mechanism of uptake of iodide appeared to be very similar to that in the thyroid gland.

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Studies in the Biochemistry of Micro-organisms

103. METABOLITES OF *ALTERNARIA TENUIS* AUCT.: CULTURE FILTRATE PRODUCTS*

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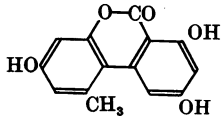
Raistrick, Stickings & Thomas (1953) have described the isolation of two metabolic products—alternariol (I) and one of its monomethyl ethers—from the mycelium of two strains of *Alternaria tenuis* auct. grown on Czapek–Dox solution. These



(I)

two compounds appear to constitute practically the whole of the ether-extractable, but petroleum-insoluble, fraction of the dried mycelium.

It was noted during this work that the metabolism solution of these strains of *A. tenuis* gave a reddish brown colour reaction with ferric chloride. While small amounts of alternariol and its methyl ether could be isolated from the solution, it was soon clear that the material responsible for this ferric reaction was a complex mixture of these two compounds with a number of new substances.

It is the purpose of this paper to describe some of the main constituents of this mixture, and their separation and characterization. The study has been extended to two other strains of *A. tenuis*. Detailed consideration of the structures of the new compounds will be published later. It will be clear from the following account that the list of metabolic products is probably very incomplete, and indeed paper chromatography indicates the presence of

* Part 102: Birkinshaw, Chaplen & Lahoz-Oliver (1957).

several other constituents not yet isolated. None of the new compounds appears to resemble alternaric acid (Grove, 1952) or the products of *Alternaria solani* described by Darpoux, Faivre-Amiot & Roux (1950).

The altenuic acids

The first experiments were carried out with strain no. 94. The products were isolated by adsorption on charcoal, followed by elution with ethanol. In this way three crystalline compounds were isolated: two colourless acidic substances, m.p. 183–184° and 245–246° respectively, which we have called altenuic acid I and altenuic acid II; and a yellow compound, m.p. 189–190°, which is discussed in the next section. Strain no. 94 deteriorated rapidly on subculturing, and attention was switched to strain no. 430, in particular to a sand-culture of this strain which produced high yields of alternariol and its methyl ether (see Raistrick *et al.* 1953).

Although varying amounts of altenuic acids I and II have been obtained from cultures of no. 430, many batches produced substantial quantities of another colourless acidic compound, whose reactions and molecular formula indicated a close relationship with altenuic acids I and II. It has been established that both the latter acids are readily converted into this new compound, simply by dissolving in aqueous sodium hydroxide and re-acidifying. The new product is therefore called altenuic acid III. All three compounds have the molecular formula $C_{15}H_{14}O_8$, containing one methoxyl and one carbon-methyl group.

Altenuic acid I gives a pale purple–brown ferric colour in ethanol, scarcely affected by adding water. The acid forms a colourless dimethyl derivative, m.p. 177–178°, which no longer shows any ferric reaction. Altenuic acid II is a very sparingly soluble substance. In dioxan–ethanol it gives a pale-brown colour with ferric chloride, again scarcely affected by addition of water. This acid also forms a colourless dimethyl compound, giving no ferric colour; the m.p. is 172–173°, depressed on admixture with the dimethyl derivative of altenuic acid I. Altenuic acid III has a variable melting point, of little value diagnostically. Its ethanolic solution gives only a pale wine colour with ethanolic ferric chloride, but on the addition of water this immediately deepens to an intense purple. The acid forms a colourless dimethyl derivative, m.p. 143·5–144·5°, soluble in cold aqueous sodium hydroxide but not in aqueous sodium carbonate, and giving a positive ferric reaction; this dimethyl compound can readily be monoacetylated. More complete methylation yields a colourless neutral trimethyl derivative, m.p. 125–126·5°, which gives no ferric colour.

Mixtures of altenuic acids I and III are difficult to separate by crystallization. Their behaviour on paper chromatography has been studied and they can be clearly separated on a buffered paper. The paper chromatograms also served as a model for separations on a preparative scale by means of a Craig countercurrent-distribution apparatus, and excellent separations were achieved.

Altenusin and dehydroaltenusin

The yellow compound, m.p. 189–190°, isolated from strain no. 94, has the molecular formula $C_{15}H_{12}O_6$. It was not obtained from other strains when solvent-extraction methods were used. However, ether extraction of the concentrated culture filtrate from no. 108 at pH 5 yielded a fraction from which a related colourless product, m.p. 202–203°, could be isolated by crystallization from chloroform. Smaller amounts of the same material were obtained from no. 430. When this substance, whose molecular formula is $C_{15}H_{14}O_6$, is dissolved in aqueous ethanol and treated carefully dropwise with dilute aqueous ferric chloride, an initial violet colour is observed which disappears on shaking, until with increasing amounts of ferric chloride a permanent deep-brown colour develops. This brown-colour reaction is identical with that given by the yellow compound isolated from no. 94, and, in fact, if the addition of ferric chloride to the colourless substance is stopped when the permanent brown colour just begins to appear, a yellow crystalline product can easily be isolated from the reaction solution. This compound is identical with the yellow substance from no. 94. It appears therefore that the colourless compound $C_{15}H_{14}O_6$ gives a violet colour with ferric chloride, but is oxidized at once to the yellow $C_{15}H_{12}O_6$, Fe^{3+} being reduced to Fe^{2+}; this is confirmed by testing with potassium ferricyanide. The yellow product can be reduced by sodium dithionite to the colourless $C_{15}H_{14}O_6$.

Although both compounds are soluble in aqueous sodium bicarbonate, the acetyl derivative of the yellow substance is insoluble even in sodium hydroxide solution, making the presence of carboxyl or other strongly acidic groups improbable. We therefore propose the name altenusin for the colourless $C_{15}H_{14}O_6$, and dehydroaltenusin for the yellow $C_{15}H_{12}O_6$. While both may be formed by the mould, and indeed their ready interconversion may play a part in the metabolism of the organism, we have no certain evidence of the presence of dehydroaltenusin in the culture filtrates. The isolation of this compound from cultures of no. 94 may well be the result of oxidation of altenusin on the charcoal (cf. Raistrick & Stickings, 1951). Unfortunately, strain no. 94 no longer produces either altenusin or dehydroaltenusin, so the question must remain open.

The molecular formula of altenusin includes one methoxyl group and one carbon-methyl group. Methylation yields a neutral, colourless tetramethyl compound, not reacting with ferric chloride. Dehydroaltenusin gives a red precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in aqueous hydrochloric acid), shown to be a mono-derivative. It forms a colourless triacetate with acetic anhydride–sodium acetate.

Altertenuol

The concentrated culture filtrate from strain no. 108, extracted at pH 7, yields a solid giving a green ferric chloride reaction; small quantities of the same material were also obtained from no. 430. A pure substance has been isolated, which shows this ferric colour; it is insoluble in aqueous sodium bicarbonate and yields a neutral triacetate. We propose for it the name altertenuol. It crystallizes from acetic acid in buff-coloured rods, m.p. 284–285°. The molecular formula, $C_{14}H_{10}O_6$, includes one methoxyl group, but no carbon-methyl group. In addition to the triacetate the compound gives a neutral colourless trimethyl compound.

Tenuazonic acid and iso*tenuazonic acid*

In early experiments with strain no. 430, it was noted that some acidic fractions gave a strong orange–red ferric colour, not attributable to any of the compounds described so far. This material is very soluble in organic solvents, even in cold light petroleum. It has been purified in various ways, but has not been obtained as a solid. The material is ketonic and strongly acidic. It is also laevorotatory; of the metabolic products described in this paper, it is the only one to show optical activity. Analyses of the compound and of its derivatives indicate the molecular formula $C_{10}H_{15}O_3N$. A green crystalline chloroform-soluble copper salt is readily obtained, and can be used for isolation and purification; on recrystallization it attains a constant rotation, $[\alpha]_{5461}^{19} - 124°$ in methanol. The acid recovered from the purified copper derivative has $[\alpha]_{5461}^{20} - 132°$ in chloroform.

