Stipitatonic Acid Biosynthesis. Incorporation of [*formyl*-^{14}C]-3-Methylorcylaldehyde and [^{14}C]Stipitaldehydic Acid, a New Tropolone Metabolite†

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ABSTRACT: [*formyl*-^{14}C]-3-Methylorcylaldehyde was incorporated into stipitatonic acid about ten times as rapidly as [*carboxyl*-^{14}C]-3-methylorsellinic acid in young still cultures of *Penicillium stipitatum*. Stipitaldehydic acid, a new tropolone metabolite, was also labeled after incubation with [*formyl*-^{14}C]-3-methylorcylaldehyde, and isolated [^{14}C]stipitaldehydic acid was shown to be incorporated into [^{14}C]stipitatonic acid upon reincubation with *P. stipitatum* cultures. Stipitaldehydic acid is assigned the structure of 4-formyl-6-hydroxytropolone-5-carboxylic acid γ-lactol on the basis of nuclear magnetic resonance (nmr), mass spectrometry, infrared and ultraviolet absorption, and biosynthetic considerations. An alternate placement of the formyl and carboxylic acid substituents on the tropolone ring cannot be ruled out on the spectral data alone. In either case, stipitaldehydic acid should be the immediate precursor of stipitatonic acid, requiring only a two-electron oxidation for conversion to stipitatonic acid. An improved isolation procedure was developed for the *P. stipitatum* aromatic metabolites. Metabolites were extracted from the culture medium by adsorption onto a column of XAD-2, and were then washed from the column with acetone. The total extract was then fractionated according to polarity by chromatography on a series of two reversed phase partition columns. No detectable quantities of radioactive or ultraviolet absorbing metabolites having a polarity between 3-methylorcylaldehyde and stipitaldehydic acid could be observed.

The discovery that 3-methylorsellinic acid (**1**) is converted into stipitatonic (**6**) and stipitatic (**7**) acids in *Penicillium* stipitatum (Scott *et al.*, 1971) provides evidence that cyclization of the proposed polyketide intermediate (**8**) occurs first to form a six-membered benzenoid ring, and that the benzenoid intermediate is subsequently metabolized by oxidative reactions leading to ring enlargement and formation of the tropolone system. Since orsellinic acid was shown not to be an intermediate in stipitatonic acid biosynthesis (Bently, 1963b), and methyl triacetic lactone has been isolated from the same organism (Acker *et al.*, 1966), the evidence also favors a methylation of the polyketide chain prior to cyclization. No intermediates have yet been established for this oxidative pathway to stipitatonic acid, although stipitalide (**4**), a new tropolone isolated and identified by Holik and Kuhr (1973), would appear to be a good candidate.

In this report we show that [*formyl*-^{14}C]-3-methylorcylaldehyde (**2**), which is incorporated into stipitatonic acid about ten times as rapidly as [*carboxyl*-^{14}C]-3-methylorsellinic acid (**1**), also labels stipitaldehydic acid (**5**), another new tropolone isolated from *P. stipitatum*. Structural data on stipitaldehydic acid suggest that it could be the immediate precursor of stipitatonic acid, differing only in having the anhydride ring reduced to a lactol ring. [^{14}C]Stipitaldehydic acid is incorporated into stipitatonic acid upon incubation with *P. stipitatum* cultures.

Experimental Section

Culturing Techniques. Cultures of *Penicillium stipitatum* NRRL 2104 were grown for 3 weeks at 25° on agar slants (2% glucose, 2% malt extract, 2% yeast extract, 0.1% peptone, and 2% agar) and stored at 5°. Spore suspensions from the slants were used to inoculate surface cultures containing the following medium: 50 g of glucose, 3.0 g of corn steep liquor, 1.0 g of yeast extract, 2.0 g of $NaNO_3$, 1.0 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 1 l. of H_2O. A mycelial mat covered the surface by the fourth day of growth at 25°.

Chromatographic Techniques. Tropolones and most other aromatic metabolites were quickly and efficiently extracted from the culture medium by adsorption onto a column of XAD-2 resin. The resin columns were packed as described by Bradlow (1968) for the extraction of steroid metabolities from urine. Filtered culture medium was acidified to pH 1.0 with addition of 6 N hydrochloric acid, since the acidic tropolones are not adsorbed by the resin from solutions above pH 2.0. The medium was poured over the resin column at a loading of 2 ml of medium/g of resin. The column was washed with 0.1 N hydrochloric acid and water (1 ml of each/g of resin) to remove unadsorbed solids. The tropolones and other adsorbed metabolites were then eluted with acetone or methanol (4 ml/g of resin). The elution could be monitored by ultraviolet absorption, thin-layer chromatography, or radioactivity assay of the eluate. All radioactive metabolites of [*carboxy*-^{14}C]-**1** or [*formyl*-^{14}C]-**2**, and 98% of the ultraviolet absorbing material in the culture medium, were adsorbed to the XAD and not eluted until the acetone wash. More than 98% of the solids in the medium, however, including glucose and salts, were not adsorbed to the resin and were therefore removed in the initial water washes. Acetone was removed *in vacuo* on a rotary evaporator, and residual water was removed by lyophilization. The residue will be referred to as the crude XAD extract. This procedure, which has been applied to a variety of urinary

† From the Department of Chemistry, Florida State University, Tallahassee, Florida 32306. *Received August 13, 1973.* Supported by Grant GB-26325 from the National Science Foundation. High-resolution mass spectrometric and nuclear magnetic resonance equipment used in this research was purchased from National Science Foundation funds.

