts to isolate the required trimethyl ether from the mixture by recrystallization from acetone only yielded a product giving an intense reddish-purple ethanolic ferric reaction, thus showing the methylation to be incomplete.

However, even partial methylation with [^{14}C]-methyl iodide appeared likely to be adequate for this purpose, since completion of the methylation with inactive dimethyl sulphate would still give rise to a labelled trimethyl ether. The activity of the 3:5-dimethoxyphthalic acid, obtained on oxidation of this ether, would likewise be dependent upon the position of the original methoxyl group in alternariol monomethyl ether. Thus the structure (IV) would give rise to labelling in ring B only, which would result in the retention of 100% of the activity in the oxidation product as before, whereas the activity of the dimethoxyphthalic acid derived from the alternative structure (III) would now depend upon the nature of the preliminary partial methylation. In this case, since the benzene ring of the phthalic acid obtained on oxidation arises from ring B of alternariol, labelling in ring A alone would lead to an oxidation product free from radioactivity. On the other hand, exclusive ^{14}C-labelling of ring B would again yield a phthalic acid containing all the original activity. Partial methylation of both rings with [^{14}C]methyl iodide would consequently produce an intermediate result, dependent upon the preferential methylation of the two free phenolic hydroxyl groups.

Accordingly, labelled alternariol trimethyl ether was prepared by sequential methylation with [^{14}C]methyl iodide and inactive dimethyl sulphate. Subsequent oxidation with alkaline permanganate yielded 3:5-dimethoxyphthalic acid, which, on assaying as the pure anhydride, proved to contain 20·4% of the activity of the parent labelled trimethyl ether. This result unequivocally favours structure (III) for alternariol monomethyl ether. From the distribution of labelling between rings A and B (approx. 4:1), it follows that the hydroxyl group on $C_{(3)}$ (ring B) is less reactive than that on $C_{(4')}$ (ring A), probably owing to its hydrogen bonding with the *ortho* lactone carbonyl group. As a consequence of the relative inactivity of the hydroxyl group on $C_{(3)}$, partial methylation of alternariol with labelled methyl iodide thus provided a greater distinction between the alternatives (III) and (IV) than would have resulted from complete methylation.

On methylation of alternariol (I) under the usual conditions for the preparation of the trimethyl ether, but with only one mole equivalent of dimethyl sulphate, a partially methylated product was readily obtained, which on recrystallization proved to be identical with the naturally occurring monomethyl ether (III). It thus appears that the ease of methylation of the three hydroxyl groups of alternariol falls in the sequence $5 > 4' > 3$, although this assumes that dimethyl sulphate and methyl iodide would methylate in the same order.

This investigation provides an instance of the use of isotopic labelling as an aid to structure determination, as opposed to its more conventional application to the study of biosynthetic pathways. This approach should prove of value in the solution of problems of a similar nature.

Among the many known phenolic constituents of fungi (cf. Raistrick, 1949), the number of recorded isolations of any individual phenol and its methyl ether from the same culture are relatively few. However, alternariol and its methyl ether are not unique in this respect and analogous examples include the *Aspergillus nidulans* depsidones, nidulin and nornidulin (Dean, Robertson, Roberts & Raper, 1953) and the *Daldinia concentrica*

products, 1:8-dihydroxynaphthalene mono- and di-methyl ethers (Allport & Bu'Lock, 1960).

In addition to alternariol and its monomethyl ether, a number of other products have been isolated from strains of *A. tenuis* and partially characterized (Rosett, Sankhala, Stickings, Taylor & Thomas, 1957). These are summarized in Table 1.