On long standing the rotation of the product slowly becomes less negative, and eventually crystallization starts. The purified crystalline substance also has the formula $C_{10}H_{15}O_3N$, and has very similar chemical properties to the original material. However, it is dextrorotatory, $[\alpha]_{5461}^{22} + 23°$ in chloroform, and its copper salt has $[\alpha]_{5461}^{22} + 24{\cdot}5°$ in methanol. There is no reason to suppose that this isomer is present in the freshly isolated metabolic material, and we regard it as an artifact.

We propose the name tenuazonic acid for the metabolic (liquid) product, and *iso*tenuazonic acid for the crystalline isomer. Tenuazonic acid is produced also by strain no. 628, which does not appear to give any substantial amounts of the other culture-filtrate products.

Tenuazonic acid titrates sharply as a monobasic acid and contains no methoxyl group. It forms a crystalline mono-derivative with Brady's reagent, and a semicarbazone, m.p. 187–189° with effervescence; the latter gives a deep-blue colour with ferric chloride. Tenuazonic acid can be converted into the *iso*-acid by boiling with aqueous alkali.

*iso*Tenuazonic acid has properties very similar to those of tenuazonic acid, but crystallizes from a small volume of low-boiling light petroleum in colourless needles, m.p. 61–63·5°. The crystals are not very stable, and frequently decompose on keeping, to a liquid which does not appear to be tenuazonic acid. It is conveniently kept as the copper salt. Brady's reagent gives an amorphous precipitate, which is a mixture containing some tenuazonic acid 2:4-dinitrophenylhydrazone. The semicarbazone, m.p. 206–206·5° with effervescence, gives the same intense blue ferric colour as the semicarbazone of tenuazonic acid.

Table 1. *Compounds produced by strains of* Alternaria tenuis

Compound	Formula	Ferric chloride colour	Produced by strain no. 94	430	108	628
Alternariol	$C_{14}H_{10}O_5$	Purple	+	+	+	*
Alternariol methyl ether	$C_{15}H_{12}O_5$	Purple	+	+	+	+
Altenuic acid I	$C_{15}H_{14}O_8$	Pale purple–brown	+	+	.	.
Altenuic acid II	$C_{15}H_{14}O_8$	Pale brown	+	+	.	.
Altenuic acid III	$C_{15}H_{14}O_8$	Deep purple	.	+	.	.
Altenusin	$C_{15}H_{14}O_6$	Violet (evanescent) ↓	†	+	+	.
Dehydroaltenusin	$C_{15}H_{12}O_6$	Brown	†	.	.	.
Altertenuol	$C_{14}H_{10}O_6$	Green	.	+	+	.
Tenuazonic acid	$C_{10}H_{15}O_3N$	Orange–red	.	+	.	+

\+ Isolated and identified.

* A very small amount of material which may have been crude alternariol was isolated from no. 628.

† Dehydroaltenusin was isolated from no. 94, but may have been produced by oxidation of altenusin during the isolation procedure.

Alternariol and its methyl ether

Alternariol and its methyl ether have been extracted from the mycelium of strains nos. 94, 430 and 108. Nos. 94 and 430 yield mixtures in which the methyl ether predominates (see Raistrick *et al.* 1953), but no. 108 gives large yields of a mixture which is mainly alternariol. No. 628 gave only a small amount of solid, from which a little impure alternariol methyl ether was isolated, with perhaps a trace of alternariol.

These substances have also been shown to be present in small amounts in the culture filtrate of no. 430.

General

The products isolated from the four strains of *Alternaria tenuis* are shown in Table 1.

It would be premature at this stage to speculate on the chemical or biochemical relationship between these compounds. It is, however, noteworthy that, with the exception of tenuazonic acid, all these products of *A. tenuis* contain either a carbon-methyl group or a methoxyl group or both, attached in each case to a C_{13} residue. Strain no. 628, from which we have isolated tenuazonic acid, but none of the C_{14} or C_{15} culture-filtrate products, likewise produces in its mycelium only a small amount of alternariol methyl ether and a negligible quantity of alternariol.

EXPERIMENTAL

All melting points are corrected, except where otherwise stated. Methoxyl, Mg, Cu, loss on drying, molecular-weight determinations and titrations were carried out in this Department. Most other determinations are by Weiler and Strauss, Oxford, with a few by Schoeller, Kronach, Germany, and by Mr F. H. Oliver of Parke, Davis and Co. Ltd., Hounslow, Middlesex.

Four strains were used in this work, bearing L.S.H.T.M. catalogue nos. S.M. 94, S.M. 108, S.M. 430 and G.A. 628.

Altenusin and altertenuol were most conveniently obtained from no. 108, from which the other culture-filtrate products have not been isolated. After the deterioration of no. 94, no. 430 was used as a source of the altenuic acids and tenuazonic acid, although the other compounds are also produced by this strain. Tenuazonic acid was also obtained from no. 628.

History of cultures

Details of strains nos. 94 and 430 have been given previously (Raistrick *et al.* 1953). We are indebted to Mr G. Smith of this department for the following mycological notes on the remaining cultures.

No. 108. This strain was isolated from a mouldy orange by Mrs S. Marcus in April 1950. It has remained morphologically very stable. Whereas many isolates of *A. tenuis* deteriorate and rapidly become sterile in laboratory cultures, no. 108 still produces abundant and typical spores.

No. 628. This was isolated by Mr G. Agosti, in December 1956, from roadside soil collected near Limerick, Eire. This also is a strongly sporulating strain.

Both isolates produce long, often branched, chains of extremely polymorphous spores, which have both cross and longitudinal septa. According to Neergaard's (1945) classification they are to be regarded as *Alternaria tenuis* auct. *sensu stricto*.

Cultural conditions

The mould was grown on Czapek–Dox medium as described previously (Raistrick *et al.* 1953), usually in batches of 100 flasks. These were harvested after 4–5 weeks, when the pH was about 7·5, and the glucose about 0·5%. The mycelium was separated from the culture filtrate and dried and extracted as in the earlier work.

Separation of mycelial products from strain no. 108

The mycelium from one batch of 100 flasks of no. 108 was extracted. The crude solid (wt. 55g.) had a methoxyl content of 2·5%, indicating 22% of alternariol methyl ether. However, the latter could not be separated by crystallization from ethanol. Recrystallization from aq. ethanol yielded faintly pink needles, m.p. 340° (decomp.) (uncorr.), undepressed on mixing with alternariol. The identity was confirmed by conversion into the trimethyl ether, m.p. and mixed m.p. 160–161° (uncorr.).

Crude solid from another batch was crystallized from dioxan, again yielding alternariol, m.p. 345° (decomp.) (uncorr.). Evaporation of the filtrate yielded two more crops of alternariol. After removal of the third crop, the solvent was removed completely and the residue recrystallized from ethanol to yield crystals, m.p. 273–274°, unchanged on admixture with the natural alternariol methyl ether (Raistrick *et al.* 1953) (Found: OMe, 11·4, 11·9. Calc. for $C_{15}H_{12}O_5$: OMe, 11·4%).

Isolation of alternariol methyl ether from the mycelium of strain no. 628

No solid separated from the ether extracts of the dried mycelium. On removal of the solvent and washing the residue with cold light petroleum, there remained a brown, sticky solid (yield 3 g./100 flasks). This was triturated with aq. Na_2CO_3 and filtered; the filtrate on acidification yielded a solid (27 mg.), m.p. >310° (decomp.), which was possibly crude alternariol. The Na_2CO_3-insoluble portion, m.p. 255–266°, was extracted in a Soxhlet apparatus with light petroleum (b.p. 40–60°) and the insoluble residue (1·5 g.) was sublimed in high vacuum. The bulk of the material sublimed below 210°; after recrystallization from ethanol and acetone it melted at 268–271°, not depressed on admixture with alternariol methyl ether, and showing the same fluorescence and ferric colour.