‡ Supported by a Biochemistry Training Grant, GM 1087, from the National Institutes of Health, National Institute of General Medical Science.

metabolites of steroids (Bradlow, 1968), prostaglandins (Granström and Samuelsson, 1971), and drugs (Mulé *et al.*, 1971), appears to be generally applicable to a variety of extractable organic microbial metabolites, even very polar ones such as stipitatonic acid and patulin (R. J. Light, unpublished results). The procedure is rapid, and the column can be reused after thorough washing with H_2O and 0.1 N hydrochloric acid.

Reversed phase partition chromatography was employed to fractionate metabolites in the crude XAD extract according to polarity. Columns were prepared with Reversil-3 as the hydrophobic support material in a manner similar to that described by Norman (1953). Solvent systems were equilibrated overnight, and a portion of the organic (stationary) phase was thoroughly mixed with Reversil-3 in a ratio of 4 ml to 10 g. This material was slurried with aqueous (moving) phase and packed in a 1.5 cm i.d. glass column. Samples were dissolved in a small amount of aqueous phase, or slurried with a small portion of aqueous phase and coated Reversil-3, applied to the column, and eluted with the remaining aqueous phase.

Sufficient retardation and reasonable separation of standard stipitatonic acid, stipitatic acid, and triacetic acid lactone could be achieved by using a solvent system consisting of 60 ml of cyclooctanone, 600 ml of water, and 4 ml of formic acid per 10 g of Reversil-3. Less polar aromatic metabolites, such as 3-methylorsellinic acid and 3-methylorcylaldehyde, were not eluted from this column by the aqueous phase but could be removed by washing the column with acetone. These less polar metabolites were then fractionated using a solvent system consisting of 15 ml of chloroform, 15 ml of 1-octanol, 2 ml of acetic acid, 90 ml of methanol, and 210 ml of water per 5 g of Reversil-3.

Thin-layer chromatography of the metabolites was carried out on plates spread with 500-μ layers of MN-cellulose powder HR-300 and developed in water–acetic acid, 97:3, v/v. Fluorescent compounds were observed under long (365 nm) and short (253.7 nm) wave ultraviolet light, and some were visualized as characteristic colored spots when the plates were sprayed with bis-diazotized benzidine reagent (Koch and Krieg, 1938).

Radioactivity Measurements. Assay of radioactivity was made with a Packard TriCarb liquid scintillation spectrometer, Model 3214. Samples were contained in glass vials along with 15 ml of scintillation solution (100 g of reagent grade naphthalene and 8 g of 2,5-diphenyloxazole per l. of redistilled dioxane) and assayed at settings giving a ^{14}C counting efficiency of 74% for unquenched samples. Quench corrections were made using [^{14}C]toluene as an internal standard. Thin-layer chromatography plates containing radioactive compounds were also assayed with a Varian Aerograph Model 6000 radiochromatogram scanner.

Chemical Syntheses. 3-Methylorcylaldehyde (**2**) and [*formyl*-^{14}C]-**2** were prepared by a modified Gatterman reaction (Adams and Levine, 1923) of β-orcinol with $Zn(CN)_2$ or [^{14}C]$Zn(CN)_2$. 3-Methylorsellinic acid (**1**) and [*carboxyl*-^{14}C]-**1** were prepared from the aldehydes by acetylation, oxidation, and deacetylation according to the method described by Robertson and Whalley (1949). β-Orcinol was prepared according to Whalley (1949). Each compound had the requisite melting point, ir, uv, nuclear magnetic resonance (nmr), and mass spectra, and the radiochemical purity was greater than 99.8% as shown by thin-layer chromatography on Adsorbosil-1.

Decarboxylation of Biosynthetic [^{14}C]*Stipitatonic Acid* (**6**). Decarboxylations of [^{14}C]-**6** in boiling water were carried out by a procedure adapted from Bentley (1963a). The $^{14}CO_2$ was passed through a saturated solution of barium hydroxide, and the [^{14}C]barium carbonate was collected, washed, and assayed by suspending in a gel of 15 ml of scintillation fluid and 0.6 g of Cab-O-Sil. Stipitatic acid (**7**) formed in the decarboxylation was extracted with ethyl acetate, and its specific activity was determined after several recrystallizations from water.

Characterization of Stipitaldehydic Acid. For larger scale isolation of stipitaldehydic acid, XAD extracts were prepared on 7-day old cultures of *P. stipitatum*, the time when the fraction C content appeared to reach a maximum level. A 2-g portion of the extract, equivalent to 1600 ml of medium, was fractionated on a 75-g Reversil column using the cyclooctanone–water–formic acid solvent system. Fraction C obtained from this column was heavily contaminated by overlapping peaks of stipitatonic acid and stipitatic acid which had also begun to build up by the seventh day. Stipitatonic acid was removed by refluxing the crude fraction C for 3 hr in water, which led to decarboxylation of stipitatonic acid and produced a sample containing stipitatic acid and stipitaldehydic acid as the major components observed by thin-layer chromatography. Stipitaldehydic acid survives these decarboxylation conditions. This sample was further purified either by fractional sublimation at 10^{-3} mm and temperatures up to 150° or by rechromatography on a smaller Reversil column. Spectral data were obtained on fractions giving a single ultraviolet-fluorescent, benzidine-positive spot on cellulose powder chromatography (R_F 0.67; developing solving water–acetic acid, 97:3, v/v). The low-resolution mass spectrum (source temperature 60°, ionization voltage 15 eV) showed the molecular ion at *m/e* 210 as the base peak and ions at *m/e* 164 (M-46) and *m/e* 136 (M-74) with relative intensities about 15% of the molecular ion. The high-resolution mass spectrum showed the molecular ion at an *m/e* of 210.0164 (calcd for $C_9H_6O_6$, 210.0162). The nmr, ultraviolet, and infrared spectral data are summarized in Table I.

