Biosynthesis of Tropolones in *Penicillium stipitatum*. VII.[1,2] The Formation of Polyketide Lactones and Other Nontropolone Compounds as a Result of Ethionine Inhibition

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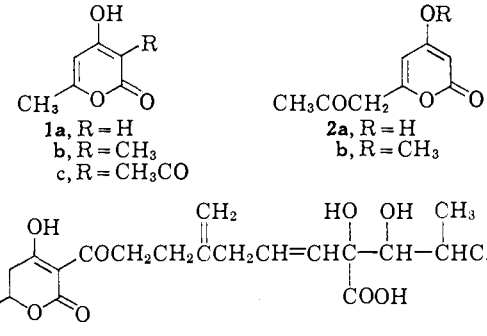
Received September 21, 1966

Abstract: When *Penicillium stipitatum* was grown in the presence of ethionine, normal tropolone biosynthesis was inhibited and other compounds were encountered. The major nontropolone metabolites were triacetic lactone and a compound, $C_8H_8O_4$, identified as tetraacetic lactone (6-(2-oxopropyl)-4-hydroxy-2-pyrone). A third pyrone, methyl triacetic lactone, was present in these cultures in much smaller amounts. Orsellinic acid and its decarboxylation product, orcinol, have also been isolated from inhibited cultures. The tetraacetic lactone was found to be much less stable than triacetic lactone. On treatment with alkali, it was rapidly converted to a mixture of orsellinic acid and orcinol. This conversion took place, albeit slowly, when the tetraacetic lactone was allowed to stand for some time under conditions of temperature and pH similar to those encountered in broth of the growing mold. The major product formed on reaction of tetraacetic lactone with ethanol was the ethyl ester of orsellinic acid.

Mold tropolones are known to be formed through the condensation of one acetate unit with three malonate units; the aromatic ring is completed by addition of a C_1 unit derived from formate or methionine.[3] In the hope of identifying possible intermediates in tropolone biosynthesis, Bentley, *et al.*, examined the effect of ethionine on intact cultures of *Penicillium stipitatum*.[1a] The formation by this organism of the major tropolone components, stipitatonic and stipitatic acids, was inhibited by the presence of ethionine. At the same time, a number of other compounds, not present in normal cultures, were encountered. One of these was identified as orsellinic acid, a compound long considered a possible intermediate likely to accept a C_1 unit. This paper now reports the characterization of further compounds accumulating under conditions of ethionine inhibition. They are triacetic lactone, **1a**, the δ lactone of 3,5,7-trioxooctanoic acid ("tetraacetic lactone"), **2a**, and orcinol. The presence of "methyltriacetic lactone," **1b**, previously reported by Tanenbaum and his colleagues,[4] has been confirmed.

Results

Isolation and Structure of Lactones. Medium from ethionine-inhibited cultures of *P. stipitatum* NRRL 2104, after 11–14 days growth, was concentrated and extracted at pH 5 successively with chloroform,[5] ether, and ethyl acetate. After adjusting the pH to 1, further extractions with ether and ethyl acetate were carried out. Compounds not present in uninhibited cultures were mainly concentrated in the extracts made at pH 5. After preliminary separation of most of the sparingly acetone soluble tropolone acids, the acidic residue was chromatographed on Celite columns moistened with H_2SO_4. Elution was carried out with chloroform and chloroform–2% *n*-butyl alcohol (CB-2).



1a, R = H
b, R = CH_3
c, R = CH_3CO

2a, R = H
b, R = CH_3

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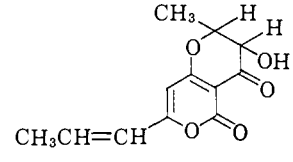

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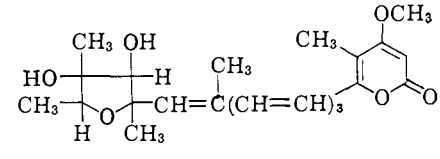

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From the pH 5 ether extract the following materials were characterized.

1. Early chloroform eluates: these fractions contained a material with R_f 0.9 on paper chromatography.[1a] The material showed a purple fluorescence under ultraviolet light and gave a bright yellow color

(1) (a) For part VI see R. Bentley, J. A. Ghaphery, and J. G. Keil, *Arch. Biochem. Biophys.*, **111**, 80 (1965); (b) for part V see R. Bentley and C. P. Thiessen, *J. Biol. Chem.*, 238, 3811 (1963).

