BIOORGANIC CHEMISTRY 3, 377–385 (1974)

The Conversion of *S*-Adenosyl-L-Methionine to Stipitatic Acid By Extracts of *Penicillium stipitatum*[1]

GERALD GOLD AND RONALD BENTLEY

Department of Biochemistry, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received January 16, 1974

Cell-free extracts prepared from acetone powders of *Penicillium stipitatum* contain an enzyme system converting [$^{14}CH_3$]-*S*-adenosyl-L-methionine to radioactive stipitatic acid; the estimated molecular weight is 105 000. The incorporated radioactivity is located at C-7 of the tropolone ring. Addition of putative acceptor compounds had little or no influence on tropolone synthesis. Methylation appears to be an early step in tropolone biosynthesis in this organism.

The primary metabolites which serve as precursors for biosynthesis of fungal tropolones—acetate, malonate, and a one-carbon unit—have been known for some time (*1*); a recent investigation of puberulonic acid formation has now brought this compound into harmony with the results obtained for stipitatonic acid and sepedonin (*2*). From a considerable amount of speculation, two rival hypotheses remain to account for the actual assembly of the seven-membered aromatic ring system. The first is the ring expansion of a benzenoid compound suitably substituted with a one-carbon unit. This suggestion originated with Robinson (*3*) in 1950, and has been elaborated by various authors (*4–10*). The second is the formation of a methyl-substituted polyketide chain followed by rearrangement to a second straight-chain intermediate which in turn undergoes cyclization to a seven-membered ring. The possible addition of a one-carbon unit to a noncyclized eight-carbon compound was noted by Bentley in 1963 (*11*), and was further considered with the discovery of tetraacetic acid lactone in ethionine-inhibited cultures of *Penicillium stipitatum* (*12*). Tanenbaum and his colleagues postulated methyltriacetic acid or lactone as the precursor, the addition of a further two-carbon unit by way of malonate being required (*13*, *14*); they also noted that the rearrangement reaction was akin to the methylmalonyl CoA to succinyl CoA isomerization.

While this second hypothesis remains attractive in view of the analogy to the isomerization noted above, evidence has recently been presented to implicate a benzene derivative, 3-methylorsellinic acid, as an intermediate in the biosynthesis of the *P. stipitatum* tropolones (*9*, *15*). This strongly suggests that the first hypothesis is correct.

The one-carbon unit is derivable in tracer experiments from formate- or methyl-labeled methionine (*5*, *6*, *11*, *14*, *16–19*) and it is presumed that the actual process is a methylation step by way of *S*-adenosylmethionine. In the first hypothesis, such a

[1] This work was supported in part by a grant from the United States Public Health Service (AM 09311).

Copyright © 1974 by Academic Press, Inc.
All rights of reproduction in any form reserved.
Printed in Great Britain

methylation could, in principle, either precede or follow cyclization to the benzene ring. In the second hypothesis, however, methylation would necessarily be an early step, perhaps directly following assembly of an octaketide chain (*12*). Since 3-methylorsellinic acid (*9*, *15*), but not orsellinic acid (*11*), is a tropolone precursor, and since methyltriacetic acid lactone has been isolated from *P. stipitatum* (*13*, *14*), methylation prior to cyclization seems to be indicated. A cell-free enzyme system, incorporating activity from *S*-adenosylmethionine into stipitatic acid, has now been obtained. The properties of this system, reported here, are consistent with methylation as an early step in the biosynthetic sequence.

EXPERIMENTAL

Penicillium stipitatum, NRRL 2104, was cultured and grown at 28°C as previously described (*20*). For routine enzyme extraction, the mycelia from stationary cultures, or the pellets from shake cultures were chilled to 4°C, filtered free of media, washed several times with deionized water, patted dry with paper towels, weighed, and then frozen quickly in an acetone–dry ice bath. The frozen cells were blended for 1 min in a steel Waring Blendor jar with 5 vol per weight of acetone at −68°C. The blended mush was continuously stirred to keep it from packing while it was filtered on a Büchner funnel. Two equal volumes of acetone at −68°C were used to wash the filtered powder, and after the powder was no longer damp from the acetone washings, diethyl ether at −68°C was used for several additional washings (15 vol per original weight). After drying in air on absorbent paper for several hours, the powder was desiccated at 4°C overnight. To prepare enzyme solution, the acetone powder (10–12 g) was extracted with 10 vol per weight of 0.02 *M*, pH 7.0, phosphate buffer in an ice-packed Virtis mixer jar (volume = 150 ml) either for five half-minute periods with half-minute pauses, or until the acetone powder was well dispersed. This preparation was centrifuged for 30 min at 13 500 rpm in a Servall SS centrifuge, and the supernatant was centrifuged for 1 h at 30 000 rpm in a Spinco centrifuge. All centrifugation was done below 4°C. The solubles from the 30 000-rpm centrifugation were dialyzed three times against 0.02 *M*, pH 7.0, phosphate buffer, with each buffer change containing greater than 100-fold the sample volume.