Some of the stipitaldehydic acid fractions from the large-scale isolation showed small amounts of a second ultraviolet-fluorescent, benzidine-positive spot on thin-layer chromatography (R_F 0.60). The mass spectrum of these samples showed an ion at *m/e* 194, and the nmr spectrum showed an additional signal at δ 5.26. These data would indicate the contaminant to be stipitalide, previously isolated and characterized by Holik and Kuhr (1973).

Spectrometric Techniques. Ultraviolet and visible absorption spectra were obtained on a Cary 15 recording spectrophotometer. Infrared spectra were obtained on a Perkin-Elmer Model 257 recording spectrophotometer. Nuclear magnetic resonance spectra were obtained on a 90-mHz or a 270-mHz Bruker nmr spectrometer (Bruker Scientific Inc., Elmsford, N. Y.) with tetramethylsilane as an internal standard. High-resolution mass spectra were obtained on a G.E.C.-A.E.I. MS-902 operated at 70 eV with a resolution of 10,000. Perfluorokerosene was used as an internal standard.

Materials. Yeast extract, malt extract, peptone, and agar were obtained from Difco. Corn steep liquor was obtained from Corn Products Sales, Atlanta, Ga. XAD-2 resin, 20–50 mesh, was obtained from Rohm and Haas Co., Philadelphia, Pa. Reversil-3 and Adsorbosil-1 were obtained from Applied Science Laboratories Inc., State College, Pa. MN-Cellulose powder HR-300 was obtained from Brinkman Instruments Inc., Westbury, N. Y. Cab-O-Sil and 2,5-diphenyloxazole were obtained from Packard Instrument Co., Downers Grove, Ill. Reagent grade naphthalene was obtained from Fisher Chemical Co. [^{14}C]$Zn(CN)_2$ was prepared from $ZnCl_2$ and [^{14}C]-NaCN, which was obtained from New England Nuclear,

TABLE I: Comparison of Spectral Data for Tropolone Acids from *P. stipitatum*.

Tropolone	Uv Absorption[a] nm	Log ε	Ir Absorption[b] (cm^{-1})	Nmr[c] δ (ppm)	Nmr[c] Line Width at Half-Height (Hz)
Stipitatonic acid (**6**)	252	4.3	1825	6.98[d]	1.3
	374	3.8	1745	6.86[d]	1.0
	437	3.5	1640		
			1240		
Stipitatic acid (**7**)	260	4.5	1700	7.42[e]	
	358	3.6	1605	7.32[e]	
			1570	6.83[e]	
			1480		
Stipitalide[f] (**4**)	254[g]	4.5[g]	1717[e]	6.88[d]	2.2
	378[g]	4.1[g]	1622[e]	6.83[d]	1.7
			1560[e]	5.26[d]	2.2
			1260[e]		
Stipitaldehydic acid (**5**)	253	4.4	1728	6.87[d]	3.8
	374	3.9	1632	6.66[d]	4.2
			1245	6.33[d]	6.0

[a] In ethanol. [b] KBr pellet. [c] In [$^2H_6$]dimethyl sulfoxide, Me_4Si reference. [d] 270-MHz instrument. [e] Reported by Holik and Kuhr (1973). [f] Sample obtained from Holik. [g] Data obtained from Holik.

Boston, Mass. An authentic sample of stipitalide was obtained from Dr. M. Holik, Research Institute of Pure Chemicals, Lachema, Brno, Czechoslovakia.

Results

Tropolone production in surface cultures of *Penicillium stipitatum* (determined by absorbance at 275 nm) began on about day 4 of growth and continued at least through day 16 when levels approached 2.0 mg/ml, a pattern very similar to that reported by Bentley and Thiessen (1963). The experiments described in this report were carried out with cultures between days 4 and 7 of growth, the period of initial synthesis of stipitatonic acid, and the period prior to substantial stipitatic acid production.

An improved extraction and purification procedure for the tropolone acids from *Penicillium stipitatum* was developed in order to study more systematically the incorporation of proposed biosynthetic intermediates and to search for the existence of new intermediates in the culture medium. The procedure described here should prove to be generally applicable in such biosynthetic studies. The extraction of metabolites from culture medium of *P. stipitatum* was achieved quickly and easily by adsorption onto columns of XAD-2 resin as described in the Experimental Section. Metabolites in this crude XAD extract were fractionated according to polarity by reversed phase partition chromatography. Two columns were necessary: a very polar one which would retard the polar tropolone acids **6** and **7**, and a less polar one to fractionate the material eluted in the acetone wash of the first column. Figure 1 illustrates the use of this method to follow the incorporation of [*carboxyl*-^{14}C]-3-methylorsellinic acid (**1**) into stipitatonic acid (**6**). Fraction A consists of a mixture of very polar materials eluting with the column void volume. Fraction B contains stipitatonic acid as the major component. Fraction C contains a new tropolone acid, stipitaldehydic acid (**5**), as the major component. Stipitatic acid (**7**), not present in appreciable quantities in this particular culture but evident in older cultures, is found in the later portion of fraction D. Fraction E was washed from the column with acetone and applied to the less polar partition column as illustrated in Figure 2. As can be seen from Figure 2, the majority of the isolated radioactivity is found in fraction G, the elution position of 3-methylorsellinic acid. Therefore, after a 12-hr incubation, most of the radioactivity recovered from the growth medium (about 60% of that added) was recovered as unchanged [*carboxyl*-^{14}C]-**1**, slightly less than 1% was incorporated into the stipitatonic acid fraction, and small amounts were found in fractions A, C, and F.