With the exception of tenuazonic acid, which was shown by Stickings (1959) to be α-acetyl-γ-*sec.*-butyltetramic acid, it is evident, even on the basis of their molecular formulae, methoxyl and carbon-methyl contents, that these compounds are probably structurally and biosynthetically interrelated. Thus Sankhala (1956) in an investigation of the chemistry of altertenuol, $C_{14}H_{10}O_6$, obtained evidence consistent with a structure related to alternariol methyl ether, which could result from the addition of a hydroxyl and the removal of a carbon-methyl group from ring A; in respect to ring B, both compounds are probably identical, although the position of the methoxyl is unknown.

It has also been demonstrated that the ring B of alternariol is present in altenuic acid I, $C_{15}H_{14}O_8$, since, on methylation with diazomethane followed by alkaline hydrolysis and oxidation with aqueous permanganate, it yielded the alternariol oxidation products, 3:5-dimethoxyphthalic acid and 4:6-dimethoxyphthalonic acid, in addition to two products of unknown constitution (Thomas, 1951). Since Rosett *et al.* (1957) showed the altenuic acids I and II to be easily convertible into altenuic acid III, the existence of a moiety identical with ring B of alternariol may be anticipated in each case.

The remaining metabolites altenusin, $C_{15}H_{14}O_6$, and its oxidation product dehydroaltenusin, $C_{15}H_{12}O_6$, can at present only be related to alternariol indirectly through altenuic acid II, on the basis of similar ultraviolet-absorption spectra (Table 2), although, as previously mentioned, analytical data alone strongly indicate a structural relationship. Thus the three absorption maxima of dehydroaltenusin closely parallel those of altenuic acid II, which in turn occur at similar wavelengths to the three peak values of orsellinic acid (2:4-dihydroxy-6-methylbenzoic acid). Since orsellinic acid is a simple derivative of ring B of alternariol and since this ring appears to be unchanged in altenuic acid I, it seems likely that its presence in dehydroaltenusin (and consequently altenusin) provides the simplest interpretation of its ultraviolet-absorption data.

The presence of additional peaks in the spectrum of alternariol (which closely approximated to the spectra of alternariol mono- and tri-methyl ethers) indicates the existence of additional conjugation to that present in orsellinic acid, as would be anticipated from the presence of a diphenyl structure.

Table 1. *Metabolites of* Alternaria tenuis *Auct.*

		M.p.	*C*-methyl
Alternariol	$C_{14}H_{10}O_5$	350°	1
Alternariol methyl ether	$C_{14}H_9O_4$ (OMe)	267°	1
Altenuic acid I	$C_{14}H_{11}O_7$ (OMe)	183–184°	1
Altenuic acid II	$C_{14}H_{11}O_7$ (OMe)	245–246°	1
Altenuic acid III	$C_{14}H_{11}O_7$ (OMe)	Variable 185–235°	1
Altenusin	$C_{14}H_{11}O_5$ (OMe)	202–203°	1
Dehydroaltenusin*	$C_{14}H_9O_5$ (OMe)	189–190°	1
Altertenuol	$C_{13}H_7O_5$ (OMe)	284–285°	0
Tenuazonic acid	$C_{10}H_{15}O_3N$	B.p. 117°/ 0·035 mm.	3

* May be an artifact of the isolation procedure (Rosett, Sankhala, Stickings, Taylor & Thomas, 1957).

Table 2. *Ultraviolet-absorption maxima of alternariol, dehydroaltenusin, altenuic acid II and orsellinic acid*

All measurements were made in ethanol solution (7–20 mg./l.), except where otherwise stated.

	$\lambda_{max.}$ (mμ), (log ε values in parentheses)		
Alternariol	335–342 (4·06)	301 (4·05)	
	290 (4·02)	257 (4·73)	220 (4·53) (shoulder)
Dehydroaltenusin	297 (3·93)	255 (4·31)	221 (4·45)
Altenuic acid II	295 (3·85)	257 (4·21)	219 (4·62)
Orsellinic acid*	297 (3·72)	262 (4·07)	216 (4·48)
Orsellinic acid (in 0·1 N-HCl)†	296 (3·50)	260 (3·99)	214 (4·29)

* Orsellinic acid synthesized from orcinol (Thomas, 1961*a*).