(2) A preliminary account of some of this work has been given: R. Bentley, J. A. Ghaphery, and P. M. Zwitkowits, Abstracts, IUPAC 4th International Symposium on The Chemistry of Natural Products, Stockholm, Sweden, 1966, p 151.

(3) For early references and a general discussion of tracer studies in tropolone biosynthesis see R. Bentley, *J. Biol. Chem.*, 238, 1895 (1963). A subsequent biosynthetic study is described by I. G. Andrew and W. Segal, *J. Chem. Soc.*, 607 (1964). An enzyme system converting acetyl CoA, malonyl CoA, and methionine to stipitatic acid has been described by S. W. Tanenbaum and E. W. Bassett, *Biochim. Biophys. Acta*, **59**, 524 (1962).

(4) P. E. Brenneisen, T. E. Acker, and S. W. Tanenbaum, *J. Am. Chem. Soc.*, 86, 1264 (1964); T. E. Acker, P. E. Brenneisen, and S. W. Tanenbaum, *ibid.*, 88, 834 (1966).

(5) The materials contained in the chloroform and ether extracts were generally similar; in a number of the experiments, the chloroform extraction was not carried out.

when sprayed with diazotized benzidine. After several recrystallizations and sublimation, the compound was obtained as a white solid, mp 204°; the nmr and ultraviolet spectra were identical with those of 3,6-dimethyl-4-hydroxy-2-pyrone, **1b**, isolated by Tanenbaum and his colleagues[4] from *P. stipitatum* NRRL 1006. The melting point for this material reported by Tanenbaum, *et al.*, was 212–214°; Jachymczyk and Chmielewska[6] quote mp 208–209° for a synthetic preparation.

2. Middle chloroform eluates: these contained principally a material with R_f 0.7–0.8 showing a purple fluorescence under ultraviolet light and a yellow-orange color with diazotized benzidine. After crystallization, the compound had mp 190°. The mass spectrum of this material possessed a parent molecular ion at *m/e* 126.0304 ($C_6H_6O_3$ required 126.0317); the spectrum was consistent with that expected for triacetic lactone, **1a**. The compound was, in fact, identical in all respects (infrared, ultraviolet, and nmr spectra, mixture melting point) with an authentic sample of triacetic lactone.

3. Final chloroform fractions: these fractions, as previously reported,[1a] contained orsellinic acid as the major component. Small amounts of the δ lactone of 3,5,7-trioxooctanoic acid and orcinol (see under 4 and 5) were also present.

4. Initial CB-2 fractions: the major component of these fractions had R_f 0.4–0.5, showed a purple fluorescence under ultraviolet light, and gave a red-brown color with diazotized benzidine. After several recrystallizations, the compound was obtained as straw-colored needles, mp 118–119°. The mass spectrum (see Figure 1A) possessed a parent molecular ion at *m/e* 168.0424 ($C_8H_8O_4$ required 168.0422). In this spectrum losses of the units CH_3, CO, and CH_2CO were clearly discernible. The spectrum had five peaks in common with that of triacetic lactone (69, 85, 98, 111, and 126). This fact, and the observation that both compounds gave abnormally high carbon–methyl values in Kuhn–Roth analysis, suggested that the new compound was "tetraacetic lactone," **2a** (δ-lactone of 3,5,7-trioxooctanoic acid, 6-(2-oxopropyl)-4-hydroxy-2-pyrone).[7] The compound had an absorption maximum at 284 mμ analogous to that of triacetic lactone (λ_{max}^{EtOH} 283 mμ). However, on addition of base, the absorption spectrum showed two peaks at 360 and 256 mμ rather than the hypsochromic displacement observed with triacetic lactone.[8] The identification was confirmed by the fact that the nmr spectrum agreed with that anticipated for structure **1a**. Furthermore, the material yielded a 2,4-dinitrophenylhydrazone and also reacted with diazomethane to form a crystalline methyl ether, **2b**.

5. Late CB-2 fractions: when all of the tetraacetic lactone had been removed from the column, the later fractions contained a material running close to the solvent front on paper chromatography (R_f 0.95–1.0). This material showed a purple fluorescence under ultraviolet light and a red-brown color with diazotized benzidine. After purification by thin layer chromatography and sublimation, this substance was obtained in a microcrystalline form (mp 109°) and was positively identified as orcinol (mixture melting point, thin layer and paper chromatography in several systems, gas chromatography–mass spectrometry).[9]

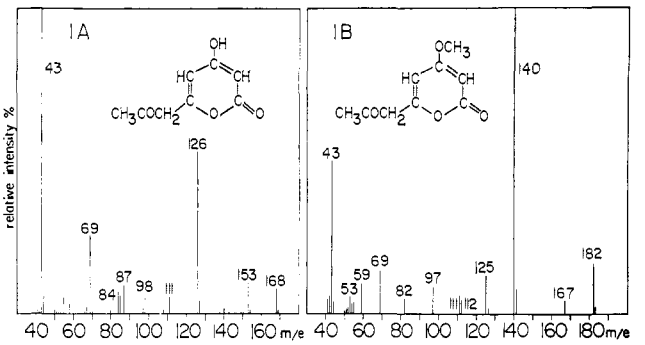


Figure 1.