Figures 3 and 4 illustrate a similar 12-hr incorporation experiment with [*formyl*-^{14}C]-3-methylorcylaldehyde (**2**). The pattern is essentially the same, except that considerably more radioactivity was incorporated into fractions A, B, and C than with the acid precursor. In this case about 70% of the added

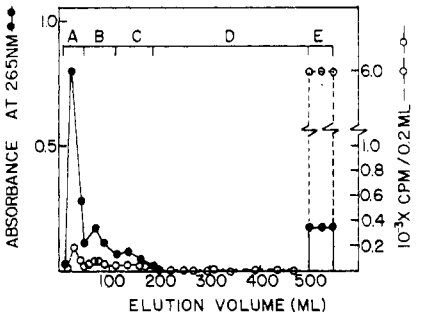


FIGURE 1: Chromatography of radioactive metabolites formed from incubation of *P. stipitatum* cultures with [*carboxyl*-^{14}C]-3-methylorsellinic acid (**1**). Two 166-ml cultures were grown in 500-ml erlenmeyer flasks for 4 days, and 1.6 μCi of [*carboxyl*-^{14}C]-**1** (426 × 10^3 dpm/μmol) was added to each flask. After further incubation for 12 hr the crude XAD extract, obtained from the combined cultures as described in the Experimental Section, contained 0.2 g and 2.3 μCi of ^{14}C. One-half of this extract was fractionated on a 10-g column of Reversil-3 as described in the Experimental Section, using the polar cyclooctanone–water–formic acid solvent system. Absorbance at 265 nm was measured on aliquots of each fraction diluted 1:20 with ethanol. Radioactivity was determined on 0.2-ml aliquots, but was not corrected for quenching. Fraction E contains material eluted from the column with acetone.

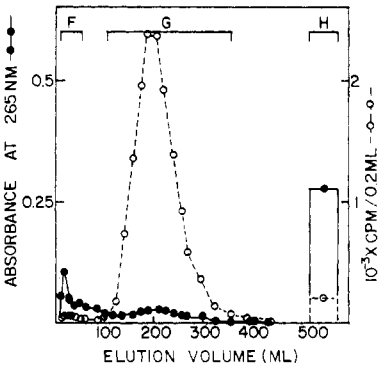


FIGURE 2: Chromatography of fraction E from Figure 1 with the less polar chloroform–octanol–water–acetic acid solvent system. An aliquot of fraction E (60 mg, 0.78 μCi) was applied to a 5-g column of Reversil-3 as described in the Experimental Section. Radioactivity and absorbance at 265 nm were determined on the eluted fractions as in Figure 1. The major radioactive peak of fraction G corresponds to the elution volume of 3-methylorsellinic acid.

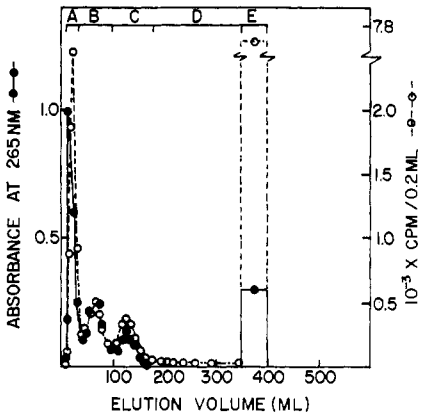


FIGURE 3: Chromatography of radioactive metabolites formed from incubation of *P. stipitatum* cultures with [*formyl*-^{14}C]-3-methylorcylaldehyde (**2**). Two 166-ml cultures were grown in 500-ml erlenmeyer flasks for 4 days, and 1.65 μCi of [*formyl*-^{14}C]-**2** (460 $\times$ 10^3 dpm/μmol) was added to each flask. After further incubation for 12 hr, the crude XAD extract was isolated from the combined culture media as described in the Experimental Section, and contained 3.1 μCi of ^{14}C. One-half of this extract was chromatographed as described in Figure 1.

radioactivity was recovered as [*formyl*-^{14}C]-**2** (Figure 4, fraction G) while about 4% was incorporated into the stipitatonic acid fraction, 6% into fraction A, 2.6% into fraction C, and less than 1% into fraction F.