† Computed from the curve obtained by Mosbach (1960).

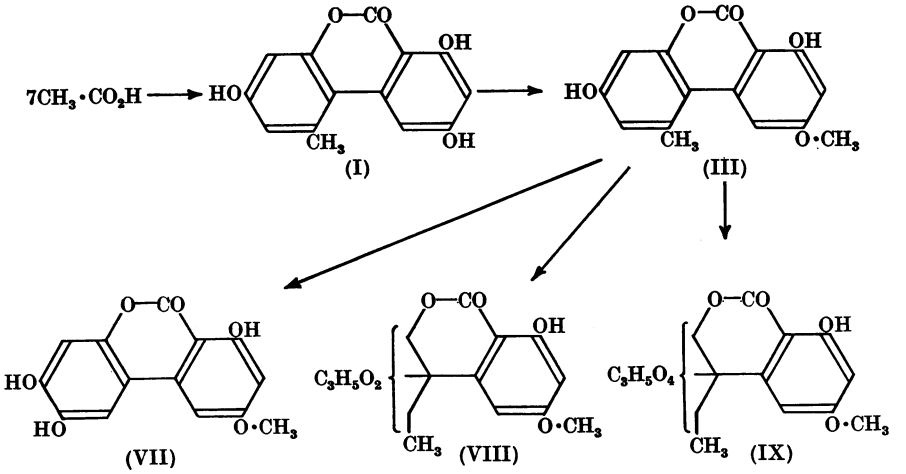

Fig. 3. Suggested biosynthetic interrelationship of *A. tenuis* phenolic metabolites: (VII), possible structure of altertenuol, $C_{14}H_{10}O_6$; (VIII), partial structure of altenusin, $C_{15}H_{14}O_6$; (IX), partial structure of altenuic acids I, II and III, $C_{15}H_{14}O_8$.

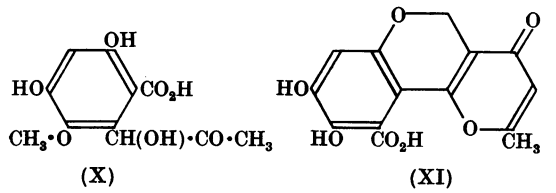

Fig. 4. Ustic acid and citromycetin.

On this basis, the simpler spectra related to orsellinic acid of altenuic acid II and dehydroaltenusin may be an indication of the modification of ring A of alternariol in these compounds.

If all the monomethoxyl-containing metabolites shown in Table 2 are biosynthetically derived from alternariol (I) via its monomethyl ether (III), then, since alternariol itself has been shown to arise from the condensation of acetate units, one could reasonably predict a biosynthetic interrelationship as illustrated in Fig. 3.

Methylation of one hydroxyl group in ring B of alternariol would be expected to increase the relative stability of this ring. Modification of ring A of alternariol has already been proposed for altertenuol as was mentioned earlier; the methyl side chain is probably removed by oxidation and decarboxylation. Although the position of the additional hydroxyl group is at present undetermined, among known examples of mould metabolites containing a trihydroxybenzene moiety the 1:3:4-orientation is the one most frequently encountered, cf. ustic acid (X) (Raistrick & Stickings, 1951), fusarubin (Ruelius & Gauhe, 1950) and citromycetin (XI) (Mackenzie, Robertson & Whalley, 1950) (Fig. 4). The last is particularly relevant inasmuch as the carboxyl group, which is acid-labile, is known to be derived biogenetically through oxidation of an acetate-methyl group (Birch *et al.* 1958). An analogous modification of ring A of alternariol followed by decarboxylation would thus lead to the structure (VII) for altertenuol. A possible common precursor of citromycetin, fusarubin and alternariol has been discussed by Thomas (1961*a*).