Isolation of products from culture filtrates of strain no. 94

A sample of the metabolism solution gave an intense brown ferric reaction, although in the later batches, when the yield of extractable crystalline material fell almost to zero, the only colour obtained on addition of $FeCl_3$ was a dull olive-green. The solution was filtered through cotton wool, then kieselguhr, and acidified to pH about 2. Adsorbent charcoal (5 g./l.) was added, and, after shaking, the solution was filtered and the charcoal washed with a little water; the filtrate no longer gave a ferric reaction. The charcoal was dried *in vacuo* over conc. H_2SO_4, then suspended in ethanol

and loosely packed in a vertical glass tube in the manner of a chromatographic column. Elution with ethanol gave an intense ruby-red eluate which was concentrated at 35–40° under reduced pressure. A crop of yellow crystals first separated, and were removed; further concentration of the filtrate yielded colourless crystals. The maximum combined yield was 5·2 g./100 flasks, but this fell rapidly in succeeding batches. The yellow crystals were readily recrystallized from ethanol, yielding dehydroaltenusin in yellow plates.

The colourless solid was boiled with water and filtered: crude altenuic acid II remained on the filter, and the filtrate on cooling deposited colourless needles of altenuic acid I. Altenuic acid I was recrystallized from water, and altenuic acid II by dissolving in boiling dioxan and adding 3 vol. of water.

Isolation of products from culture filtrates of strain no. 108

When a sample (10 ml.) of the culture filtrate was treated with $FeCl_3$, the first drops produced a greenish colour, with a precipitate, but the addition of more $FeCl_3$ changed the green to brown.

The filtered solution was acidified to pH 6·5 with conc. HCl, then concentrated to 1·5 l. in a climbing-film evaporator (internal temperature 35–40°). Four volumes of ethanol were added, and the sticky precipitate (which gave no ferric reaction) was removed by filtration through glass wool. The ethanol was removed in the climbing-film evaporator, and the pH adjusted to 7·0 with NaOH. This procedure helped to reduce emulsification during the subsequent extractions. The concentrate was then extracted continuously with ether until the extract no longer gave a green ferric reaction (about 24 hr.). The pH was adjusted to 5 with HCl, and the solution further extracted continuously with ether until the extracts no longer gave an evanescent violet colour with $FeCl_3$ (about 40 hr.); the pH was readjusted to 5 when necessary.

Extract at pH 7. Evaporation yielded a brown solid, which was washed with ether; wt. 1–2 g./100-flask batch. It gave an intense green colour with $FeCl_3$ and consisted mainly of altertenuol. The presence of other substances, including probably alternariol and its methyl ether, made the compound difficult to purify. In one experiment, the solid (1·26 g.) was triturated first with ether, which removed a little material giving a purple ferric colour, and then dissolved as far as possible in boiling ethyl acetate. The hot solution was filtered quickly. The insoluble part gave a purple ferric reaction and melted above 300°. The filtrate was concentrated under reduced pressure until it became turbid. After 16 hr. the crystalline altertenuol was collected; a second crop was obtained from the filtrate (total yield, 0·7 g., m.p. 284–287°).

Extract at pH 5. Removal of the ether gave a slightly coloured crystalline solid. It was triturated with cold $CHCl_3$ and filtered. The crude altenusin was dissolved in ether, and passed through a column of acid-washed alumina. Washing was continued with ether until the eluate ceased to give the typical ferric reaction. The ethereal solution was reduced to a small volume, and $CHCl_3$ was added until a slight turbidity resulted. On allowing to stand, colourless crystals separated. These were filtered, and the filtrate concentrated to give further crops. The altenusin, fairly pure at this stage, weighed 2·5–6·5 g./100-flask batch.

Isolation of products from culture filtrates of strain no. 430

The culture filtrate behaved towards $FeCl_3$ in a manner similar to the filtrate from no. 108, but the final colour was a deeper brown. Earlier batches were concentrated as described for no. 108. Later, it was found convenient to acidify to pH 1–2 with HCl before evaporation; the dark amorphous material precipitated was removed by filtration (fraction *A*), and the filtrate was readjusted to pH 6·5 with NaOH. After evaporation, the concentrated culture filtrate was adjusted to pH 7, and extracted continuously with ether for 30–35 hr. A cream-coloured solid separated from the boiling ether; this was filtered (fraction *B*) and the filtrate evaporated (fraction *C*).

The solution was then acidified to pH 1–2 with conc. HCl, and extracted several times with half-volumes of benzene, until the extracts no longer gave an appreciable orange colour with $FeCl_3$. Evaporation of the benzene yielded fraction *D*.

The aqueous concentrate was then further extracted three times with ether and the ether evaporated to give fraction *E*.

Fraction A. This gave a brown ferric colour. It has not yet been investigated.

Fraction B. The solid, m.p. 280° (decomp.), filtered off from the continuous ether extracts gave the ferric colour of tenuazonic acid. When dissolved in a minimum quantity of ethanol and treated with Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in 2N-HCl), a crystalline derivative soon separated. Qualitative tests showed the presence of Mg and the absence of Na and K (Found, after drying at 100° in high vacuum: Mg, 5·4. $(C_{10}H_{14}O_3N)_2Mg$ requires Mg, 5·85%).

Fraction *B* (0·1 g.) dissolved in ethanol (1 ml.) was diluted with water (9 ml.) and acidified to Congo red with 2N-HCl. The solution was extracted three times with light petroleum (b.p. 60–80°). The petroleum was evaporated, leaving a colourless gum (0·05 g.), with the properties of tenuazonic acid as isolated from fraction *D*. The solid is therefore almost certainly a magnesium salt of tenuazonic acid. The yield was 0·6–0·9 g./batch of 100 flasks.

Fraction C. The material removed during the first few hours of continuous extraction was largely solid, and was worked up separately. In the earlier batches, this gave a green ferric reaction, no doubt due to altertenuol (see below). In the case of the later batches, in which the preliminary acidification was carried out, the ferric reaction was purple, indicative of alternariol or its methyl ether. This part of fraction *C* has not been studied extensively, but the isolation of altertenuol, alternariol and alternariol methyl ether, by somewhat different separation procedures, is described below.

After the first few hours the continuous extracts yielded gums on evaporation, but some of these slowly crystallized. The crystals were very soluble in ether but much less so in $CHCl_3$, from which they could be recrystallized. The fractions were therefore allowed to stand with $CHCl_3$ (10 vol.) until crystallization was complete, and then filtered; further crystalline material was obtained on evaporation of the filtrate. In this way 2–3 g. of crude altenusin was obtained per batch of 100 flasks. After purification, it melted at 201·5–203° (decomp.) and did not depress the m.p. of altenusin from strain no. 108.

Fraction D. The benzene extract, after removal of the solvent under reduced pressure, left an orange-coloured gum. It was dissolved in a little ethanol, titrated to pH 7 with aq. N-NaOH, then treated with a chemically equivalent volume of 0·1 N-copper acetate. The mixture was extracted with $CHCl_3$ until the extract no longer gave the orange ferric reaction. Evaporation of the $CHCl_3$ yielded a green gum, which was dissolved in warm methanol (20 vol.) and treated with warm water (50 vol.). On cooling, the green copper salt crystallized; further quantities were obtained from the filtrate. The yield was 5–7 g./batch of 100 flasks. If prepared from freshly isolated tenuazonic acid, it was fairly pure. If necessary it was recrystallized from aq. methanol.

It is essential for this method of purification that altenusin should first be removed, since this substance is oxidized by copper acetate, resulting in pH changes and contamination of the copper tenuazonate. Exhaustive continuous extraction at pH 7 must therefore be carried out, to remove fraction *C* completely.

Tenuazonic acid is conveniently stored as the copper salt, which is quite stable. The free acid is readily regenerated by shaking the copper salt with $CHCl_3$ and 2 N-HCl until all the solid has disappeared, and all the blue-green colour is in the aqueous layer. The $CHCl_3$ layer is separated and the aqueous layer further extracted with $CHCl_3$. The combined $CHCl_3$ extracts are washed with 2 N-HCl and water. Evaporation of the solvent leaves the tenuazonic acid as a nearly colourless gum, not crystallizing even on standing for some days at 0–5°.