Enzyme assay was performed in a 12-ml centrifuge tube containing the following: 1.00 ml of protein solution (8–10 mg of biuret protein); 0.10 ml of 0.02 *M*, pH 7.0, phosphate buffer (containing as needed a variety of test compounds); and 0.10 ml of pH 3.5 sulfuric acid (0.005 *N*) containing 100 nmoles of [$^{14}CH_3$]*S*-adenosyl-L-methionine (18.4×10^3 dpm). These components were mixed at 0°C, then incubated at 28°C in a water bath for 30–60 min. The incubation was terminated and the proteins precipitated with 1 drop of concentrated hydrochloric acid. The acidified incubation solution was extracted four times with a total of 20 vol/vol of diethyl ether; a clinical centrifuge was used to break the ether–water emulsion. The combined ether layers were dried over anhydrous magnesium sulfate for several hours, then filtered through a tightly packed glass or cotton wool plug. The filtrate was evaporated in a stream of nitrogen, and assayed for radioactivity and stipitatic acid (absorbance at 270 nm).

Proteins were determined according to Gornall et al. (*21*) except for fractions from Sephadex columns when a modified, small-scale Lowry technique was used. Two

scintillator cocktails were used; either 4.0 g PPO (2,5-diphenyloxazole), 0.05 g POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene), to 1 liter of toluene, or 60.0 g naphthalene, 4.0 g PPO, 0.2 g POPOP, 100 ml methanol, 20 ml ethylene glycol, a 1 liter of 1,4-dioxane (for aqueous solutions). Sephadex chromatography was carried out under the usual conditions: blue dextran, equine apo-ferritin, lactic acid dehydrogenase, and bovine serum albumin were used as standards. Each protein was detected by the procedures of Andrews (*22*).

RESULTS

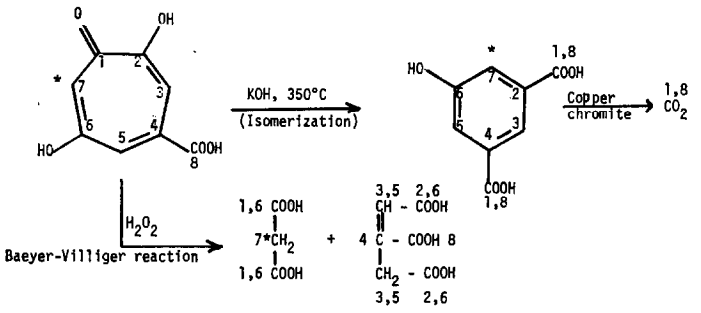
Since the substrate for methylation in tropolone biosynthesis had not been identified, it seemed logical to search for a preparation which would incorporate activity from the commercially available [$^{14}CH_3$]*S*-adenosyl-L-methionine into tropolones. Attempts to obtain active preparations from 7-day-old surface cultures of *P. stipitatum* by the following techniques were unsuccessful; grinding in a mortar either alone or with glass beads, freeze–thaw cycling followed by mortar grinding with glass beads, homogenization in a Blendor, use of ultrasonic vibrations. Only the use of acetone powder preparations (see Experimental) gave consistent results.

