810. *The Biosynthesis of Phenols. Part IV.* A New Metabolic Product of* Aspergillus terreus *Thom.*

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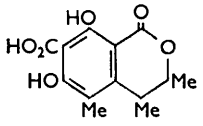
A mutant strain derived from *Aspergillus terreus* Thom produces 3,4-dihydro-6,8-dihydroxy-3,4,5-trimethylisocoumarin-7-carboxylic acid (I). The structure of this compound follows from degradative evidence and from comparison of a derivative with a degradation product of citrinin.

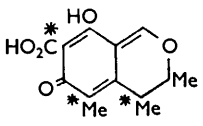
It has been shown that several mutants derived from *Aspergillus terreus* Thom synthesise different phenolic compounds.[1] Consideration of the relations between the molecular structures of these phenols may be expected to contribute to our knowledge of the manner in which such compounds are synthesised by fungi. This has in fact been realised in the elucidation of a section of the pathway of biosynthesis by *A. terreus* of geodoxin.[2]

In the course of these studies it was observed, in agreement with Raistrick and Smith,[3] that the wild type of *A. terreus* regularly produced citrinin (II), but some of the mutants did not. One of these mutants produces, in the culture fluid, a phenol with a molecular formula differing from that of citrinin only in the oxygen content. This optically active compound, $C_{13}H_{14}O_6$, behaves as a monobasic acid, contains three *C*-methyl groups, three active hydrogen atoms, and no methoxyl function. Methylation with diazomethane gives a neutral trimethyl derivative which is converted by careful hydrolysis with alkali into an acid, $C_{15}H_{18}O_6$. This evidence, together with the characteristics of the infrared absorption spectra of these compounds, defined the nature of four of the oxygen atoms: two in a carboxyl function and two in phenolic hydroxyl groups. These phenolic hydroxyl groups are evidently in a *meta*-relationship since the phenol, $C_{12}H_{14}O_4$, obtained through decarboxylation of the original compound, gave a strong red-brown coloration with calcium hypochlorite solution. Furthermore, since the original phenolic acid gave no such coloration it appeared likely that it was in the γ-resorcylic acid series. This was supported by the acid strength. The pK_a, after correction for the methanol in the solvent mixture,[4] was 2·3; this is similar to the value for γ-resorcylic acid (pK_a 2·3 [5]) whereas α- and β-resorcylic acid have pK_a 4·04 [6] and 3·3,[7] respectively.

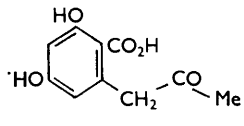
The presence of a band at 1730 cm.$^{-1}$ in addition to one at 1667 cm.$^{-1}$ (carboxyl) in the infrared absorption spectrum of the acid, $C_{15}H_{18}O_6$, which has been mentioned above, indicated that the remaining two oxygen atoms were probably in a six-membered lactone



(I)



(II)



(III)

ring. Furthermore, since the lactone band was shifted to 1645 cm.$^{-1}$ in the product, $C_{12}H_{14}O_4$, obtained when the original metabolite was decarboxylated, it appeared that there was a phenolic hydroxyl group *ortho* to the point of attachment of the carbonyl function of the lactone.

* Part III, *J.*, 1961, 2312.

[1] Hassall and McMorris, *J.*, 1959, 2831.
[2] Curtis, Hassall, Jones, and Williams, *J.*, 1960, 4838; Hassall and Lewis, *J.*, 1961, 2312.
[3] Raistrick and Smith, *Biochem. J.*, 1935, **29**, 606.
[4] Halford, *J. Amer. Chem. Soc.*, 1933, **55**, 2272; Hisao Hukamanai, *J. Pharm. Soc. Japan*, 1940, **60**, 209.
[5] Brown, McDaniel, and Hafliger, in Braude and Nachod, "Determination of Organic Structures by Physical Methods," Academic Press Inc., New York, 1955, p. 629.
[6] Ostwald, *Z. phys. Chem.*, 1889, **3**, 251.
[7] Süss, *Monatsh.*, 1905, **26**, 1331.

The evidence is most readily interpreted in terms of structure (I); this has been confirmed by showing that the methylation product of the metabolite is identical with the compound obtained from citrinin [8] by catalytic hydrogenation, methylation, and oxidation with chromic acid, in turn.

By using radioactive tracers, Birch and his co-workers have shown that the carbon atoms marked with an asterisk in formula (II) differ from others in citrinin in being derived from formic acid.[9] This leads us to suggest that citrinin may be derived from compound (III), a known metabolite of *Penicillium brevicompactum*,[10] through a series of steps involving methylation, oxidation, and reduction. The identification of the phenolic acid (I) as a metabolite of a mutant of *A. terreus* that no longer produces citrinin suggests that the reduction of the carboxyl function of the β-resorcylic acid derivative (III) occurs at a late stage in the biosynthesis of citrinin. Further studies using mutants for the elucidation of the biosynthesis of citrinin are in progress.

Experimental

M. p.s were determined on a Kofler block. Ultraviolet spectra were measured in ethanol on a Unicam spectrophotometer. Infrared spectra were measured by Dr. H. E. Hallam and Mr. D. Jones for potassium bromide discs with a Grubb–Parsons double-beam spectrometer.

Isolation of 3,4-Dihydro-6,8-dihydroxy-3,4,5-trimethylisocoumarin-7-carboxylic Acid (I).—The strain of *Aspergillus terreus* Thom which was used in these experiments was obtained, by selection, from a mixture of variants produced when *A. terreus* Thom (Ac 100, L.T.C.C.) was grown at 30° on Czapek–Dox agar containing 2% of glucose, with subculturing at three-monthly intervals for 5 years. Ten-day slopes of the selected strain were shaken with sterilised water, and the spore suspension was used to inoculate sterile Czapek–Dox medium (Raistrick and Smith modification), approx. 200 c.c. in flat-sided bottles (approx. 1 l. capacity). These liquid cultures were allowed to grow for 40 days at 30° ± 1°.