Fraction E. The brown gum remaining after evaporation of the solvent was treated with dry ether (about 50 ml.) and allowed to stand. Nearly colourless crystals separated and were filtered. The filtrate was allowed to evaporate slowly in air, and was further treated with small volumes of ether and filtered. In this way crude mixed altenuic acids were obtained; the yields were variable, but usually ranged from 1 to 4 g./batch of 100 flasks.

Usually the first crop was fairly pure altenuic acid III, and could be recrystallized directly from aq. methanol. Some later crops gave little sign of the presence of altenuic acid III, judged by the $FeCl_3$ test. These were separated by extraction with ether in a Soxhlet apparatus: this removed altenuic acid I, leaving the insoluble altenuic acid II in the thimble. The altenuic acid I could also be extracted with boiling water as described above. The altenuic acid I was recrystallized from water or ethanol, and altenuic acid II from aqueous dioxan.

Mixtures of altenuic acids I and III are difficult to separate by crystallization. However, the presence of these acids in mixtures could be detected by paper chromatography, which also separated them from alternariol and its methyl ether, present as impurities. The system used was butanol equilibrated with citrate or phosphate buffer (M/15) on Whatman no. 3 paper previously sprayed with the same buffer and dried (we are grateful to Mr N. Spencer for suggesting this system). Buffers of various pH values between 5 and 6·5 were used, but the most convenient was found to be pH 5·25. Very good separation of altenuic acids I and III was achieved (R_F values 0·50 and 0·36 at pH 5·25, citrate buffer), while the phenolic impurities had $R_F > 0·9$. The spray used was diazotized sulphanilic acid followed by Na_2CO_3; the acids showed up as clearly defined brown spots.

These mixtures could also be analysed by using ethyl acetate–citrate buffer (pH 5·25), and this served as a model for separation on a preparative scale (0·5 g.) by means of a Craig countercurrent-distribution apparatus (capacity of tubes: 25 ml. + 25 ml.). Thus in one example a mixture (0·45 g.) was distributed through twenty tubes. One drop from the ethyl acetate layer of each tube was spotted on filter paper and detected with the above spray: this showed that there had been a clear separation into three zones, identified by paper chromatography as altenuic acid III (tubes 1–3), altenuic acid I (tubes 6–13), and alternariol and its methyl ether (tubes 17–20). Acidification and extraction of the first two fractions yielded practically pure altenuic acid III (0·25 g.) and altenuic acid I (0·10 g.).

Isolation of altertenuol. One of the earlier batches was extracted by hand at pH 7 with ether (3 × 1 vol.). After removal of the solvent there remained a solid, which gave a green ferric reaction. Crystallization first from ethanol, then twice from 90% acetic acid, yielded slightly buff-coloured needles, m.p. 283–285° with darkening and sublimation. The substance did not depress the m.p. of altertenuol isolated from strain no. 108, and the colour reaction with $FeCl_3$ was identical.

Isolation and identification of alternariol and alternariol methyl ether. The first batch of 100 flasks of this strain was filtered and concentrated as described above. Inorganic material was filtered off, and the filtrate acidified to Congo red. The brown sludge formed was separated by centrifuging and dried (19·7 g.); the filtrate yielded in the manner already described a mixture of altenuic acids. The deposit (19·7 g.) was treated with dry ether (insoluble residue 3·05 g.), and the ether solution was extracted with aq. 2% $NaHCO_3$. The ether layer was then evaporated, leaving a neutral, partly crystalline gum (1·45 g.). Most of the gum was removed on washing with ether, leaving a pale-brown solid (1·0 g.), which gave the ferric reaction of alternariol. A sample heated in a high vacuum at 220° partially sublimed, yielding a colourless sublimate, m.p. 255° (decomp.); on raising the sublimation temperature to 250° a second colourless sublimate formed, m.p. 340–350° (decomp.). The first sublimate crystallized from ethanol in needles, m.p. 263–264° (decomp.) (uncorr.), undepressed on admixture with the alternariol methyl ether obtained from the mycelium, and giving the typical colour reactions with $FeCl_3$ and conc. H_2SO_4 (Raistrick *et al.* 1953). The second sublimate, after crystallization from aq. ethanol, was similarly shown to consist of alternariol. The ratio of alternariol to its methyl ether was approximately 1:4.

Isolation of products from culture filtrates of strain no. 628

The ferric reaction of this filtrate was similar to that of no. 430, but the final colour was redder. Preliminary tests showed that a substantial amount of tenuazonic acid was present, but the other compounds appeared to be absent.

The culture filtrate was concentrated as already described, then extracted by hand at pH 7 with ether. Evaporation yielded only a small residue, which gave a brown ferric reaction.

The pH was adjusted to 1–2 with HCl, and the solution again extracted by hand with ether until the extract no longer gave the orange ferric colour. Evaporation of the ether yielded a brown gum (11·2 g.). This was not completely petroleum-soluble, so was subjected to a preliminary purification by dissolving in ether (60 ml.) and adding light

petroleum (b.p. 40–60°, 200 ml.), which precipitated a gum. The solvents were decanted and evaporated. The residue (6·4 g.) was again dissolved in ether and the procedure repeated. After a third treatment, the crude tenuazonic acid (4·6 g.) was converted into the copper salt, which was purified as already described (yield, 4·1 g.); $[\alpha]_{5461}^{20} - 117 \pm 5°$ in methanol (*c*, 0·2); a further quantity (0·4 g.) was obtained as a second crop. The precipitated gums were combined and retreated in the same way, to give a further quantity of copper salt (0·8 g.).

The altenuic acids

Properties of altenuic acid I. Altenuic acid I crystallizes from water or ethanol in colourless needles, m.p. 183–184° with effervescence; if heated further, it resets at about 200° and remelts at 224–230° with further decomposition. The compound retains solvent even when dried to constant weight at 100° in high vacuum (Found, on a sample crystallized from water and dried at 100° in high vacuum: C, 54·2, 54·4; H, 4·55, 4·6; OMe, 9·9; equiv. by titration, 161, 164, 166. $C_{15}H_{14}O_8, \frac{1}{2}H_2O$ requires C, 54·4; H, 4·6; 1 OMe, 9·4%; equiv., titrating as a dibasic acid, 165·5). To obtain solvent-free material, it was necessary to dry for 8 hr. at 120° in a high vacuum (Found, C, 55·7; H, 4·6; OMe, 9·9; *C*-Me, 5·45. $C_{15}H_{14}O_8$ requires C, 55·9; H, 4·4; 1 OMe, 9·6; 1 *C*-Me, 4·65%). It dissolves on heating in methanol, ethanol, dioxan, acetic acid and water, but is not very soluble in the cold. An ethanolic solution gives only a pale purple-brown colour on addition of ethanolic $FeCl_3$, essentially unchanged on adding water (cf. altenuic acid III). Its aqueous solution gives no precipitate with Brady's reagent.

Dimethyl derivative of altenuic acid I. Excess of ethereal diazomethane was added to altenuic acid I, and the suspension was allowed to stand overnight. The insoluble *dimethyl derivative* was filtered, and recrystallized from ethanol, from which it separated in colourless, narrow hexagonal plates; after two recrystallizations, the m.p. was constant at 177–178° (uncorr.) [Found: C, 58·2, 58·6; H, 5·35, 5·05; OMe, 26·0, 26·55; mol.wt. (Rast), 330. $C_{17}H_{18}O_8$ requires C, 58·3; H, 5·2; 3OMe, 26·6%; mol.wt. 350]. The product is only slowly soluble in cold dilute NaOH, but readily dissolves on warming. An ethanolic solution gives no ferric reaction.

Properties of altenuic acid II. Altenuic acid II crystallizes from aq. dioxan in small colourless rectangular plates, m.p. 245–246° (decomp.) (uncorr.). It can also be crystallized from aq. acetic acid (Found: C, 55·7; H, 4·7; OMe, 9·4; *C*-Me, 6·0; equiv. by titration, 160. $C_{15}H_{14}O_8$ requires C, 55·9; H, 4·4; 1OMe, 9·6; 1*C*-Me, 4·65%; equiv., titrating as a dibasic acid, 161). Altenuic acid II is very sparingly soluble in water and the commoner organic solvents. A solution in dioxan–ethanol (1:1) gives only a pale-brown ferric colour, essentially unchanged on adding water.