The tropolone nature of the product from enzymatic experiments was established as follows. In a large-scale run, 180 ml of nondialyzed extract in 0.01 *M*, pH 7.0, phosphate buffer was incubated with 5 μCi of [$^{14}CH_3$]*S*-adenosyl-L-methionine for 3 h at 28°C. After acidification to pH 3.0 (concd HCl) and extraction with ether, it was possible to isolate stipitatic acid without addition of carrier. The specific activity of the stipitatic acid after five recrystallizations from water was 1.1 μCi/mmole, and thereafter it remained constant (1.0 μCi/mmole). The stipitatic acid was examined chromatographically with radioactivity being determined on a Vanguard 880 Autoscanner or Packard 7021 Radiochromatogram scanner and the tropolone being detected by ferric chloride spray and examination in ultraviolet light. In the following systems (all solvent compositions are v/v) there was only a single peak of radioactivity and the R_f value of the sample spot (first number quoted) agreed with the R_f value of the radioactive material (second number quoted): Whatman No. 4 paper, butanol :acetic acid : water, 4 : 1 : 1, $R_f = 0.63, 0.61$; thin layer, silica gel, butanol : acetic acid : water, 17 : 2 : 1, $R_f = 0.65, 0.63$; thin layer, silica gel, chloroform : acetic acid, 19 : 1, $R_f = 0.05, 0.05$. Furthermore, after addition of carrier stipitatic acid, recrystallization, and preparation of the diacetyl derivative, radioactivity on chromatography was likewise associated only with the diacetylstipitatic acid spot: Whatman No. 4 paper, butanol : acetic acid : water, 45 : 1 : 4, $R_f = 0.70, 0.68$; thin layer, silica gel, chloroform : acetic acid, 19 : 1, $R_f = 0.50, 0.45$.

The stipitatic acid labeled by incubation of [$^{14}CH_3$]*S*-adenosyl-L-methionine and the enzyme extract, was degraded in separate experiments, either by isomerization to 5-hydroxyisophthalic acid or, by treatment with H_2O_2, to a mixture of malonic and aconitic acids (*11*). Since C-1 of stipitatic acid is extruded to become one of the carboxyl groups of the phthalate (*8*), and since the two carboxyl groups were found not to be radioactive (see Table 1) neither C-1 nor C-8 of stipitatic acid had become labeled. In the peroxide degradation almost all of the radioactivity in stipitatic acid was associated

TABLE 1

DEGRADATION OF ENZYMATICALLY SYNTHESIZED STIPITATIC ACID



Reaction and products	Specific activity (μCi/mmole × 100)	% specific activity
Isomerization		
Stipitatic acid	1.5	100
5-Hydroxyisophthalic acid[a]	1.5	100
CO_2 from 5-hydroxyisophthalic acid	0.0018	1
Baeyer–Villiger		
Stipitatic acid	1.7	100
Malonic acid[b]	0.75	43
Aconitic acid	0.01	1

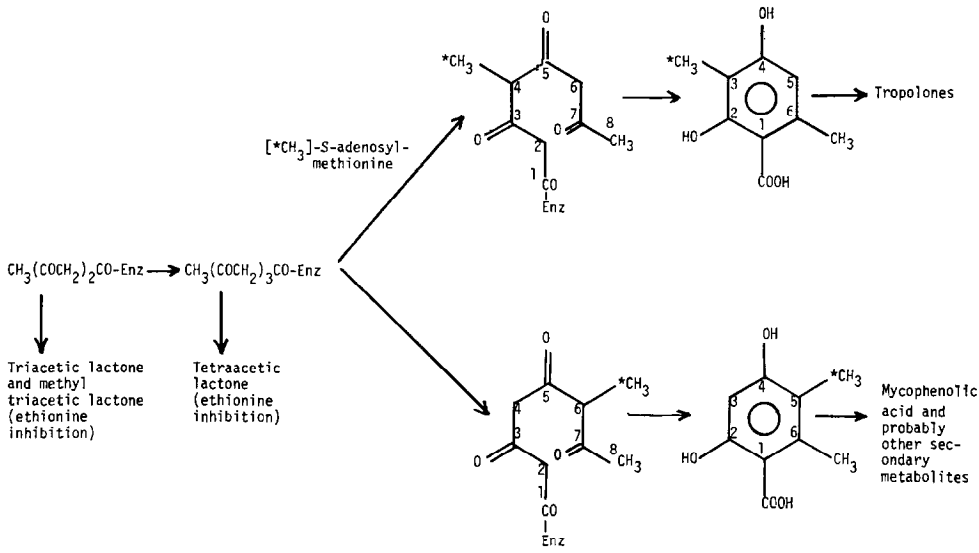
[a] This material was twice recrystallized from water, then sublimed.