The existing altertenuol degradation data is also satisfied by the formulation of ring A as a 1:2:3-trihydroxybenzene (pyrogallol) derivative. This grouping, although relatively rare among fungal metabolites, is known to be present in dihydrofuscin and flavipin (Raistrick & Rudman, 1956). Further, all three compounds may share a common acetate origin leading to a 1:3-dihydroxybenzene intermediate, the additional hydroxylation occurring after cyclization. However, both dihydrofuscin and flavipin differ from altertenuol in being trisubstituted pyrogallols, and it is conceivable that their unusual 1:2:3-trihydroxy orientation is necessitated by the formation of the three vicinal carbon side chains before the final hydroxylation step.

In order to convert alternariol monomethyl ether, $C_{15}H_{12}O_5$, into the altenuic acids, $C_{15}H_{14}O_8$, it would be necessary to add H_2O_3, without either the modification of ring B or the loss of a carbon-methyl group. Although this may in theory be effected without cleavage of ring A, in view of the known properties of these acids, a modification of this nature merits serious consideration. Such cleavages of aromatic rings without loss of carbon

are well known, for example, the conversion of 3-hydroxyanthranilic acid into quinolinic acid and the metabolism of protocatechuic acid to β-carboxymuconic acid. Each of these examples involves the fission of a shikimic acid-derived benzenoid derivative.

Birkinshaw (1953) postulated an analogous mode of formation of the heterocyclic mould product patulin, through oxidative ring fission of gentisic aldehyde. It was subsequently shown that patulin can arise from 6-methylsalicylic acid, both of which occur together with gentisyl alcohol in *Penicillium patulum* (Bu'Lock & Ryan, 1958; Tanenbaum & Bassett, 1959). Similarly, orsellinic acid (XII) was converted by ring fission into penicillic acid (XIV) (Mosbach, 1960) (see Fig. 5). These two examples require the oxidative metabolism of benzenoid products which are known to arise in fungi via the acetate pathway. Further, they could both involve fission of the $C_{(3)}$–$C_{(4)}$ bond of a substituted 1:3:4-trihydroxybenzene intermediate, which may be significant in relation to altertenuol as a possible intermediary metabolite of alternariol. Of the two possible alternative modes of cleavage of orsellinic acid (XII), the one favoured by the above hypothesis was in fact that which was shown to take place. On the basis of the preceding discussion, a hypothetical intermediate similar to (XIII) would be anticipated.

No analogous fission of a dibenzenoid product appears to have been investigated, but a ring cleavage closely parallel to that involved in protocatechuic acid metabolism is strongly indicated on structural grounds for ellagic acid, $C_{14}H_6O_8$ (XV), and chebulic acid, $C_{14}H_{12}O_{11}$ (XVI) (Schmidt & Mayer, 1951). This could further undergo a cyclical condensation accompanied by decarboxylation and dehydrogenation to yield brevifolincarboxylic acid, $C_{13}H_8O_8$ (XVII) (Haworth & Grimshaw, 1955; Schmidt, 1956; Schmidt & Eckert, 1956) as shown in Fig. 6.

It is possible that the formation of the *cyclo*pentenone ring of brevifolincarboxylic acid in this way is analogous to the biosynthesis of the *cyclo*pentadiol derivative caldariomycin from β-oxoadipic acid (Shaw, Beckwith & Hager, 1959) since this precursor is a known metabolite of catechol.

The oxidative cleavage of ring A of alternariol, either in the suggested manner or at some other point, is therefore a possible source of the altenuic acids, the interconvertibility of which could be explained on the basis of the alternative possible modes of lactonization of the resulting hydroxyl and carboxyl groups. Thus all three altenuic acids titrated as dibasic acids, and it has been shown that altenuic acid I dimethyl ether, $C_{17}H_{18}O_8$, which contains three methoxyl groups and is insoluble in cold aqueous alkali, dissolves on heating with the production of three equivalents of acid.