Dimethyl derivative of altenuic acid II. Altenuic acid II (60 mg.) was treated with ethereal diazomethane for two days at room temp. After evaporating to dryness, the *dimethyl derivative* was crystallized from methanol, forming large colourless prisms (32 mg.), m.p. 172–172·5°, raised on further recrystallization to 172–173° [Found: C, 58·4; H, 5·1; OMe, 26·5; mol.wt. (Rast), 372. $C_{17}H_{18}O_8$ requires C, 58·3; H, 5·2; 3OMe, 26·6%; mol.wt., 350]. A mixture with the dimethyl derivative of altenuic acid I melted at 158–160°. The compound gave no ferric reaction and was only slowly soluble in cold dilute NaOH.

Properties of altenuic acid III. Altenuic acid III crystallizes from aq. methanol or glacial acetic acid in colourless prisms. The m.p. is variable; material from aqueous methanol usually melts at about 185° with effervescence, followed by resetting, complete by about 195°; a further melt occurs between 215° and 235° with effervescence. When crystallized from acetic acid, the substance melts first in the range 198–202° with effervescence, resetting immediately, and remelting at about 225° with effervescence. In addition, the acid crystallizes from ethyl acetate and water; the latter is not to be recommended for purification, since altenuic acid III is partly decomposed on boiling with water (Found: C, 55·8, 55·75; H, 4·5, 4·8; OMe, 9·7; *C*-Me, 4·75; equiv. by titration, 157. $C_{15}H_{14}O_8$ requires C, 55·9; H, 4·4; 1OMe, 9·6; 1*C*-Me, 4·65%; equiv., titrating as a dibasic acid, 161). Altenuic acid III is readily soluble in ethanol and methanol, sparingly soluble in ether, and even less soluble in $CHCl_3$, benzene or light petroleum. An ethanolic solution gives a pale-wine colour with ethanolic $FeCl_3$, but on the addition of water this deepens to an intense purple. An aqueous solution gives no precipitate with Brady's reagent.

Dimethyl derivative of altenuic acid III. Altenuic acid III (50 mg.) was suspended in ether (5 ml.) and ground finely with a glass rod. Excess of ethereal diazomethane was added, and the mixture was allowed to stand with occasional shaking for 0·5 hr. After evaporation, the residual solid was recrystallized from methanol (2 ml.) to give the *dimethyl derivative* (45 mg.) in well-formed rhombic tablets, m.p. 143·5–144·5°, unchanged on further recrystallization (Found: C, 58·5; H, 5·5; OMe, 26·6. $C_{17}H_{18}O_8$ requires C, 58·3; H, 5·2; 3OMe, 26·6%). The compound is insoluble in 2N-Na_2CO_3, but dissolves in cold 2N-NaOH. In ethanolic solution it gives with ethanolic $FeCl_3$ a pale orange–brown colour, scarcely affected by addition of water.

Monoacetyl dimethyl derivative of altenuic acid III. The dimethyl compound was treated with acetic anhydride and one drop of conc. H_2SO_4. The solid dissolved slowly. The reaction mixture was neutralized with $NaHCO_3$ solution, then extracted with ether. The ether extract was evaporated to dryness, leaving a gum which crystallized from 50% ethanol in large colourless prisms, m.p. 107–110°. After further recrystallizations from aq. ethanol and ethyl acetate, the *monoacetyl dimethyl derivative* melted at 108–109° (Found: C, 58·3; H, 5·1; OMe, 23·8, 23·3. $C_{19}H_{20}O_9$ requires C, 58·2; H, 5·1; OMe, 23·8%). The compound is insoluble in 2N-NaOH, and gives no ferric colour.

Trimethyl derivative of altenuic acid III. A mixture of altenuic acid III (0·20 g.), dimethyl sulphate (1 ml.), anhydrous K_2CO_3 (1 g.) and dry acetone (25 ml.) was boiled under reflux for 1 hr. Acetone was removed under reduced pressure and water was added to the residue. Vigorous shaking for 10 min. destroyed the excess of dimethyl sulphate, and after trituration the residual gum hardened and was filtered. The crude *trimethyl derivative* (0·22 g.) was recrystallized from methanol (3 ml.), with charcoal, to give colourless, well-formed prisms (0·19 g.), m.p. 125–126·5°; the m.p. was unchanged on recrystallization [Found: C, 59·3; H, 5·6; OMe, 33·5, 34·1; mol.wt. (Rast), 368. $C_{18}H_{20}O_8$ requires C, 59·3; H, 5·5; 4OMe, 34·1%; mol.wt. 364]. The compound gives no ferric colour and is insoluble in cold aq. NaOH.

Conversion of altenuic acid I into altenuic acid III. Altenuic acid I (19 mg.) was dissolved in a slight excess of 2N-NaOH, giving a pale-yellow solution. On acidifying to

pH 2 with 2N-HCl a white precipitate formed, which was filtered (5 mg.). Ether extraction of the filtrate gave a further quantity (10 mg.). Unlike the starting material, which gives a pale purple–brown ferric colour in aqueous ethanol, this gave an intense purple coloration. The product crystallized from aq. methanol in prisms, m.p. 175–181° with effervescence, resetting on further heating and remelting at 227–232° with further decomposition. The m.p. was essentially unchanged on admixture with altenuic acid III, but in view of the indefinite nature of this m.p. the product was methylated, to provide further evidence of identity.

The product (7 mg.) was treated with ethereal diazomethane at 0° for 0·5 hr. The solvent was removed and the residue crystallized from methanol to give colourless tablets, m.p. 142–145°, which did not depress the m.p. of the dimethyl derivative of altenuic acid III.

Additional confirmation of the conversion was provided by paper chromatography, with the system described above [butanol–citrate buffer (pH 5·25) on buffered paper]. The R_F values were: altenuic acid I, 0·50; the above product, 0·37; altenuic acid III, 0·36.

Conversion of altenuic acid II into altenuic acid III. Altenuic acid II, similarly treated, gave an identical product, m.p. 175–181° with effervescence, resetting on further heating and remelting at 227–234° with further decomposition, and essentially unchanged on admixture with altenuic acid III. The methylation product melted at 142–142·5°, not depressed when mixed with a sample of the dimethyl derivative of altenuic acid III.

Confirmation was again provided by paper chromatography. Altenuic acid II does not give a coloured spot with the diazotized sulphanilic acid–Na_2CO_3 spray, but after treatment with alkali and reacidification it gives a brown spot, R_F 0·35, at pH 5·25.

Altenusin and dehydroaltenusin

Properties of altenusin. Altenusin crystallizes from $CHCl_3$ in colourless prisms. After drying at 100° under reduced pressure, it melts at 202–203° with effervescence to a yellow liquid; if not dried, the crystals melt at about 95°, resolidify, and remelt at the higher temperature. Altenusin can also be crystallized from benzene or water (Found, after drying at 100° in a high vacuum: C, 62·1; H, 5·2; OMe, 10·4; *C*-Me, 5·2. $C_{15}H_{14}O_6$ requires C, 62·1; H, 4·9; 1OMe, 10·7; 1*C*-Me, 5·2%). The substance is readily soluble in ether, ethanol and methanol; it also dissolves in aq. $NaHCO_3$. Its ethanolic solution, treated with ethanolic $FeCl_3$, gives a pale-grey colour, turning to deep brown with excess. If the altenusin is dissolved in aq. ethanol, and aq. $FeCl_3$ is added dropwise, the first drops give a violet colour, quickly fading to leave a colourless solution; with increasing amounts of $FeCl_3$ this reaction is no longer observed, but a permanent intense brown colour appears. During the first stages of this reaction the presence of ferrous ions can be shown by the usual ferricyanide test. Altenusin does not reduce Fehling's solution in the cold, though the solution turns green; on boiling, the usual reduction takes place. However, cold aqueous copper acetate is immediately reduced on addition of an ethanolic solution of altenusin. An aqueous solution gives no precipitate with Brady's reagent after several hours.