[b] Aconitic acid and malonic acid were isolated from this peroxidation reaction by chromatography on acidified Celite, using first butanol:chloroform; 13:87, and then diethyl ether. In both cases the solvents were pre-equilibrated with 0.5 *N* sulfuric acid before use.

with the malonic acid fragment. Malonic acid in this reaction is derived from C-1, -6, and -7 of stipitatic acid.[2] Since C-1 can be eliminated as the labeled carbon, the radioactivity must be at C-6 and/or C-7. In addition to malonic acid, the peroxidation also yielded aconitic acid. One of the carboxyl groups of this aconitic acid is derived from C-6 of stipitatic acid. Because aconitic acid was virtually inactive, C-6 must be devoid of radioactivity; therefore, C-7 must be the only carbon of stipitatic acid labeled by incubation of [$^{14}CH_3$]*S*-adenosyl-L-methionine with the cell-free extracts.

The standard enzyme assay took advantage of the fact that the ether extract of the enzyme incubations contained radioactivity almost exclusively as stipitatic acid. With this assumption, and a second assumption that stipitatic acid was the only compound in the ether extract with an absorption at 270 nm in the ultraviolet, a rapid assay was possible at the sacrifice of little accuracy. At this wavelength, there was a linear and

[2] The malonate obtained from the peroxidation reaction had only 43% of the specific activity of the stipitatic acid from which it was derived. However, similar results were found earlier, and were attributed to malonate being partly derived from carbons other than C-1, -6, and -7 of stipitatic acid (*23*).



SCHEME 1.

sensitive response over the range of 1–10 mg stipitatic acid. The extinction coefficient, E_{270}, was calculated to be 2.3×10^4 liter/cm/mole. The incubation and ether-extraction procedure described in the Methods section, when run on boiled-enzyme blanks, gave assays of 15–30 cpm above background, and assays of 1500–2500 cpm above background for active preparations. Using this assay, the reaction was found to be linear with time for at least 40 min (see Fig. 1).

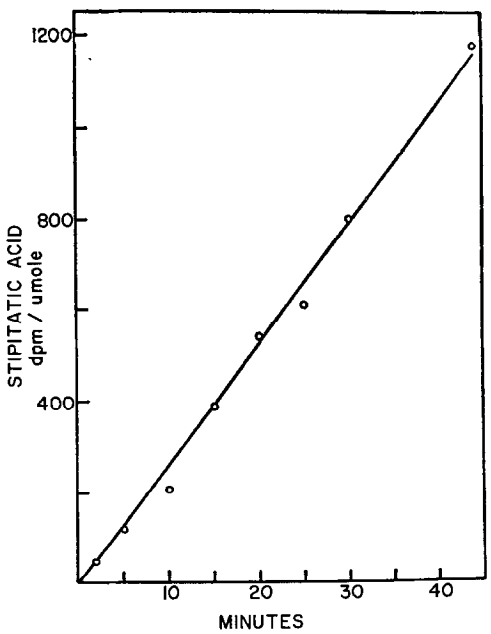


FIG. 1. Enzymatic synthesis of stipitatic acid as a function of time. The standard assay conditions were used.

A convenient enzyme unit was defined as the amount of enzyme converting 1 nmole of [$^{14}CH_3$]*S*-adenosyl-L-methionine to ether-extractable compounds in 30 min. From the data shown in Fig. 1 it was calculated that approximately 10% of the added radioactivity (i.e., approximately 10 nmoles of *S*-adenosyl-L-methionine) was converted to ether solubles (i.e., stipitatic acid) in 30 min. Because approximately 10 mg of biuret protein carried out this conversion, a typical enzyme extract contained approximately 1 unit of activity per milligram of biuret protein.

In a study of the pH optimum, a broad plateau of maximum activity was obtained between pH 6.9 and 7.9. At both extremes the incorporated ^{14}C was shown chromatographically (tlc in chloroform : acetic acid, 19 : 1, $R_f = 0.05$; and 2-butanol : water : acetic acid 17 : 2 : 1, $R_f = 0.65$) to be associated with standard stipitatic acid. All subsequent work was carried out at pH 6.9. As far as buffer composition and concentration were concerned, Tris buffer was not better than phosphate and 0.02 *M* phosphate buffer gave maximal methylation activity.