Chromatography of the acidic material of the pH 5 ethyl acetate extract similarly gave triacetic and tetraacetic lactones and orsellinic acid. The total yields of materials obtained from 1 l. of culture medium were approximately as follows: triacetic lactone, 385 mg; tetraacetic lactone, 215 mg; methyl triacetic lactone, 15 mg; orsellinic acid, 15 mg; orcinol, 17 mg.

Since Tanenbaum and his colleagues[4] had isolated methyl triacetic lactone from *P. stipitatum* NRRL 1006 the effect of ethionine on this organism was also examined. This strain was grown at 37° as in the work of Tanenbaum, with the medium used for NRRL 2104. Paper chromatography of the pH 5 ether and ethyl acetate extracts showed that this strain also produced triacetic and tetraacetic lactone as the major nontropolone metabolites, along with smaller amounts of orsellinic acid and orcinol. After column chromatography, methyl triacetic lactone was also detected. The concentration of this latter substance in the broth of both strains NRRL 1006 and 2104 was such that it could not be detected simply by paper chromatography of the initial extracts.

Cyclization of Tetraacetic Lactone. Tetraacetic lactone was readily converted to orsellinic acid and orcinol under various conditions of pH. As shown in Table I, the proportions of these aromatic compounds depended upon the reaction conditions. For example, after a 4-hr reflux period with alcoholic KOH there was no unchanged lactone present, and orcinol was the only product. Orsellinic acid itself was unstable under these conditions and was converted quantitatively to orcinol. The conversion to aromatic compounds also occurred, albeit slowly, under "physiological" conditions (Table I, expt 5 and 6).

When tetraacetic lactone and ethanol were heated at 110° in a sealed tube for 4–16 hr, the major component was found to be orsellinic ester. No evidence for the presence of the ethyl ester of 3,5,7-trioxooctanoic acid was obtained. Orsellinic acid itself was converted mainly to orcinol under these conditions, and no orsellinic ester was formed.

(6) W. Jachymczyk and I. Chmielewska, *Bull. Acad. Polon. Sci., Ser. Sci. Chim.*, 8, 155 (1960).

(7) It is of interest to recall that this structure, in the diketone form, was originally proposed for dehydroacetic acid by J. N. Collie, *J. Chem. Soc.*, **59**, 617 (1891).

(8) J. A. Berson, *J. Am. Chem. Soc.*, 74, 5172 (1952); J. D. Bu'Lock and H. G. Smith, *J. Chem. Soc.*, 502, 1960; A. I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products," Pergamon Press, New York, N. Y., 1964, p 140.

(9) A more convenient method for the isolation of orcinol from the inhibited cultures is described in the following paper.

Table I. Cyclization of Tetraacetic Lactone to Aromatic Products

Expt no.	Reagents	Temp, °C	Time	Major components	Minor components
1a	Lactone (28 mg), KOH (180 mg), EtOH (2 ml)	Reflux	30 min	Orcinol	Lactone, orsellinic acid in *ca.* 1:1 ratio
1b		Reflux	4 hr	Orcinol	None
2	Lactone (4 mg), 0.5 *M* KOH (1 ml)	30	16 hr	Lactone, orcinol, orsellinic acid	
3	Lactone (4 mg), 0.1 *M* KOH (1 ml)	60	1 hr	Orcinol	Orsellinic acid
4	Lactone (4 mg), 63% H_2SO_4 (0.1 ml)	100	1 min	Orcinol	Traces of lactone and orsellinic acid
5a	Lactone (10 mg), culture medium at pH 5 (100 ml)	28	7 days	Lactone	None
5b		28	14 days	Lactone, orcinol in *ca.* 1:1 ratio	Orsellinic acid
5c		28	21 days	Orcinol	Orsellinic acid, lactone
6a	Lactone (2 mg), acetate buffer at pH 5 (1 ml)	21	12 days	Lactone	Traces of orsellinic acid and orcinol
6b		21	21 days	Lactone	Orsellinic acid, orcinol