Tetramethylaltenusin. (*a*) A mixture of altenusin (0·55 g.), dimethyl sulphate (2 ml.), anhydrous K_2CO_3 (2 g.) and dry acetone (50 ml.) was boiled under reflux for 24 hr. The acetone was removed by distillation under reduced pressure, and the residue was treated with water. After shaking, and rubbing the residual oil with a rod, a solid was obtained. This was filtered and dried (0·59 g.) and repeatedly recrystallized from methanol to give *tetramethylaltenusin* as colourless prisms, m.p. 116–117° (0·40 g.) [Found: C, 65·7; H, 6·2; OMe, 44·2; mol.wt. (Rast), 342. $C_{19}H_{22}O_6$ requires C, 65·9; H, 6·4; 5OMe, 44·8%; mol.wt., 346].

(*b*) Altenusin was dissolved in a little methanol, cooled in an ice bath and treated with excess of ethereal diazomethane. After standing overnight at 0–5°, the ether was evaporated. The gummy residue was dissolved in ether and the solution was filtered, washed with 2N-NaOH, then water, dried and again evaporated to dryness. The pale-brown gum was recrystallized several times from methanol to give colourless prisms, m.p. 116–117°, unchanged on admixture with the derivative above.

Tetramethylaltenusin shows no ferric reaction, is insoluble in cold 2N-NaOH, and gives no precipitate with Brady's reagent.

Properties of dehydroaltenusin. Dehydroaltenusin obtained from strain no. 94 crystallized from ethanol in yellow needles, m.p. 189–190° (decomp.) (Found: C, 62·4, 62·3; H, 4·4, 4·5; OMe, 11·1, 11·1. $C_{15}H_{12}O_6$ requires C, 62·5; H, 4·2; 1OMe, 10·8%). Dehydroaltenusin is only slightly soluble in water. Aq. $NaHCO_3$ readily extracts the compound from ethyl acetate solution but dissolves the solid only slowly; the bicarbonate solution is coloured yellow. In aqueous NaOH the colour is yellow–green, soon changing to yellow. An ethanolic solution gives with $FeCl_3$ an intense brown colour, identical with that produced by the action of excess of $FeCl_3$ on altenusin; the colour is essentially unchanged by the addition of water. An ethanolic solution yields a red precipitate with Brady's reagent (see below).

Oxidation of altenusin to dehydroaltenusin with ferric chloride. Altenusin (100 mg.) was dissolved in a minimum volume of ethanol and an equal volume of water was added. Aq. $FeCl_3$ was added dropwise with shaking until the evanescent violet colour was no longer produced, and a slight permanent brown could be seen. By this time a yellow precipitate had formed. After standing this was filtered, washed with water, dried (87 mg.) and recrystallized from methanol, from which it separated in yellow needles, m.p. 189–190° (decomp.), not depressed on admixture with the material obtained from strain no. 94 (Found: C, 62·2; H, 4·4; OMe, 11·1, 11·2, 11·1. Calc. for $C_{15}H_{12}O_6$: C, 62·5; H, 4·2; 1OMe, 10·8%). The properties of this substance were identical with those of dehydroaltenusin, given above.

2:4-Dinitrophenylhydrazone of dehydroaltenusin. A solution of dehydroaltenusin in ethanol was treated with excess of Brady's reagent. After standing overnight at room temperature the red precipitate was filtered, and recrystallized from ethanol to give the 2:4-dinitrophenylhydrazone as red needles, m.p. 231–232° (decomp.) (Found: C, 53·9, 53·9; H, 3·8, 3·6; N, 11·4. $C_{21}H_{16}O_9N_4$ requires C, 53·8; H, 3·4; N, 12·0%). When the derivative was dissolved in ethanol and treated with a drop of aq. NaOH, a blue colour was observed.

Triacetyldehydroaltenusin. Dehydroaltenusin (0·3 g.) was well mixed with fused sodium acetate (0·3 g.) and acetic anhydride (3·0 ml.) was added. The mixture was boiled for 1 hr. under reflux, then poured into water (250 ml.). After

standing, the solid was filtered and dried, and the crude derivative (0·45 g.) recrystallized several times from benzene, from which it separates in colourless plates, m.p. about 144° with effervescence, resetting and remelting at 178–179° (uncorr.) (Found, after drying at 65° at 16 mm.: C, 63·6; H, 4·7; OMe, 6·6; Ac, 30·2. $C_{21}H_{18}O_9$, $\frac{1}{2}C_6H_6$ requires C, 63·6; H, 4·7; 1OMe, 6·8; 3Ac, 28·5%). When dried at 160° in a high vacuum, *triacetyldehydroaltenusin* melts at 178–179° (uncorr.) [Found: C, 60·8; H, 4·4; OMe, 7·8; mol.wt. (Rast), 410. $C_{21}H_{18}O_9$ requires C, 60·9; H, 4·4; 1OMe, 7·5%; mol.wt., 414]. The triacetate is insoluble in cold 2N-NaOH and does not react with $FeCl_3$ or Brady's reagent in the cold.

Reduction of dehydroaltenusin to altenusin. Dehydroaltenusin was dissolved in the minimum quantity of boiling ethanol and excess of saturated aqueous $Na_2S_2O_4$ was added; the yellow colour was immediately discharged. The mixture was cooled and water was added. A colourless solid separated, and was filtered, dried and recrystallized from $CHCl_3$. Colourless crystals were obtained, which possessed the properties of altenusin; after drying at 100° they melted at 198° (decomp.) (uncorr.), rising to 201° (decomp.) (uncorr.) after admixture with altenusin.

Altertenuol

Properties. Altertenuol separates from acetic acid or ethyl acetate in buff-coloured rods, m.p. 284–285° with darkening and sublimation; even after vacuum sublimation the buff colour persists. For analysis a sample crystallized from ethyl acetate was dried at 100° in high vacuum (Found: C, 60·9, 61·2; H, 3·5, 3·9; OMe, 11·5; *C*-Me, nil. $C_{14}H_{10}O_6$ requires C, 61·3; H, 3·7; 1OMe, 11·3%). Altertenuol is readily soluble in acetone and moderately soluble in alcohols, acetic acid, ethyl acetate and dioxan, but very sparingly soluble in ether, $CHCl_3$, hydrocarbons and water. It dissolves in conc. H_2SO_4 to give a lime-green solution which shows a blue fluorescence in u.v. light; solutions in ethanol are also fluorescent. It is insoluble in aqueous $NaHCO_3$, but gives a yellow solution in aqueous Na_2CO_3 or NaOH, deepening to orange on standing. In ethanolic solution $FeCl_3$ gives a grey–green colour, which becomes a more intense bottle-green on adding water. Altertenuol does not react with Brady's reagent.

Triacetylaltertenuol. Altertenuol (0·14 g.), anhydrous sodium acetate (0·20 g.) and acetic anhydride (1 ml.) were boiled under reflux for 2 hr. After cooling, the reaction mixture was poured into water (100 ml.). The oil which separated solidified on rubbing, and was filtered, washed and dried [0·18 g., m.p. 225–229° (decomp.)]. After crystallization twice from ethanol, with charcoal, and once from ethyl acetate, pure *triacetylaltertenuol* melted at 245–246° [Found: C, 59·7; H, 4·4; OMe, 7·8%; mol.wt. (Rast), 413. $C_{20}H_{16}O_9$ requires C, 60·0; H, 4·0; 1OMe, 7·8%; mol.wt., 400]. The compound gives no colour with $FeCl_3$, and is insoluble in cold aq. 2N-NaOH.

Trimethylaltertenuol. Altertenuol (0·35 g.), dimethyl sulphate (1·5 ml.), anhydrous K_2CO_3 (1·5 g.) and dry acetone (75 ml.) were boiled under reflux for 24 hr. Acetone was removed by distillation and water was added to the residue. After shaking, the insoluble product was filtered, washed and dried (0·38 g.), then crystallized repeatedly from benzene, from which *trimethylaltertenuol* separated in colourless crystals, m.p. 211–212° (Found: C, 64·6; H, 5·1; OMe, 39·0. $C_{17}H_{16}O_6$ requires C, 64·6; H, 5·1; 4OMe, 39·2%). The compound gives no colour with $FeCl_3$ and is insoluble in cold aq. 2N-NaOH.