The enzyme system was heat labile; a very rapid loss of methylation activity occurred from 40°C on and total inactivation occurred with a 5-min preincubation at or above 55°C. Exogenous *S*-adenosyl-L-methionine did not affect the heat lability.

When chromatographed on Sephadex G-100 the enzymatically active fraction followed just behind the void volume; on Sephadex G-200 (elution with 0.02 *M* pH 7.0 phosphate buffer) the active fraction was eluted between bovine serum albumin monomer and lactic acid dehydrogenase; from the plot of elution volume vs logarithm of molecular weight a value of about 105 000 was estimated.

Enzyme preparations dialyzed against a large volume of 0.2 *M*, pH 7.0, phosphate buffer containing 1 m*M* Versene EDTA were stimulated 2- to 3-fold by addition of 0.1 ml of 0.01 *M* solutions of cobalt, magnesium, and manganese chlorides (decreasing order of activity). Iron had little effect. Furthermore, addition of $NADP^+$, NADH, and ATP had little or no effect on the system.

It was never possible to identify the acceptor for the transferred methyl group. It appeared to be an enzyme-bound material since extensive dialysis and Sephadex chromatography did not lead to loss of tropolone synthesis. Likewise, any acceptor was not removed by EDTA or by treatment with $NaBH_4$. The following compounds added to the usual assay system showed activity between 80 and 120% of that with water alone: 6-methylsalicylic acid, orsellinic acid, 2,6-dihydroxy-4-methylbenzoic acid, 3,5-dihydroxyphenylacetic acid, diacetylacetone, 3,5,7-trioxo-7-phenylheptanoic acid, stipitatic acid, tetraacetic acid (3,5,7-trioxooctanoic acid). No evidence for the formation of stipitatonic acid was obtained so the system is presumed to contain an active stipitatonic acid decarboxylase (*24*).

DISCUSSION

In 1962, Tanenbaum and Bassett described the preparation of a cell-free extract from *P. stipitatum* which was very active in converting [1,3-$^{14}C_2$]malonyl CoA into stipitatic acid (*19*). The organism was grown on a modified Czapek Dox medium (containing ammonium ion) and the initial homogenate from a Blendor was stood overnight at 0°C at pH 8.5. The system was assayed by use of paper chromatography and radioactivity determination; in one experiment using [2-^{14}C]diethyl malonate as precursor,

carrier stipitatic acid was added to the eluant from the final chromatogram and the mixture was recrystallized[3] to constant specific activity. An isotopic conversion factor f, was defined as

$$\frac{\text{dpm stipitatic acid isolated} \times 10^5}{\text{dpm added precursor}}.$$

The best precursor was [1,3-$^{14}C_2$]malonyl CoA for which $f = 17$. The two 1-carbon donors tested in this system were as follows: formate, $f = 0.015$,[4] and [$^{14}CH_3$]methionine, $f = 0.32$. Although these factors are arbitrary and do not represent actual conversions, it is clear that the system was much less efficient in incorporating activity from these 1-carbon units than from the polyketide-forming units. The *S*-adenosyl derivative of L-methionine was not examined.

In the present work, exhaustively dialyzed enzyme preparations yielded radioactive stipitatic acid by addition of [$^{14}CH_3$]*S*-adenosyl-L-methionine without any other substrate, and the presence of potential acceptors for the methyl group had little or no effect on the system. These findings invite comparison with the C-methylating enzyme of Gatenbeck et al. (*25*). From *Aspergillus flaviceps* (sic—probably *flavipes* is meant) these workers isolated an enzyme preparation incorporating activity from added *S*-adenosyl-L-methionine into 5-methylorsellinic acid (isolated as the decarboxylated product, 5,6-dimethylresorcinol). No significant difference was noted in experiments with and without added orsellinic acid. Furthermore, tentative evidence was obtained to indicate that the methyl acceptor was an enzyme-bound derivative of tetraacetic acid. Methylation was thus regarded as necessarily preceding cyclization. Similarly, in our system, preliminary evidence was also obtained that activity from labeled acetic acid was incorporated into the protein fraction which was enzymatically active, suggesting the presence of enzyme-bound polyketide.