Discussion

Polyketo acids ("polyketides") have long been considered as possible intermediates in the biosynthesis of a variety of mold secondary metabolites. There has, however, been no rigorous proof of the existence of these acids, or their biologically active derivatives, during biosynthetic processes. It has been considered that the enzymes leading to secondary metabolites are organized as a multienzyme complex, similar to that of the yeast fatty acid synthesizing system.[10] In agreement with this view, intermediates between acetyl and malonyl CoA and the terminal metabolites have seldom been detected. The isolation of the methyl triacetic lactone, **1b**, initially by Tanenbaum and his colleagues,[4] and of the tri- and tetraacetic lactones, **1a** and **2a**, reported here, represent some of the closest approaches to simple polyketides that have yet been made for mold metabolites. These lactones are unique as nonaromatic dehydration products of the C_6 and C_8 polyketides, 3,5-dioxohexanoic acid[11] and 3,5,7-trioxooctanoic acid (**6**), respectively. Using carrier techniques, Light, Harris, and Harris[12] have also obtained evidence for the presence of small amounts of triacetic lactone is normal cultures of *Penicillium patulum*. It has also been reported that triacetic acid and lactone are formed when an organism, thought to be a *Pseudomonas*, was allowed to metabolize dehydroacetic acid, **1c**.[13] The isolation of these various lactones considerably strengthens the hypothesis that acids such as 3,5-dioxohexanoic acid and 3,5,7-trioxooctanoic acid (or their CoA derivatives) are actually involved in the secondary metabolism of these molds.

Two other fungal metabolites are related, at least structurally, to the tri- and tetraacetic lactones. Alternaric acid, **3**, known to be an acetate-derived product with added C_1 units,[14] may be regarded as a dihydrotriacetic lactone, acylated at position 3 with a branched side chain. Radicinin,[15] **4**, may be regarded as being derived from tetraacetic lactone; the CH_3COCH_2 side chain has to be modified to $CH_3CH{=}CH$ and, in addition, an acylation with an acetoacetyl unit at position 3 must be postulated. Citreoviridin,[16] **5**, is apparently an example of pyrone formation at the carboxyl end of a long polyacetate chain.

Our observation that tetraacetic lactone is readily converted to orsellinic acid, **7**, and its decarboxylation product, orcinol, **8**, not only provides further evidence for the correctness of the structure assigned to the lactone, but also has other implications. The instability of tetraacetic lactone may be contrasted with the marked stability of triacetic lactone. Witter and Stotz[17] pointed out that there is no evidence to indicate that triacetic lactone and 3,5-dioxohexanoic acid are readily interconvertible or are in an equilibrium state in solution. Under conditions from which triacetic lactone can be recovered quantitatively (1 *M* alkali at 30° for 16 hr, or 0.2 *M* alkali at 60° for 1 hr) tetraacetic lactone is either partially or completely cyclized. Another difference between the two compounds is observed on reaction with ethanol. When triacetic lactone was heated with ethanol in a sealed tube for 48 hr at 110°, Witter and Stotz[17] found that 43% of the lactone remained unchanged and that 47% was converted to the ethyl ester of 3,5-dioxohexanoic acid. When tetraacetic lactone was heated with ethanol in the same way, it was partly converted to orsellinic ester and orcinol after only 4 hr. Since orsellinic acid itself does not form the ester under these conditions, ester formation must occur prior to aromatization. The observed result, therefore, may be attributed to a difference in the stability of 3,5,7-trioxooctanoic ester relative to 3,5-dioxohexanoic ester.

While our work was in progress, Harris and Carney[18] described the carboxylation of diacetylacetone (2,4,6-

(10) F. Lynen and M. Tada, *Angew. Chem.*, 73, 513 (1961).

(11) A radioactive peak with the chromatographic properties of 3,5-dioxohexanoic acid was observed by J. D. Brodie, G. Wasson, and J. W. Porter, *J. Biol. Chem.*, 239, 1346 (1964), when a pigeon liver fatty acid synthesizing system was incubated with radioactive malonyl CoA and acetyl CoA in the absence of NADPH. D. J. H. Brock and K. Bloch, *Biochem. Biophys. Res. Commun.*, 23, 775 (1966), observed that long-chain fatty acid synthesis by the *E. coli* synthetase was suppressed by addition of certain thiols (glutathione, mercaptoethanol, dithiothreitol). Under these conditions triacetic lactone was isolated.