Tenuazonic acid and iso*tenuazonic acid*

Properties of tenuazonic acid. Tenuazonic acid has not been obtained in crystalline form. When recovered from the pure copper salt it remains as a pale-brown viscous gum, which can be distilled in a high vacuum without essential change in properties or loss of optical activity. The distillate is a faintly straw-coloured gum, b.p. about 117°/0·035 mm.; $[\alpha]_{5461}^{20}$ $-136\pm5°$, $-132\pm2°$ in $CHCl_3$ (*c*, 0·2, 0·5) (Found: C, 60·6; H, 7·9; N, 7·1%; equivalent by titration, 197. $C_{10}H_{15}O_3N$ requires C, 60·9; H, 7·7; N, 7·1%; equivalent (monobasic), 197). The compound is readily soluble in all the usual organic solvents, including light petroleum, but is sparingly soluble in water. Its aqueous or aqueous ethanolic solution is strongly acidic. Addition of ethanolic $FeCl_3$ to an ethanolic solution of tenuazonic acid produces a brilliant orange–red colour, unaltered by the addition of water. An ethanolic solution treated with excess of aqueous Brady's reagent soon clouds and gives a microcrystalline yellow precipitate. When tenuazonic acid is dissolved in an equivalent of aq. NaOH and treated dropwise with aqueous copper acetate, a green precipitate is formed, soluble in excess of copper acetate, and also soluble in $CHCl_3$.

Tenuazonic acid slowly changes on long standing. The optical rotation becomes less negative, and eventually crystallization begins, owing to separation of *iso*tenuazonic acid (see below). It is difficult to separate pure tenuazonic acid from its mixtures with any substantial quantity of the *iso*-acid, and it is better to convert it immediately into the copper salt, which is stable.

Copper salt of tenuazonic acid. Freshly isolated tenuazonic acid was converted into the copper salt as described in the isolation procedure. The salt was dissolved in methanol (20 vol.), and water (50 vol.) was added to the hot solution. On cooling, the *copper salt* separated in green needles, $[\alpha]_{5461}^{20}$ $-117°$ in methanol. Further crystallization raised the optical rotation to a constant value, $[\alpha]_{5461}^{19}$ $-124\pm5°$ in methanol (air-dried; *c*, 0·2). Copper tenuazonate melts indistinctly from about 175° [Found, on air-dried material: loss in weight at 100° in a high vacuum, 7·2. $Cu(C_{10}H_{14}O_3N)_2$, $3H_2O$ requires loss of $2H_2O$, 7·1%. Found, on material dried at 100° in a high vacuum: C, 51·0; H, 6·5; N, 5·75; Cu, 13·2. $Cu(C_{10}H_{14}O_3N)_2,H_2O$ requires C, 50·7; H, 6·4; N, 5·9; Cu, 13·4%]. The compound is soluble in $CHCl_3$, methanol, ethanol and acetone, sparingly soluble in water and insoluble in benzene.

2:4-*Dinitrophenylhydrazone of tenuazonic acid.* (*a*) Tenuazonic acid (0·10 g.), 2:4-dinitrophenylhydrazine (0·09 g.) and methanol (5 ml.) were boiled under reflux. The solid soon dissolved, and after 1 hr. yellow crystals began to separate from the boiling solution. After a further 15 min. the mixture was cooled, allowed to stand, then filtered, washed with methanol and dried. The 2:4-*dinitrophenylhydrazone* (0·11 g.), m.p. 197–200°, was recrystallized from methanol to yield glistening yellow needles, m.p. 199–200°; $[\alpha]_{5461}^{23}$ $-140\pm10°$ in methanol (*c*, 0·1) [Found: C, 50·95, 50·7; H, 5·2, 5·1; N, 18·35%; mol.wt. (Rast), 394. $C_{16}H_{12}O_6N_5$ requires C, 50·9; H, 5·1; N, 18·6%; mol.wt. 377].

(*b*) Tenuazonic acid (0·22 g.) was dissolved in methanol (2 ml.), and Brady's reagent (120 ml.) was added rapidly.

The solution soon clouded, and yellow crystals separated overnight. These were filtered, washed with water and dried (0·37 g.), m.p. 190–194°. Recrystallization from methanol yielded the pure compound (0·25 g.), identical in appearance, m.p. and mixed m.p. with the derivative obtained by method (*a*); $[\alpha]_{5461}^{20}$ − 136 ± 5° in methanol (*c*, 0·2).

The compound dissolves slowly in aq. $NaHCO_3$ to give a dull yellow solution. A dilute ethanolic solution treated with a drop of aq. NaOH yields a deep-red colour. Addition of $FeCl_3$ to an ethanolic solution produces only a slight deepening of colour.

Semicarbazone of tenuazonic acid. Tenuazonic acid (0·49 g.), hydrated sodium acetate (2·5 g.) and water (2·5 ml.) were warmed slightly to give a clear solution, cooled, and treated with semicarbazide hydrochloride (0·5 g.), which dissolved at once. After some hours a gum separated, which hardened on rubbing; the mixture was then left for some days at 0–5° to complete the separation of the derivative. The brownish solid was then filtered, washed with a minimum amount of ice-cold water and dried. The crude derivative (0·59 g.) was recrystallized from boiling water (7 ml.), cooling slowly with seeding to avoid separation as an oil. After 2–3 hr. a pale-brown first crop was filtered (0·18 g.), m.p. 186–187° with effervescence, and the filtrate produced a nearly white crop on standing at 0–5°. This material (0·22 g.), m.p. 188–189° with effervescence, was recrystallized from water (5 ml.) to give the *semicarbazone* as cream-coloured nodular crystals of indistinct form (0·14 g.), m.p. 187–189° with effervescence; $[\alpha]_{5461}^{22}$ − 184 ± 5° in water (*c*, 0·2) (Found, on material dried at 100° in a high vacuum: C, 51·5; H, 7·3; N, 22·2. $C_{11}H_{18}O_3N_4$ requires C, 51·95; H, 7·1; N, 22·0). The semicarbazone gives a deep-blue colour with $FeCl_3$ in aq. or ethanolic solution.

iso*Tenuazonic acid.* (*a*) Crude tenuazonic acid (1·37 g.) which had been standing at room temperature for 2½ years, had darkened in colour, and was largely crystalline. It was dissolved in benzene, and some brown flocculent insoluble matter removed by filtration. The filtrate (15 ml.) was added slowly to light petroleum (b.p. 60–80°, 100 ml.) with shaking. More amorphous solid was precipitated, and was removed by filtration. After evaporation of the solvents, there remained a pale-yellow oil (1·13 g.) which soon began to crystallize, and which was dissolved without residue in light petroleum (b.p. 60–80°, 6 ml.). The solution was set aside to crystallize at 0–5° for 3 days, and then filtered, and the crystals were washed with a minimum volume of ice-cold light petroleum. The crude *iso*tenuazonic acid (0·63 g.), m.p. 47–55°, was dissolved in light petroleum (b.p. 60–80°, 20 ml.) and passed through a short alumina column (4·5 cm. × 1·5 cm.) to remove impurities: this involved considerable loss, the eluate yielding on evaporation only 0·12 g. of nearly colourless crystalline material. Repeated recrystallization from small volumes of light petroleum gave pure iso*tenuazonic acid* (0·059 g.) as colourless needles, m.p. 61–63·5°; a further quantity (0·25 g.), m.p. 61–62·5°, was obtained from the various residues; $[\alpha]_{5461}^{22}$ + 23 ± 2° in $CHCl_3$ (*c*, 0·5). [Found: C, 61·2; H, 7·8; N, 6·85%; equiv. by titration, 203; mol.wt. (Rast), 225. $C_{10}H_{15}O_3N$ requires C, 60·9; H, 7·7; N, 7·1%; mol.wt., 197].