Similar fractionations from cultures incubated for longer periods of time were carried out in order to compare the rates of incorporation of [*carboxyl*-^{14}C]-**1** and [*formyl*-^{14}C]-**2** into fractions A, B, and C (Figure 5). The radioactivity in each fraction was estimated from the area of the curve under the chromatographic peaks. Since the radioactivity assayed in the fractions from these columns was not corrected for quenching, actual incorporation values were a little higher than the relative ones shown in Figure 5. Both [*carboxyl*-^{14}C]-**1** and [*formyl*-^{14}C]-**2** disappear from the culture medium over the 3-day period of the experiment. The loss of the aldehyde appears to be less at 12 hr, but its rate of disappearance from 12 hr to 3 days is about twice as great as that of the acid. Furthermore, the rate of incorporation of the aldehyde into fractions A, B, and C is about ten times greater than the incorporation of the acid into these fractions. Approximately 20% of the radioactivity from [*formyl*-^{14}C]-**2** was found in fraction B after the 3-day incubation.

The radioactivity incorporated into fraction B from both precursors was shown to be contained in stipitatonic acid (**6**) by addition of authentic carrier **6** and recrystallization from acetone to constant specific activity. The incorporation was shown to be specific when chemical decarboxylation (Scheme I, reaction e) of the purified [^{14}C]-**6** yielded unlabeled stipitatic acid (**7**) and [^{14}C]CO_2 of the same specific activity as the [^{14}C]-**6**. Fraction B (6 mg) from [*carboxyl*-^{14}C]-**1** diluted with 40 mg of carrier **6** to a specific activity of 73.6 dpm/μmol gave specific activities of 66.5, 61.8, and 58.5 dpm/μmol upon subsequent recrystallizations. Upon decarboxylation the recrystallized sample yielded CO_2 with a specific activity of 53.4 dpm/μmol and stipitatic acid with a specific activity of 0.0 dpm/μmol. Fraction B (7 mg) from [*formyl*-^{14}C]-**2** diluted with 40 mg of carrier **6** to a specific activity of 447 dpm/μmol gave specific activities of 433, 433, and 416 dpm/μmol upon subsequent recrystallizations. Upon decarboxylation the recrystallized sample yielded CO_2 with a specific activity of 372 dpm/μmol and stipitatic acid with a specific activity of 4 dpm/μmol.

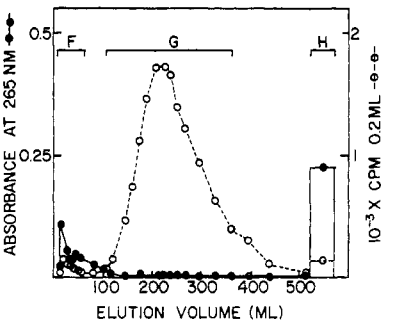


FIGURE 4: Chromatography of fraction E from Figure 3 with the less polar chloroform–octanol–water–acetic acid system. An aliquot of fraction E (50 mg, 0.75 μCi) was chromatographed as described in Figure 2. The major radioactive peak of fraction G corresponds to the elution volume of 3-methylorcylaldehyde.

From the schemes proposed for the conversion of **1** to **6** (Scott *et al.*, 1971), one would expect that intermediates should have polarities intermediate between these two compounds, and should be found in the reversed phase chromatographic eluate between fractions B and G. The only additional fractions labeled appreciably by [*formyl*-^{14}C]-**2** were A and C. The small amount of radioactivity found in fraction F (Figure 4) was no greater than that found in experiments with the [*carboxyl*-^{14}C]-**1**, and was not enough to investigate further. In order to check the possibility that radioactive intermediates might have remained in the mycelium, mycelia from two different [*formyl*-^{14}C]-**2** incorporation experiments were extracted with ether. Essentially all of the radioactivity recovered in these extracts chromatographed on reverse phase columns as unreacted [*formyl*-^{14}C]-**2**.

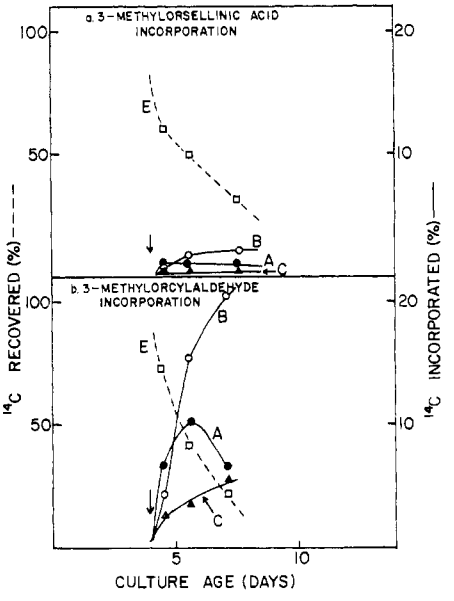
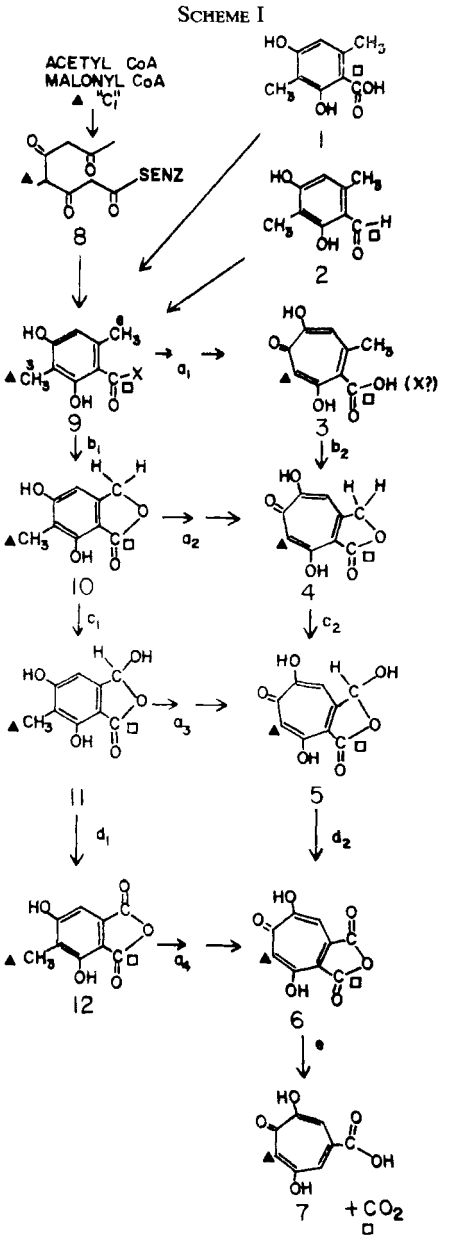