If enzyme systems capable of cleaving aromatic rings (cf. Bassett & Tanenbaum, 1960) are relatively widespread among plants and micro-organisms, then it would seem reasonable to anticipate that a number of those organisms known to produce phenolic metabolites may also yield significant amounts of their ring-fission products.

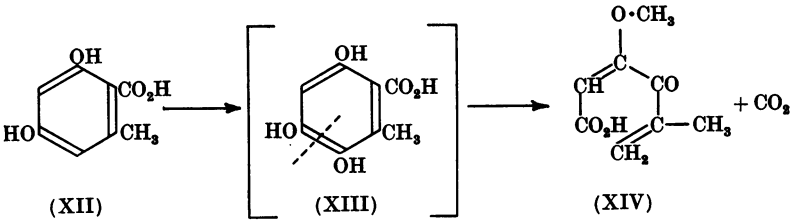

Fig. 5. Conversion of orsellinic acid into penicillic acid.

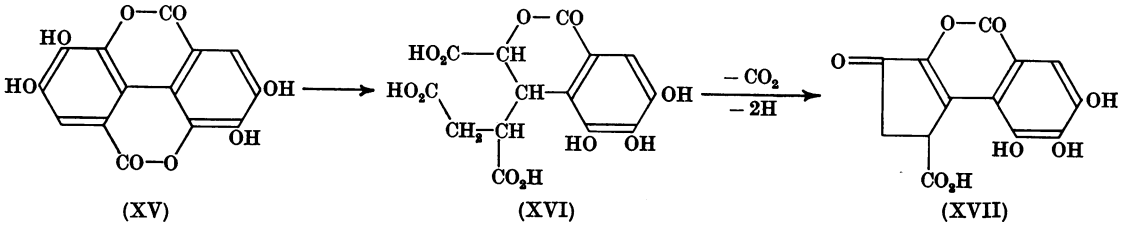

Fig. 6. Possible interrelation of ellagic, chebulic and brevifolincarboxylic acid.

EXPERIMENTAL

All melting points are uncorrected.

Spectroscopic determinations. Infrared measurements with Nujol mulls were carried out in a Perkin–Elmer Double Beam Spectrophotometer with an NaCl prism, and were obtained through the courtesy of the Division of Applied Microbiology, National Research Council, Ottawa. Subsequent measurements on chloroform solutions of alternariol and alternariol monomethyl ether made with a Perkin–Elmer Infracord Spectrophotometer were necessarily less accurate.

Ultraviolet-absorption spectra (Table 3) were measured in ethanol solution. The determinations were carried out on a Cary recording spectrophotometer.

Alternariol monomethyl ether. Alternariol methyl ether was prepared as described by Raistrick *et al.* (1953) and purified by recrystallization from ethanol. The resulting colourless needles, m.p. 267–268°, proved to be free from alternariol on paper chromatographic examination under ultraviolet light, when only a single fluorescent component was discernible. The system used consisted of Whatman no. 1 paper impregnated with 0·5 M-sodium borate buffer, pH 9·2, with a 1:4 mixture of the same buffer and propan-1-ol as solvent (personal communication from Dr C. E. Stickings).

Preparation of labelled alternariol trimethyl ether. Alternariol monomethyl ether (0·500 g.) in dry acetone (25 ml.) was refluxed with freshly distilled methyl iodide (0·20 ml.) containing 50 μc of $^{14}CH_3I$ (purchased from The Radiochemical Centre, Amersham, Bucks.) in the presence of excess of anhydrous K_2CO_3 (5·0 g.) for 6 hr. At this stage dry pure dimethyl sulphate (2·0 ml.) in acetone (25 ml.) was added to the mixture, and refluxing was continued for a further 16 hr.