(*b*) A solution of tenuazonic acid (0·48 g.) in 0·1 N-NaOH (49 ml.) was boiled under reflux. At intervals the solution was cooled and the optical rotation observed. The initial α_{5461} was − 2·4° (*l*, 1 dm.); after 1 hr., − 0·6°; after 4 hr., + 0·4°; after 7 hr., + 0·6°; after 10 hr., + 0·6°. After 10·3 hr. at the boiling point the solution was cooled and acidified (3 ml. of 2 N-HCl) and extracted with ether. The ether was evaporated, leaving a pale-brown gum (0·48 g.). Direct crystallization from light petroleum gave a mixture of crystals and brown gum; hence the product was first distilled in high vacuum in small portions on to a cold finger (bath temp., 50–70°). The nearly colourless distillate, which slowly crystallized, was recrystallized from light petroleum to yield colourless needles (0·25 g.), m.p. 57·5–63·5°. Further recrystallization brought the m.p. to a constant 59·5–62°, not depressed on admixture with the pure *iso*-tenuazonic acid from method (*a*); $[\alpha]_{5461}^{23}$ + 23 ± 2° in $CHCl_3$ (*c*, 0·5). For confirmation it was converted into the copper salt; after crystallization this had $[\alpha]_{5461}^{23}$ + 23 ± 1° in methanol (*c*, 1·0).

*iso*Tenuazonic acid is very similar in chemical properties to its isomer. The two compounds are indistinguishable by the $FeCl_3$ and copper acetate reactions, but Brady's reagent gives a yellow precipitate which is mainly amorphous. *iso*Tenuazonic acid is unstable; even when kept in a desiccator it tends to liquefy slowly.

Copper salt of iso*tenuazonic acid.* Prepared in the same way as copper tenuazonate, the *copper salt* separates from aq. methanol in green needles, m.p. from 175°; $[\alpha]_{5461}^{22}$ + 24·5 ± 1° in methanol (on air-dried material; *c*, 1·0) [Found, on air-dried material: OCH_3, nil; loss in weight at 100° in a high vacuum, 6·8, 7·0. $Cu(C_{10}H_{14}O_3N)_2,3H_2O$ requires loss of $2H_2O$, 7·1%. Found, on material dried at 100° in high vacuum: C, 51·0; H, 6·3; N, 6·1; Cu, 13·1. $Cu(C_{10}H_{14}O_3N)_2$, H_2O requires C, 50·7; H, 6·4; N, 5·9; Cu, 13·4%]. The solubility of the salt is similar to that of copper tenuazonate, but it can be crystallized from $CHCl_3$.

Action of 2:4-*dinitrophenylhydrazine on* iso*tenuazonic acid.* (*a*) *iso*Tenuazonic acid (0·05 g.) was dissolved in ethanol (3 ml.) and treated with Brady's reagent (100 ml.). After 24 hr. the solid was filtered (0·07 g.), m.p. 120–130°. When crystallized from methanol (7 ml.) it yielded a small quantity (0·02 g.) of yellow needles, m.p. 187–190°, raised to 188–193° on admixture with the 2:4-dinitrophenylhydrazone of tenuazonic acid.

(*b*) *iso*Tenuazonic acid (0·10 g.), 2:4-dinitrophenylhydrazine (0·09 g.) and methanol (5 ml.) were boiled under reflux for 1·5 hr. On cooling and scratching, crystallization commenced. After 3 hr., the mixture was filtered, yielding yellow needles (0·05 g.), m.p. 198·5–200·5°, $[\alpha]_{5461}^{22}$ − 140 ± 10° in methanol (*c*, 0·1); a mixture with the derivative of tenuazonic acid melted at 197·5–199·5°. The filtrate was allowed to evaporate to dryness in air, and the largely crystalline residue was moistened with methanol and filtered, yielding yellow needles (0·10 g.), m.p. 145–155°, raised on further recrystallization to 155–175°. This material is probably not pure, but has $[\alpha]_{5461}^{22}$ + 38 ± 10° in methanol (*c*, 0·1).

Semicarbazone of iso*tenuazonic acid.* *iso*Tenuazonic acid (72 mg.) and hydrated sodium acetate (0·5 g.) were dissolved in water (0·5 ml.), and semicarbazide hydrochloride (0·1 g.) was added. The solution was allowed to stand at room temperature. Solid separated overnight, and after 4 days it was collected (84 mg.), m.p. 198–199° with effervescence. When recrystallized from water, the *semicarbazone* separated as nodular crystals, m.p. 206–206·5° with effervescence; $[\alpha]_{5461}^{23}$ − 37 ± 5° in water (*c*, 0·2) [Found: C, 51·6, 51·9; H, 7·1, 7·2; N, 22·4. $C_{11}H_{18}O_3N_4$ requires C, 51·95; H, 7·1; N, 22·0%]. The compound gives a deep-blue colour with aq. $FeCl_3$.

SUMMARY

1. The culture filtrates of each of four strains of *Alternaria tenuis* auct., grown on Czapek–Dox medium, have been shown to contain one or more of five metabolic products or groups of products.

2. One group, alternariol and alternariol methyl ether, has previously been shown to occur in the mycelium of this species; the remaining compounds have not previously been described.

3. A second group, the altenuic acids, consists of three closely related isomeric colourless substances, $C_{15}H_{14}O_8$, containing one carbon-methyl and one methoxyl group. Altenuic acid I, m.p. 183–184°, and altenuic acid II, m.p. 245–246°, are readily converted into altenuic acid III, which has a variable melting point and gives a strong purple colour with ferric chloride in aqueous ethanol.

4. Thirdly, a colourless reducing compound, altenusin, $C_{15}H_{14}O_6$, m.p. 202–203°, has been isolated from two of the strains. It is oxidized by ferric chloride to the yellow dehydroaltenusin, $C_{15}H_{12}O_6$, m.p. 189–190°, which has a deep-brown ferric reaction. Both compounds contain one carbon-methyl and one methoxyl group.

5. Fourthly, altertenuol, $C_{14}H_{10}O_6$, buff-coloured needles, m.p. 284–285°, has been isolated in small amounts from two strains. It contains one methoxyl group, but no carbon-methyl group, and gives an intense green ferric colour.

6. Lastly, two strains produce tenuazonic acid, $C_{10}H_{15}O_3N$, b.p. about 117°/0·035 mm., $[\alpha]_{5461}^{20} - 132°$ in chloroform, which has not been obtained as a solid. On long standing, or more rapidly in boiling aqueous alkali, it isomerizes partly to *iso*tenuazonic acid, $[\alpha]_{5461}^{22} + 23°$ in chloroform, which forms colourless needles, m.p. 61–63·5°. Both compounds are ketonic, and give a strong orange–red ferric reaction.

7. Characteristic derivatives of all these new compounds are described.

8. The molecular formulae indicate some relationship between all these metabolic products except tenuazonic acid.

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The Conversion of Casein into Microbial Proteins in the Rumen

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In an earlier paper McDonald (1954*a*) reviewed the problem of determining the extent to which the microbes of the rumen of the sheep were capable of utilizing the nitrogen of the host's diet for the synthesis of their own proteins; the technical difficulties were discussed in some detail and results given for the fate of the ethanol-soluble protein, zein, in the rumen. For reasons given, it was considered that zein would be less effectively utilized by the microbes than a more soluble protein. An effort was therefore made to devise a procedure for the study of casein, as it had already been shown that this protein was readily attacked in the rumen (McDonald, 1952; Chalmers, Cuthbertson & Synge, 1954).

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The characteristic feature of casein as a phosphoprotein has been exploited to yield a direct assessment of the fraction of the dietary casein that escapes digestion in the rumen, and hence an assessment of the synthesis of microbial protein in the rumen.

MATERIALS AND METHODS

Animals. The experimental sheep were prepared with fistulae into the rumen and duodenum. The reasons for collecting abomasal contents immediately after passage into the duodenum have previously been recorded (McDonald, 1954*a*). The duodenal fistula was prepared by McDonald's (1953) modification of the surgical technique of Ward, Young & Huffman (1950) and Young (1951), and the rumen fistula by the procedure of Phillipson & Innes (1939). The fistulae were closed with pliable cannulae made from polyvinyl chloride (Welvic Paste P.B. supplied by Imperial Chemical Industries Ltd.).