FIGURE 5: Effect of incubation time on the extent of incorporation of (a) [*carboxyl*-^{14}C]-3-methylorsellinic acid (**1**) and (b) [*formyl*-^{14}C]-3-methylorcylaldehyde (**2**) into metabolite fractions. [*carboxyl*-^{14}C]-**1** (1.6 μCi/166 ml of culture) or [*formyl*-^{14}C]-**2** (1.65 μCi/155 ml of culture) was added to cultures on day 4 (arrow), and duplicate cultures were harvested at the times indicated. The culture media were extracted and chromatographed as described in Figures 1 and 3. The radioactivity in each fraction was calculated from the area under the curve of the elution profiles, and normalized to a percentage of the radioactivity originally added to the cultures. Metabolites found in the reversed phase column fractions are as follows: stipitatonic acid, fraction B; stipitaldehydic acid, fraction C; reisolated 3-methylorsellinic acid or 3-methylorcylaldehyde, fraction E.

SCHEME I



Fractions A and C were chromatographed on cellulose powder thin-layer plates (developing solvent, water–acetic acid, 97:3, v/v). In each case the radioactivity was associated

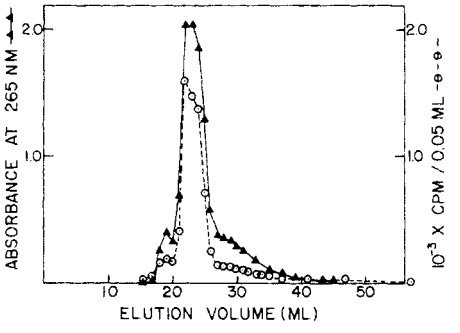


FIGURE 6: Cellulose powder column chromatography of metabolite fraction C labeled by incorporation of [*formyl*-^{14}C]-3-methylorcylaldehyde (**2**). An aliquot of fraction C (25 ml, 0.091 μCi) obtained from cultures incubated for 4 days with [*formyl*-^{14}C]-**2** was applied to a 5-g column of cellulose powder packed in water–acetic acid, 97:3, *v/v*. The column was eluted with the same solvent. Radioactivity was determined on 0.05-ml aliquots, and the absorbance was determined on aliquots diluted 1:40 in ethanol.

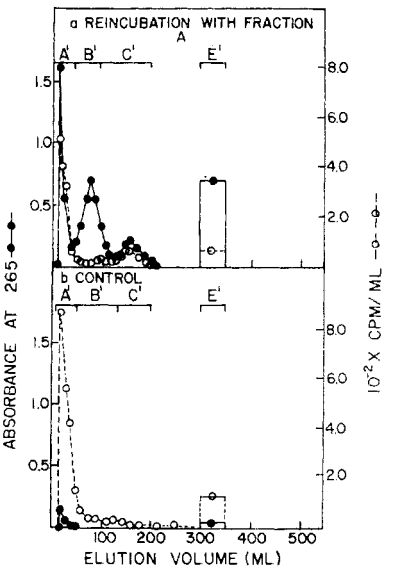


FIGURE 7: Incubation of ^{14}C fraction A with cultures of *P. stipitatum*. (a) Aliquots of fraction A (60 mg, 0.08 μCi) obtained from cultures incubated for 1 day with [*formyl*-^{14}C]-**2** as described in Figure 3 were added to duplicate 166-ml cultures of *P. stipitatum* on day 4, and the cultures were incubated an additional 2 days. Metabolites were extracted with an XAD column as described in the Experimental Section and chromatographed as described in Figure 1. (b) For the control, an aliquot of fraction A (30 mg, 0.04 μCi) was rechromatographed as described in Figure 1.

with the major bis-diazotized benzidine positive spot, although several ultraviolet fluorescent spots were also observed in each fraction. The radioactive component in fraction A had an R_F of 0.9 in this solvent system, while the radioactive component in fraction C had an R_F of 0.67. For comparison, stipitatonic acid had an R_F of 0.73. Fraction C was further analyzed by chromatography on a column of cellulose powder as shown in Figure 6. Here it can be seen that the peak of radioactivity is associated with the major ultraviolet absorbing component in the fraction.

In order to determine whether either radioactive component was an intermediate in stipitatonic acid biosynthesis, aliquots of fraction A and fraction C were incubated with separate cultures of *P. stipitatum*. Metabolites from these incubations were isolated and fractionated by reversed phase chromatography as previously described. Figure 7a shows the polar products from a culture incubated with fraction A, and Figure 7b shows a control in which a sample of fraction A was rechromatographed. Only a trace of radioactivity is evident in fraction B′, no more than is found in the control chromatogram. Therefore, the very polar radioactive material in fraction A does not appear to be an intermediate in the synthesis of stipitatonic acid.