All of the prior evidence and the properties of the enzyme extract are consistent with methylation at the prearomatic level as an early step in tropolone biosynthesis. It appears probable that in *P. stipitatum* the enzyme system directs the incoming methyl group to C-4 of tetraacetic acid forming 3-methylorsellinic acid; in the *A. flaviceps* enzyme preparation the methyl is directed to C-6 of tetraacetic acid (see Scheme 1). As a result of the isolation of methyltriacetic acid lactone from *P. stipitatum* Tannenbaum and his colleagues had suggested methylation of a 6-carbon polyketide rather than the intrinsically more likely 8-carbon product (*13*, *14*). This isolation, however, was achieved from cultures grown under rather unusual conditions—namely, the use of a special basal inorganic salt mixture in place of the normal iron component of Czapek-Dox medium (*26*), and with growth at an elevated temperature (37°C rather than 28°C used by other workers). With ethionine-inhibited cultures, small amounts of methyltriacetic acid lactone are also formed, along with larger amounts of triacetic acid lactone

[3] Individual incubations utilized very high levels of activity. In this particular experiment, 5×10^8 dpm (i.e., 227 μCi) were employed in a 10-ml incubation volume.

[4] Although as defined, f is a ratio of total activity recovered to total activity added, (i.e., a measure of overall conversion) the figures given in Table 1 of Ref. *19* appear to have been calculated from

$$\frac{\text{dpm}/\mu\text{mole stipitatic acid} \times 10^5}{\text{dpm added precursor}}$$

There are apparent errors in this Table for calculating f for formate and acetyl CoA.

and tetraacetic acid lactone (*27*). Thus, these three lactones may be regarded as accumulating when the usual pathway (presumably methyl addition to an 8-carbon polyketide) is subject to interference.

Both 3- and 5-methylorsellinic acid have been implicated as precursors to other secondary metabolites, with methylation occurring at the prearomatic level, by experiments with intact organisms. In the lichen, *Parmelia tinctorum*, tritiated 3-methylorsellinic acid but not tritiated orsellinic acid is incorporated into lecanoric acid (*28*). 5-Methylorsellinic acid, but not orsellinic acid functions as a precursor to mycophenolic acid in *Penicillium brevicompactum* (*29*, *30*). Similarly, [1′-^{14}C]2,4,6-trihydroxy-3-methylacetophenone but not [1′-^{14}C]2,4,6-trihydroxyacetophenone was incorporated into usnic acid by the lichens, *Cladonia mitis* and *Parmelia caperata* (*31*). In *Aspergillus clavatus*, neither [1′-^{14}C]2,4-dihydroxyacetophenone nor [1′-^{14}C]2,4-dihydroxy-3-methylacetophenone was converted to clavatol although both were metabolized by the organism (*32*).

While the prearomatic methylation may be an important feature in many cases of secondary metabolism, aromatic methylation (albeit of shikimate-derived units) appears well established for members of the ubiquinone and menaquinone series (*33*). Methylation reactions in steroids and triterpenes also appear to occur after the basic isoprene skeleton has been assembled (*34*). In several instances the available evidence is insufficient or contradictory. In *Aspergillus flavipes*, both orsellinic and 5-methylorsellinic acids were converted to flavipin [and without degradation to acetate] (*35*). Hence, methylation of added orsellinic acid was presumed to occur, but it is not clear whether the "normal" biosynthetic sequence involves methylation before or after cyclization of the polyketide. Both 5-methylorsellinic acid and orsellinic acid itself were isolated from wild-type and mutant strains of *Aspergillus terreus* I.M.I. 16043 (*36*); in view of the co-occurrence of both acids, methylation after cyclization was postulated but prearomatic methylation was not ruled out. [It is remarkable that another, unspecified strain of this same organism produces the 3-methyl derivative (*37*).] 5-Methylorsellinic acid was occasionally isolated during work with *Gliocladium roseum* and was thought to be derived from orsellinic acid (*38*); other work in this same organism indicates methylation at the polyketide level (*39*). In replacement cultures of *Streptomyces rimosus*, 2,4-dihydroxyacetophenone and 2,4-dihydroxy-3-methylacetophenone were further methylated to clavatol, although the conditions were not readily reproducible (*32*). In this organism, the only compounds known to be produced by C-methylation are the tetracyclines; in other Streptomycetes, however, it is accepted that in tetracycline formation, methylation occurs at a prearomatic stage (*40*).