The dark brown culture fluid (47 l. batch) was separated from mycelium and treated with activated charcoal according to the procedure already described.[2] When the charcoal was extracted with methanol no citrinin was found in the mixture of products whereas substantial yields of citrinin were found when this procedure was applied to similar cultures of the original wild strain of *A. terreus* Thom (Ac 100, L.T.C.C.). The amber-coloured solution remaining after the treatment with charcoal was evaporated to dryness at a temperature not exceeding 35°. The dry residue was extracted continuously with methanol. The crude oil which was obtained by evaporation of methanol was extracted to completion with successive amounts of light petroleum (b. p. 60—80°). The semisolid residue (123 g.) gave crystals of a potassium salt (2·2 g.), m. p. 206—210° (from ethanol–water), which, after acidification and recrystallisation from light petroleum (b. p. 60—80°), gave the *acid* as needles (1·52 g.), m. p. 137—138°, $[\alpha]_D^{15}$ +104° (*c* 1·1 in chloroform) [Found: C, 58·8; H, 5·2; O, 35·8; *C*-Me, 16·4, OMe, 0%; equiv. (potentiometric), 268; *M* (Rast), 266. $C_{13}H_{14}O_6$ requires C, 58·6; H, 5·3; O, 36·1; 3*C*-Me, 16·9%; *M* and equiv. (monobasic acid), 266]. The infrared spectrum showed bands at 1695 (lactone carbonyl), 1650 (*o*-hydroxyphenyl-COOH), and 1613 cm.$^{-1}$ (aromatic C=C). $\lambda_{max.}$ 233, 323 mμ (log ε 4·4, 3·83). The compound dissolved in sodium hydrogen carbonate solution with effervescence and gave a positive test for carboxylic acids with Nomura's reagent.[11] It failed to couple with diazotised sulphanilic acid, and gave a pale transient yellow colour with calcium hypochlorite solution.

Decarboxylation of the Acid (I).—The acid (I) (100 mg.) was boiled under reflux in methanol (3 c.c.) and 2N-sodium hydroxide (7 c.c.) for 1·5 hr. Removal of methanol and acidification gave a solid (93 mg.) which was purified by sublimation at 140° in a high vacuum to give the product as prisms, m. p. 217° [Found: C, 64·5; H, 6·3; O, 29·2%; *M* (Rast), 208. $C_{12}H_{14}O_4$ requires C, 64·8; H, 6·35; O, 28·8%; *M*, 222], $\lambda_{max.}$ 270, 312 mμ (log ε 3·75, 3·49). The infrared spectrum showed bands at 1645 (hydrogen-bonded carbonyl of lactone) and 1618 cm.$^{-1}$ (aromatic C=C). The product was insoluble in sodium hydrogen carbonate solution. It gave a dark

[8] Cartwright, Brown, Robertson, and Whalley, *J.*, 1949, 867.
[9] Birch, Fitton, Pride, Ryan, Smith, and Whalley, *J.*, 1958, 4576.
[10] Clutterbuck, Oxford, Raistrick, and Smith, *Biochem. J.*, 1932, **26**, 1441.
[11] Nomura, *Bull. Chem. Soc. Japan*, 1959, **32**, 536.

colour with ethanolic ferric chloride and a strong red-brown colour with a solution of bleaching powder. The same product was obtained when the metabolite (I) was heated with hydrogen iodide solution on a boiling-water bath for 2 hr.

Methylation of this product (50 mg.), in methanol (10 c.c.), with excess of diazomethane during 24 hr. yielded prisms (40 mg.), m. p. 117—118° (Found: C, 65·5; H, 7·0; O, 27·3; OMe, 12·9. $C_{13}H_{16}O_4$ requires C, 66·0; H, 6·8; O, 27·1; OMe, 13·0%), $\lambda_{max.}$ 267, 312 mμ (log ε 3·91, 3·65). The infrared spectrum showed a band at 1647 $cm.^{-1}$ which is attributed to the hydrogen-bonded carbonyl group of the lactone function. The compound gave a strong blue colour with ethanolic ferric chloride. It is evident that this compound is **3,4-*dihydro*-8-*hydroxy*-6-*methoxy*-3,4,5-*trimethylisocoumarin***. Following common experience, the 8-hydroxyl group, which is involved in hydrogen bonding, is relatively resistant to methylation.

Methylation of the Metabolite (I).—The phenol (I) (20 mg.) in methanol (20 c.c.) was treated with an excess of ethereal diazomethane during 12 hr. Working up in the usual way gave a neutral product (14 mg.) as needles, m. p. 103—104° (Found: C, 61·9; H, 6·4; O, 31·5; OMe, 29·2. Calc. for $C_{16}H_{20}O_6$: C, 62·3; H, 6·5; O, 31·1; 3OMe, 30·2%), $\lambda_{max.}$ 252, 297 mμ (log ε 4·9, 4·42). The infrared spectrum includes bands at 1736 (aryl ester) and 1712 $cm.^{-1}$ (lactone carbonyl).

A sample of methyl 3,4-dihydro-6,8-dimethoxy-3,4,5-trimethylisocoumarin-7-carboxylate prepared from citrinin [8] had an identical infrared spectrum. The m. p., 103—104°, was not depressed by admixture with the methylation product of (I).

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