Incubation of cultures with fraction C, containing a single major radioactive component as shown in Figure 6, did result in incorporation of radioactivity into fraction B′ as shown in Figure 8a. About 55% of the added radioactivity was recovered, and at least one-half of this was found in fraction B′. (These radioactive determinations were not corrected for quenching, which is a bit greater for the very yellow fraction B′ than for the slightly yellow fraction C′.) A control is shown in Figure 8b in which an aliquot of fraction C was incubated with sterile medium, then combined with a nonradioactive culture extract and chromatographed. The control is important since it indicates only a small amount of [^{14}C]stipitatonic acid had trailed into fraction C during its isolation, and

therefore that most of the radioactivity in fraction B′ of Figure 8a was formed during the reincubation with *P. stipitatum*. Radiopurity of the product was established by addition of carrier stipitatonic acid to an aliquot of fraction B′ (diluted specific activity, 55.7 dpm/μmol) and recrystallization twice from acetone (recrystallized specific activities, 52.5 and 59.3 dpm/μmol). Decarboxylation yielded 94% of the radioactivity as $^{14}CO_2$, and none as stipitatic acid.

For identification of the chemical nature of the major radioactive component of fraction C, a larger scale isolation of unlabeled material was attempted. Inspection of the quantity of ultraviolet absorbing material in fraction C obtained from cultures of different age, data available from the chromatograms used to generate Figure 5, showed that the level of fraction C plateaued about day 7, while the quantity of material in fraction B (stipitatonic acid) and fraction D (stipitatic acid) continued to increase past day 10. Hence fraction C was isolated from 7-day cultures and further purified as described in the Experimental Section.

Table I compares the spectroscopic data obtained on this compound, which we have designated stipitaldehydic acid (**5**), with other tropolone acids obtained from this organism. The ultraviolet and infrared absorption bands are very characteristic of tropolones. Only three protons are visible in the nmr spectrum, all somewhat broad singlets. The upfield proton is sharpened somewhat upon the addition of D_2O, and hence can be assigned to the lactol ring hydrogen. The mass spectrum is also consistent with structure **5**, giving a molecular formula of $C_9H_6O_6$ and principal fragment ions at M-46 (loss of CH_2O_2) and M-74 (loss of $C_2H_2O_3$). The spectral data do not unambiguously locate the aldehyde carbon of the lactol ring, *i.e.*, they cannot distinguish between structures **5** and **14**. The tentative assignment of structure **5** is based upon the most reasonable biosynthetic relationship among the tropolones, shown in Scheme I. The relationship between this assignment, and the assignment of structure **4** to stipitalide by Holik and Kuhr (1973), is considered below in the Discussion. The ultimate distinction between structures **5** and **14** must await isolation of sufficient material for chemical modification and degradation, and possibly chemical synthesis.

Some partially purified samples of stipitaldehydic acid contained a contaminant with an R_F on thin-layer chromatography identical with that of authentic stipitalide, an ion in the mass spectrum at *m/e* of 194, and an nmr signal at δ 5.26. Hence smaller amounts of stipitalide also appear to be present in fraction C, (less than 10–15%) which account for some of the tailing of the radioactivity peak in Figure 6. The sizable incorporation of radioactivity from fraction C into stipitatonic acid (Figure 8), can only be accounted for if it came predominantly from the major radioactive component, stipitaldehydic acid, but some bioconversion of contaminating stipitalide could also have occurred.

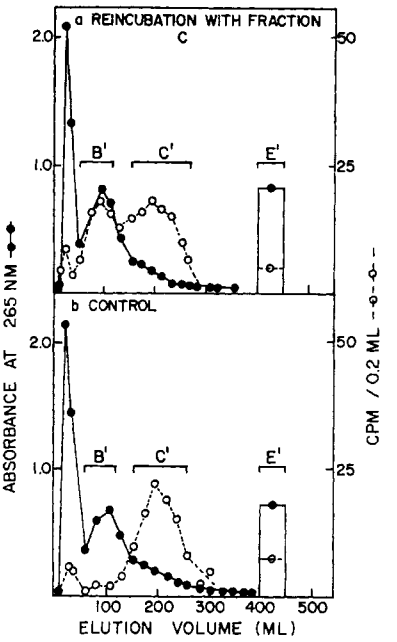


FIGURE 8: Incubation of ^{14}C fraction C with cultures of *P. stipitatum*. (a) Aliquots of fraction C (10 mg, 0.03 μCi) obtained from cultures incubated for 4 days with [*formyl*-^{14}C]-**2** as described in Figure 3 were added to duplicate 166-ml cultures of *P. stipitatum* on day 4, and the cultures were incubated an additional 3 days. Metabolites were extracted with an XAD column as described in the Experimental Section and chromatographed as described in Figure 1. (b) For the control, aliquots of ^{14}C fraction C were incubated with sterile medium for 3 days and combined with nonradioactive medium from 7-day cultures, and the metabolites were extracted and chromatographed as in (a).