After this time the solution gave a negative ferric reaction, showing methylation to be complete. The mixture was filtered and concentrated to yield alternariol trimethyl ether, $C_{17}H_{16}O_5$ (0·52 g.), m.p. 161° after recrystallization from acetone. On assaying for ^{14}C at infinite thickness (cf. Chaplen & Thomas, 1960), the product was observed to have an activity of 46·2 μc/g., corresponding to the incorporation of 48% of the added $^{14}CH_3I$ into the alternariol trimethyl ether.

Potassium permanganate oxidation of alternariol trimethyl ether. The labelled alternariol trimethyl ether was diluted approximately 20-fold with inactive product by dissolving the mixture in hot acetone. The crystals obtained on cooling were then assayed for ^{14}C (Found: activity, 2·55 μc/g. = 765 μc/mole).

Diluted trimethyl ether (3·85 g.) was hydrolysed by refluxing with N-NaOH (65 ml.) for 15 min. The resulting solution was cooled and treated with excess of aqueous $KMnO_4$ (26 g. in 260 ml.) and kept at room temperature overnight. After the MnO_2 precipitate which had formed had been filtered off, the filtrate was adjusted to pH 7 with conc. HCl and the excess of $KMnO_4$ reduced with sodium dithionite. The solution was then acidified to pH 2, before extraction with ether (4 × 1 vol.). Evaporation of the ether and crystallization of the residue from water yielded characteristic very pale-yellow rectangular plates of 3:5-dimethoxyphthalic acid (0·50 g.).

The 4:6-dimethoxyphthalonic acid remaining in the extraction mother liquor was not recovered.

3:5-Dimethoxyphthalic anhydride. The recrystallized 3:5-dimethoxyphthalic acid was melted in a sublimation tube and maintained at 190° for 5 min., by which time the evolution of water was complete. The liquid was then allowed to cool, when it immediately crystallized, yielding colourless needles of 3:5-dimethoxyphthalic anhydride. This was purified by sublimation in a high vacuum, yielding a product, m.p. 148–149°, which was assayed for ^{14}C (Found: activity, 156 μc/mole, equivalent to 20·4% of the activity of the starting product, $C_{17}H_{16}O_5$).

Synthesis of alternariol monomethyl ether. Alternariol (0·516 g., 2 m-moles), free from methyl ether, was gently refluxed with freshly distilled dimethyl sulphate (0·10 ml., approx. 1 m-mole) in dry acetone (20 ml.) in the presence of a slight excess of anhydrous K_2CO_3 (0·150 g.). After 6 hr., the mixture was treated with sufficient diluted HCl to decompose the residual K_2CO_3 and precipitate the methylation product, which was then collected by filtration, yield 0·502 g.

Whereas alternariol does not normally crystallize from ethanol, this methylation product readily did so, forming colourless needles, melting at the same temperature as the naturally occurring alternariol monomethyl ether, m.p. 267° (decomp.), and not depressed on admixture with it. Further, both the synthetic and natural methyl ethers were insoluble in cold saturated Na_2CO_3 solution, whereas alternariol, m.p. 345–350° (decomp.), rapidly dissolves forming a greenish-yellow solution.

Alkaline hydrolysis of altenuic acid I dimethyl ether. Altenuic acid I dimethyl ether was prepared from altenuic acid I by reaction with excess of diazomethane (Rosett *et al.* 1957). The ethanol-crystallized product, $C_{17}H_{18}O_8$, m.p. 177–178° (0·602 g.), was gently refluxed with 0·5 N-NaOH in an atmosphere of O_2- and CO_2-free N_2. After 30 min. the solution was cooled and neutralized to phenolphthalein with N-HCl (Found: equiv. by titration, 118. $C_{17}H_{18}O_8$ requires equiv. titrating as a tribasic acid, 117). The pale-yellow neutral solution on addition of aqueous $FeCl_3$ yielded only a slight yellow–brown precipitate and gave a negative reaction to Brady's reagent. On acidification and ether extraction (4 × 1 vol.) a colourless acidic gum was produced in 90% yield, which failed to crystallize.