(12) R. J. Light, T. M. Harris, and C. M. Harris, *Federation Proc., Abstr.*, 25, 768 (1966); T. M. Harris, C. M. Harris, and R. J. Light, *Biochim. Biophys. Acta*, 120, 420 (1966).

(13) T. Kotani, S. Nonomura, and C. Tatsumi, *Nippon Nogeikagaku Kaishi*, 38, 585 (1964).

(14) W. B. Turner, *J. Chem. Soc.*, 522 (1961).

(15) J. F. Grove, *ibid.*, 3234 (1964).

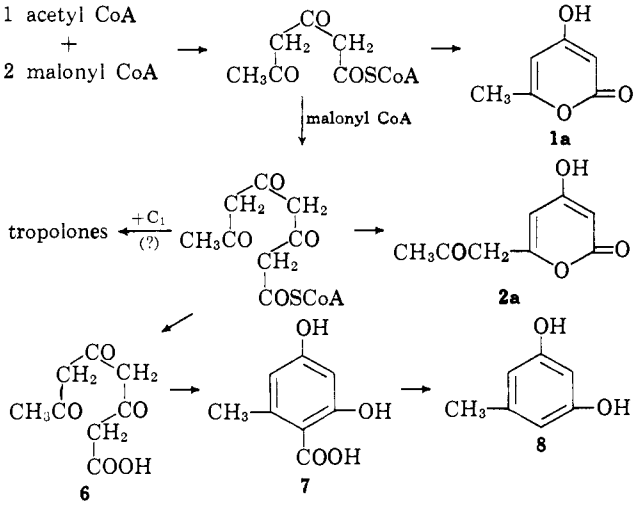
(16) N. Sakabe, T. Goto, and Y. Hirata, *Tetrahedron Letters*, 1825 (1964).

(17) R. F. Witter and E. Stotz, *J. Biol. Chem.*, 176, 485 (1948).

(18) T. M. Harris and R. L. Carney, *J. Am. Chem. Soc.*, 88, 2053 (1966).

trioxoheptane) to yield trace amounts of 3,5,7-trioxooctanoic acid, **6**. This acid, which is the parent of tetraacetic lactone, was obtained in amounts too small for purification and complete characterization. The most important evidence for its formation was the fact that on treatment with aqueous buffer at pH 5 (16 hr, 25°) the crude acid was converted to orsellinic acid, **7**, apparently in good yield.[19] Under these conditions, tetraacetic lactone is stable, requiring periods of about 12 days before the formation of orsellinic acid can be detected (see Table I).

Our use of ethionine as an inhibitor stemmed from the hope that interference with the C_1 addition process of tropolone biosynthesis would lead to the accumulation of possible intermediates. Although the isolation of orsellinic acid from the inhibited cultures initially tended to support the long postulated precursor role for this acid, the facile aromatization of tetraacetic acid and its lactone most probably indicates that the formation of orsellinic acid is not directly related to tropolone biosynthesis. Since the free tetraacetic acid is more readily cyclized than the lactone, the latter is not likely to be a major source of orsellinic acid in the inhibited cultures. Possibly the formation of tetraacetic lactone and orsellinic acid represent branch pathways from the CoA derivative (or other activated form) of tetraacetic acid as shown below. Orsellinic acid is presumably the direct precursor of the orcinol, **8**, which we have also identified as a component of the ethionine-inhibited cultures.[20]



The effects of triacetic lactone and of dehydroacetic acid on various fungi have been the subject of some reports. Ehrensvärd[22] originally noted that triacetic lactone stimulated the formation of aromatic compounds by *Penicillium urticae* while dehydroacetic acid had an inhibitory effect. Triacetic lactone has also been reported to stimulate the synthesis of 6-methylsalicylic acid from acetate-1-C^{14} in *Penicillium patulum*.[12] Tanenbaum[23] has reported that triacetic lactone (at 1.3×10^{-5} *M*) causes an increase from an average of 10.5 mg of stipitatic acid/100 mg of mycelium to an average of 37.8 mg/100 mg of mycelium in *P. stipitatum* growing on Czapek–Dox medium. At this concentration of triacetic lactone, the mycelial weight was almost exactly half of that of the controls. No results for stipitatonic acid were reported. In these experiments dehydroacetic acid was without effect.