Discussion

In this report we have confirmed the previously reported incorporation of [*carboxyl*-^{14}C]-3-methylosellinic acid (**1**) into stipitatonic acid (Scott *et al.*, 1971). We have also found that [*formyl*-^{14}C]-3-methylorcylaldehyde (**2**) is much more efficient as a tropolone precursor, being incorporated about ten times as rapidly as the acid. A similar observation has been made by Steward and Packter (1968) studying gliorosein biosynthesis. In that case, 5-methylorcylaldehyde was incorporated into glioresein whereas 5-methylosellinic acid was not. Several explanations for the preference of aldehyde **2** over acid **1** as a precursor are possible: (a) the actual intermediate may be **2** rather than **1**; (b) the actual intermediate may be a covalent derivative of **1** or **2**, with **2** more easily converted to this derivative than **1**; (c) the actual intermediate may be **1** located in a metabolic pool more easily accessible to external **2** than external **1**. Current data do not allow a distinction to be made among these possibilities.

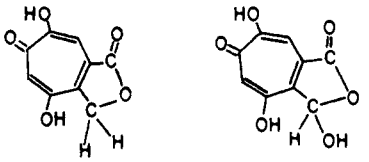
Use of aldehyde **2** as a precursor, however, does lead to sufficient incorporation levels to allow for the detection of further intermediates in the stipitatonic acid pathway. Scheme I shows some possible intermediates in this pathway. Structure **9** is meant to reflect the uncertainty just mentioned with regard to the nature of the actual benzenoid intermediate. Oxidation at either methyl group of this intermediate is possible. The 6-methyl eventually becomes part of the anhydride ring of stipitatonic acid, whereas oxidative attack at the 3-methyl group leads to ring expansion to the tropolone ring system (reactions a_1, a_2, a_3, or a_4). Additional intermediates are possible, depending upon the mechanism of this ring expansion (Scott *et al.*, 1971; Scott and Wiesner, 1972). The actual intermediates observed will depend upon the sequence in which these reactions occur. Multiple pathways are possible.

The isolation techniques reported in this paper were designed to screen for such intermediates appearing in the culture medium. These intermediates should occur in greatest *relative* abundance in young cultures about day 4 or 5 when stipitatonic acid synthesis is just beginning, and should be of a polarity intermediate between **1** and **6**. Stipitaldehydic acid (**5**), the major component of fraction C, was the only compound meeting these criteria observed in appreciable concentrations in the chromatograms, and the only compound less polar than stipitatonic acid which was detectably labeled from

[*formyl*-^{14}C]-3-methylorcylaldehyde. The kinetics of labeling of **5** and **6** (fractions C and B, respectively, Figure 5b) are more in accord with **5** as a precursor of **6** rather than a product of 6. Reincubation of cultures with isolated [^{14}C]-**5** also led to labeling of **6**, another property expected of an intermediate.

Holik and Kuhr (1973) recently isolated and characterized stipitalide (**4**) from this organism. We cited evidence above that some samples of fraction C appeared to contain minor amounts of **4**, hence this compound is also a potential intermediate. The evidence therefore favors a pathway involving ring expansion as an early step. Further proof of such a pathway will have to await detection of other possible intermediates and work with isolated enzyme preparations.

The spectral data obtained on stipitaldehydic acid does not distinguish between structure **5** and its isomer **14**. By the same token, data presented by Holik and Kuhr (1973) for stipitalide do not conclusively distinguish between structures **4** and **13**.



13 14

Their assignment of isomer **4** is based upon long range coupling between one of the aromatic protons and the methylene protons, and upon very small differences in chemical shifts in aromatic protons, somewhat dangerous because of the possibility of tautomeric forms. Coupling might still be possible in structure **13**. In the case of stipitaldehydic acid, we observed no coupling between the δ 6.33 proton and either the δ 6.66 or 6.87 protons, and the broadness of line widths (Table I) suggests some tautomeric shifts may occur. The lactol carbonyl absorption (1728 cm^{-1}) of stipitaldehydic acid, and the lactone carbonyl absorption of stipitalide (1716 cm^{-1}) are consistent with hydrogen-bonded structures **5** and **4**, but would have more significance if the isomeric structures were available for comparison. The isomeric *o*-hydroxyphthalides show carbonyl absorptions which differ as a result of hydrogen bonding: 1735 cm^{-1} for the isomer with the hydroxyl ortho to the carbonyl group; 1760 cm^{-1} for the isomer with the hydroxyl ortho to the methylene group (Geissman and Crout, 1969).

Support for the structural assignment in both cases therefore rests upon the biosynthetic argument proposed in Scheme I. The possibility remains, however, that the carboxyl group of 3-methylorsellinic acid is reduced during some stage of the biosynthetic pathway, and reoxidized at a later time, allowing for structures **13** and **14** as intermediates. Such a reduction at the benzenoid level, as discussed above, does represent one possible explanation for the better incorporation of 3-methylorcylaldehyde.

Use of the XAD-2 resin for extraction of metabolites from the growth medium is much less involved than exhaustive extraction with organic solvents, and may have an advantage for unstable compounds in avoiding the reflux temperature necessary for continuous extraction methods. Its general applicability to fungal metabolites must await testing with a variety of systems.

Acknowledgments

The authors would like to thank M. Holik for providing a sample of stipitalide, and Mrs. Gayle Travis for expert technical assistance.

Added in Proof

The authors have received a private communication from Professor A. I. Scott, Yale University, that Dr. E. Lee (1974) has also recently isolated stipitaldehydic acid and assigned structure **5** on the basis of infrared, nmr, and mass spectroscopic data which confirm that reported here.

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