Potassium permanganate oxidation of altenuic acid I dimethyl ether. Altenuic acid I dimethyl ether (0·40 g.) was dissolved in boiling 0·5 N-NaOH (32 ml.) and after addition of $KMnO_4$ (2 g. in 40 ml. of water) the mixture was refluxed for 30 min. The resulting MnO_2 was separated by filtration, and the filtrate acidified with 2 N-H_2SO_4 (20 ml.) and then extracted with ether, initially by shaking (4 × 1 vol.) and subsequently in a continuous-extraction apparatus.

The continuous extract on evaporation yielded a colourless crystalline solid (0·098 g.) which crystallized from water in needles (0·060 g.), m.p. 172°; above this temperature the melt darkened to a deep-red colour and partially reset at 230–240°. This melting behaviour corresponded exactly to that of 4:6-dimethoxyphthalonic acid, m.p. 173–174° (decomp.), and on admixture the m.p. was not depressed. Further, both substances dissolved in cold conc. H_2SO_4 giving a pale-yellow solution, which on heating changed to an intense wine-red colour.

The initial ether extract obtained by shaking yielded a partly crystalline gum (0·069 g.), which on crystallization from water gave rise to a further quantity of the same oxidation product.

On carrying out the above oxidation at room temperature for 24 hr., approx. 0·50 g. of starting product yielded (from the initial ether extract) an unknown acid (0·15 g.), which, after washing with ether, crystallized from water in colourless needles, m.p. 142–144° (decomp.). From the ether washings, pale-yellow prisms (9 mg.), m.p. 175°, were obtained, which were identified as 3:5-dimethoxyphthalic acid by comparison of colour reactions (Raistrick *et al.* 1953) and also by methylation of a sample (6 mg.) with diazomethane. Sublimation of the resulting ester in a high vacuum produced colourless needles (4·5 mg.), m.p. 93°, undepressed on admixture with authentic dimethyl 3:5-dimethoxyphthalate.

The major product of the room temperature oxidation was obtained on evaporation of the continuous ether extract, which yielded a colourless crystalline residue (0·37 g.) which crystallized from water in needles, m.p. 180–185° (decomp.). On admixture with 4:6-dimethoxyphthalonic acid, m.p. 174°, the m.p. was depressed.

SUMMARY

1. Alternariol monomethyl ether, $C_{15}H_{12}O_5$, a metabolite of *Alternaria tenuis* Auct., has been shown to be 3:4′-dihydroxy-5-methoxy-6′-methyldibenzo-α-pyrone (III).

2. The position of the methoxyl group was established (*a*) by comparison of the infrared carbonyl frequencies of alternariol and alternariol monomethyl ether with the fully methylated product, and (*b*) by sequential methylation of alternariol monomethyl ether with [^{14}C]methyl iodide and dimethyl sulphate, followed by oxidative degradation of the resulting labelled trimethyl ether.

3. A tentative biosynthetic interrelationship between alternariol monomethyl ether and the other phenolic constituents of *A. tenuis* is proposed on the basis of their known properties.

Isotopic assaying equipment was generously loaned by Imperial Chemical Industries Ltd., who also provided a grant for the purchase of [^{14}C]methyl iodide. This investigation was carried out during the tenure of a Wellcome Research Fellowship, the award of which is gratefully acknowledged. The author wishes to thank Professor J. H. Birkinshaw for his interest in this work.

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Estimation of Succinic Acid in Biological Materials

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(*Received* 9 *December* 1960)

The method described in this paper determines between 0·02 and 0·20 μmole of succinate in biological material. It is based on the reduction of 2:6-dichlorophenol-indophenol by succinate in the presence of succinic dehydrogenase (Green, Mii & Kohout, 1955) and the measurement of the extinction change of the dye at 600 mμ in a spectrophotometer.

It has the advantage over similar methods that the enzyme is more easily prepared than that used