In the work reported here, both tri- and tetraacetic lactone appeared to have only minor effects on *Penicillium stipitatum* when the components were added at the fifth day of growth. In one experiment, using triacetic lactone at concentrations of 8.5×10^{-5} *M* and 1.7×10^{-4} *M*, there was a slight decrease in mycelial weight and a slight increase in the amount of total tropolones.[24] On the other hand, in experiments[25] in which 100 mg of triacetic lactone or 55 mg of tetraacetic lactone was added on days 5, 6, 7, and 8 to single cultures, the amounts of tropolones in the treated and normal cultures were the same and the mycelial weights showed only slight variations. The reason for the different results obtained by Tanenbaum and ourselves may stem from the time at which the lactone was added to the cultures. In our work, the additions were made at the fifth day of growth when a complete mycelial pad was present, whereas in Tanenbaum's experiments, the lactone was apparently added at the time of inoculation.

Experimental Section

Isolation of Metabolites. Portions of the previously described "growth medium" (700 ml)[26] were autoclaved in Fernbach flasks (2.8 l.). DL-Ethionine (1.1 g) was suspended in water (80 ml) and dissolved by addition of 2 *N* HCl to pH 5. The volume was made to 100 ml and this solution was separately autoclaved. To each flask was added 5 ml of the ethionine solution, prior to inoculation with *Penicillium stipitatum* NRRL 2104. The organism was allowed to grow as a surface culture for 11–14 days at 28–30°; the filtered culture medium was then evaporated *in vacuo* to about $^1/_{20}$th of the original volume. The concentrated medium was extracted successively with chloroform,[5] ether, and ethyl acetate. Two extractions were made with each solvent, and the total volume of each solvent was 1.5 times that of the concentrated medium. The medium was then acidified to pH 1 with HCl and two further extractions with ether and ethyl acetate were made in the same way. The weight of each extract was determined after removal of the solvent. In a typical batch the medium obtained from 14 flasks amounted to 8400 ml; it was concentrated to 300 ml. The weights of the three extracts prior to acidification were as follows: chloroform, 4.5 g; ether, 5.6 g; ethyl acetate, 6.0 g. After acidification, the extract weights were 1.3 g for the ether extraction and 3.2 g for the ethyl acetate.

Prior to chromatography, each extract was dissolved in ethyl acetate (80 ml/g) and the solution was extracted with saturated sodium bicarbonate solution (2 × 150 ml). The aqueous extracts

(19) Biogenetic type syntheses of aromatic compounds by cyclization of model polyketo compounds have recently received attention; see, for example, T. Money, J. L. Douglas, and A. I. Scott, *J. Am. Chem. Soc.*, 88, 624 (1966); L. Crombie, D. E. Games, and M. H. Knight, *Chem. Commun.*, 355 (1966); L. Crombie and A. W. G. James, *ibid.*, 357 (1966).

(20) Orcinol has only been infrequently reported as a product of mold metabolism. Preliminary evidence for its presence in *Penicillium griseofulvum* was reported by Reio;[21] it has been detected in *Aspergillus fumigatus* and *Gliocladium roseum* by G. Pettersson, *Acta Chem. Scand.*, 18, 1202 (1964); **19**, 414 (1965), and in mutants of *Aspergillus terreus* by R. F. Curtis, P. C. Harries, C. H. Hassall, and J. D. Levi, *Biochem. J.*, 90, 43 (1964), and R. F. Curtis, P. C. Harries, C. H. Hassall, J. D. Levi, and D. M. Phillips, *J. Chem. Soc., Org. Sect.*, 168 (1966). The enzyme, orsellinic acid decarboxylase, has been described in a lichen (*Umbilicaria pustulata*) and in its algal phycobiont (*Trebouxia*) by K. Mosbach and U. Ehrensvärd, *Biochem. Biophys. Res. Commun.*, **22**, 145 (1966), and in *Gliocladium roseum* by G. Pettersson, *Acta Chem. Scand.*, **19**, 2013 (1965).

(21) L. Reio, *J. Chromatog.*, **1**, 338 (1958).

(22) G. Ehrensvärd, *Exptl. Cell Res., Suppl.*, **3**, 102 (1955).

(23) S. W. Tanenbaum, "Biogenesis of Antibiotic Substances," Publishing House of the Czechoslovak Academy of Science, Prague, 1965, p 143.

(24) The observed values were as follows; the mycelial weight (g) is given first followed by total tropolone concentration (mmole/g mycelium). Controls, 7.63, 1.52; 8.5×10^{-5} *M* lactone, 7.22, 1.58; 1.7×10^{-4} *M* lactone, 6.65, 1.85.

(25) See following paper.

(26) R. Bentley and C. P. Thiessen, *J. Biol. Chem.*, **238**, 1880 (1963).

were acidified with concentrated HCl to pH 1 and reextracted with ethyl acetate. After drying over sodium sulfate, the solvent was removed under vacuum. The residue was then stirred several times with acetone; the solid tropolone acids which separated out were removed by filtration, and the filtrates were evaporated under vacuum.

To exemplify the methods used in chromatography of the extracts, the procedure used in fractionating the acidic material contained in the pH 5 ether extract will be described. A portion (3.0 g) of the material obtained by removal of the acetone was chromatographed on a column (45 × 500 mm) containing Celite (150 g) treated with 0.5 N H_2SO_4.[27] Elution was begun with chloroform; 150 fractions (10–12 ml each) were collected. Sixty fractions were next collected with CB-2, and material remaining on the column was then removed with 500 ml of chloroform–10% n-butyl alcohol and 1000 ml of chloroform–33% n-butyl alcohol. Fractions were examined by paper chromatography using Whatman No. 1 paper buffered at pH 6.7 and developed with water-saturated n-butyl alcohol.[1a]

Fractions 19–30. After several crystallizations from ethyl acetate–petroleum ether (bp 38–52°) and subsequent sublimation (0.1 mm, 90°) the major component was obtained as micro crystals (30 mg, mp 204°, R_f 0.9), λ_{max}^{EtOH} 289 mμ (ε 8300); nmr [$(CD_3)_2SO$] τ 3.92, 7.85, and 8.25. These values agree with those reported for methyl triacetic lactone.[4]

Fractions 31–70. The major component (R_f 0.7–0.8) was found to be contaminated with a small amount of orsellinic acid (R_f 0.5–0.55). The crude product (700 mg) was crystallized from ethyl acetate–petroleum ether (bp 38–52°) (charcoal treatment) to give 400 mg of a white crystalline solid, mp 190°; there was no melting point depression in admixture with authentic triacetic lactone;[28] λ_{max}^{KBr} 2.95 (small), 3.5 (broad), 5.92, and 6.10 μ; λ_{max}^{EtOH} 283 mμ (ε 7500) changed by addition of NaOH (pH 8) to 278 mμ (ε 8200); nmr [$(CD_3)_2SO$] τ 4.04 (multiplet), 4.76 (J = 2.5 cps; exchangeable with deuterium), 7.84, relative intensity 1:1:3. *Anal.* Calcd for $C_6H_6O_3$: C, 57.14; H, 4.76. Found: C, 57.28; H, 4.58.

Fractions 71–150. These fractions contained principally orsellinic acid, together with small amounts of tetraacetic lactone (see below) and orcinol.

Fractions 150–210 (CB-2). The major component (R_f 0.4–0.5) was accompanied by small amounts of orcinol (R_f 0.95), particularly in the latter fractions. After crystallization from ethyl acetate–petroleum ether (bp 38–52°) (charcoal treatment) the yield of slightly straw-colored tetraacetic lactone, **2a**, mp 118–119°, was 80 mg; λ_{max}^{KBr} 2.95, 3.4 (broad), 3.85 (broad), 5.83, 6.02, and 6.20 μ; λ_{max}^{EtOH} 284 mμ (ε 8000) changed by addition of NaOH (pH 8) to 360 mμ (ε 23,000) and 256 mμ (ε 15,400); nmr [$(CD_3)_2SO$] τ 3.91 (J = 2.5 cps), 4.71 (J = 2.5 cps; exchangeable with deuterium), 6.24 (exchangeable with deuterium), 7.83, relative intensity 1:1:2:3. *Anal.* Calcd for $C_8H_8O_4 \cdot H_2O$: C, 51.61; H, 5.37. Found: C, 51.57; H, 5.47.

In another fractionation of a smaller amount of the crude pH 5 ether extract, some of the later CB-2 fractions contained mainly the material with an R_f 0.9–1.0 and only small amounts of tetraacetic lactone. After preparative thin layer chromatography on silica gel using the solvent C described by Reio for paper chromatography,[21] material with the same R_f as orcinol was further purified by sublimation (25 mm, 100°), The crystalline sublimate (mp 109°) was shown to be identical with orcinol (paper and thin layer chromatography, mixture melting point, gas chromatography–mass spectrometry).

The acidic material contained in the pH 5 ethyl acetate extract could also be fractionated on the Celite columns. In a typical run, 1.5 g of the crude material was chromatographed on 75 g of Celite (column size, 30 × 400 mm); the first 80 fractions were eluted with chloroform and the next 70 with CB-2. Fractions 27–50 yielded 400 mg of triacetic lactone, fractions 60–75 contained small amounts of orsellinic acid, and fractions 95–140 yielded 350 mg of tetraacetic lactone. Small amounts of the two lactones could also be obtained by chromatography of the pH 1 ether and ethyl acetate extracts.

6-(2-Oxopropyl)-4-methoxy-2-pyrone (2b). Tetraacetic lactone (80 mg) was added to 15 ml of an ether solution of diazomethane. After standing for 30 min at 0°, the ether solution was decanted from a small amount of undissolved material, and the solvent was removed under vacuum. The solid residue, crystallized from chloroform–ether, formed white needles of 6-(2-oxopropyl)-4-methoxy-2-pyrone, mp 80–81° (60 mg); λ_{max}^{KBr} 2.88, 5.78, 5.83, and 6.08 μ; λ_{max}^{EtOH} 282 mμ (ε 6250); nmr ($CDCl_3$) τ 4.07 (J = 2.5 cps), 4.54 (J = 2.5 cps), 6.19, 6.46, and 7.74, relative intensity 1:1:3:2:3. Gas chromatography–mass spectrometry: a single peak was observed on a column of 3% SE-30 (100–120 mesh Gaschrom S) which gave the mass spectrum shown in Figure 1B. *Anal.* Calcd for $C_9H_{10}O_4$: C, 59.34; H, 5.49. Found: C, 59.94; H, 5.76.

2,4-Dinitrophenylhydrazone of Tetraacetic Lactone. A solution of the lactone (20 mg) in water (10 ml), prepared with slight warming, was cooled and treated with 10 ml of 2 N HCl saturated with 2,4-dinitrophenylhydrazine. A yellow precipitate which formed immediately was filtered off after the mixture had been allowed to stand for 30 min at room temperature. The 2,4-dinitrophenylhydrazone of 6-(2-oxopropyl)-4-hydroxy-2-pyrone was crystallized from ethyl acetate–petroleum ether (bp 38–52°); mp 205–206°. *Anal.* Calcd for $C_{14}H_{12}O_7N_4 \cdot H_2O$: N, 15.21. Found: N, 15.09.

Experiments on the Cyclization of 6-(2-Oxopropyl)-4-hydroxy-2-pyrone (2a). Following treatment of the tetraacetic lactone with acid or base under the conditions specified in Table I, the reaction mixture was acidified and either evaporated to dryness or extracted with ether. The products were identified by paper chromatography; the chromatograms were inspected under ultraviolet light and were then sprayed with diazotized benzidine.

For isolation of orcinol, 10 mg of lactone, 90 mg of KOH, and 1 ml of ethanol were refluxed for 3 hr. Following acidification and evaporation to dryness, the residue was taken up in acetone and was subjected to preparative thin layer chromatography (silica gel, solvent C).[21] The major spot had the same R_f (~0.3) as orcinol. After sublimation (25 mm, 100°), the crystalline product was shown to be identical with orcinol (melting point, mixture melting point, gas chromatography–mass spectrometry). Treatment of orsellinic acid with alcoholic KOH under these same conditions also gave orcinol in almost quantitative yield.

The reaction of 10 mg of tetraacetic lactone with 0.3 ml of ethanol (sealed tube) at 110° for 4 hr gave a dark brown solution. Analysis by thin layer chromatography with solvent C of Reio[21] showed that the main spot had the same R_f (0.85) as that given by orsellinic acid ethyl ester.[29] No orsellinic acid could be detected. A little unchanged lactone remained at the origin, a small spot with the R_f (0.3) of orcinol was present, and a faint spot (R_f 0.15) was given by an unidentified material. When the reaction time was 16 hr, the spots at the origin and with R_f 0.3 were fainter, and the spot corresponding to orsellinic ester was more pronounced. Three very small spots, R_f 0.15, 0.5, and 0.75, were also observed. When orsellinic acid was treated with ethanol at 110° for 18 hr, the solution remained colorless. Analysis by thin layer chromatography showed no remaining orsellinic acid and no orsellinic ester; the major spot had the characteristics of orcinol and in addition there was a faint spot with R_f 0.75.

Acknowledgments. The authors thank S. E. Dreher and Lisa Hall for assistance in growing large-scale cultures of *P. stipitatum*, and J. A. Ghaphery for carrying out tropolone assays. We are very much indebted to Dr. R. J. Highet, Laboratory of Metabolism, National Heart Institute, for obtaining the high-resolution mass spectra reported in this paper, and to Dr. C. C. Sweeley of this Department for gas chromatography–mass spectrometry. This work was supported, in part, by Research Grants GM 08477 and AM 09311 from the National Institutes of Health, U. S. Public Health Service.

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