ACKNOWLEDGEMENTS

We are grateful to Dr. T. M. Harris, Chemistry Department, Vanderbilt University, for samples of 3,5,7-trioxooctanoic acid and 7-phenyl-3,5,7-trioxoheptanoic acid. Radioactive *S*-adenosyl-L-methionine was obtained commercially.

REFERENCES

1. W. B. TURNER, "Fungal Metabolites," pp. 111, 134. Academic Press, New York, 1971.
2. A. I. SCOTT AND E. LEE, *J. Chem. Soc. Chem. Commun.*, 655 (1972).

3. R. ROBINSON, *Chem. Ind. (London)*, **12** (1951).
4. T. R. SESHADRI, *J. Sci. Ind. Res. (India)*, **14B**, 248 (1955).
5. J. H. RICHARDS AND L. D. FERRETTI, *Biochem. Biophys. Res. Commun.*, **2**, 107 (1960).
6. L. D. FERRETTI AND J. H. RICHARDS, *Proc. Nat. Acad. Sci.* USA, **46**, 1438 (1960).
7. R. BENTLEY, *Biochem. Biophys. Res. Commun.*, **3**, 215 (1960).
8. I. G. ANDREW AND W. SEGAL, *J. Chem. Soc.*, 607 (1964).
9. A. I. SCOTT, H. GUILFORD, AND E. LEE, *J. Amer. Chem. Soc.*, **93**, 3534 (1971).
10. A. I. SCOTT AND K. J. WIESNER, *J. Chem. Soc. Chem. Commun.*, 1075 (1972).
11. R. BENTLEY, *J. Biol. Chem.*, **238**, 1895 (1963).
12. R. BENTLEY AND P. M. ZWITKOWITS, *J. Amer. Chem. Soc.*, **89**, 681 (1967).
13. T. E. ACKER, P. E. BRENNEISEN, AND S. W. TANENBAUM, *J. Amer. Chem. Soc.*, **88**, 834 (1966).
14. G. S. MARX AND S. W. TANENBAUM, *J. Amer. Chem. Soc.*, **90**, 5302 (1968).
15. R. W. BRYANT, JR. AND R. J. LIGHT, *Biochemistry*, **13**, 1516 (1974).
16. R. BENTLEY, *Biochim. Biophys. Acta*, **29**, 666 (1958).
17. A. G. MCINNES, D. G. SMITH, L. C. VINING, AND J. L. C. WRIGHT, *Chem. Commun.*, 1669 (1968).
18. J. WRIGHT, D. G. SMITH, A. G. MCINNES, L. C. VINING, AND D. W. S. WESTLAKE, *Can. J. Biochem.*, **47**, 945 (1969).
19. S. W. TANENBAUM AND E. W. BASSETT, *Biochim. Biophys. Acta*, **59**, 524 (1962).
20. R. BENTLEY AND C. P. THIESSEN, *J. Biol. Chem.*, **238**, 1880 (1963).
21. A. G. GORNALL, C. J. BARDAWILL, AND M. M. DAVID, *J. Biol. Chem.*, **177**, 751 (1949).
22. P. ANDREWS, *Biochem. J.*, **96**, 595 (1965).
23. R. BENTLEY, *J. Biol. Chem.*, **238**, 1889 (1963).
24. R. BENTLEY AND C. P. THIESSEN, *J. Biol. Chem.*, **238**, 3811 (1963).
25. S. GATENBECK, P. O. ERIKSSON, AND Y. HANSSON, *Acta Chem. Scand.*, **23**, 699 (1969).
26. P. V. DIVEKAR, P. E. BRENNEISEN, AND S. W. TANENBAUM, *Biochim. Biophys. Acta*, **50**, 588 (1961).
27. R. BENTLEY AND P. M. ZWITKOWITS, *J. Amer. Chem. Soc.*, **89**, 676 (1967).
28. M. YAMAZAKI AND S. SHIBATA, *Chem. Pharm. Bull. (Tokyo)*, **14**, 96 (1966).
29. L. CANONICA, W. KROSZCZYNSKI, B. M. RANZI, B. RINDONE, AND C. SCOLASTICO, *Chem. Commun.*, 257 (1971).
30. C. T. BEDFORD, J. C. FAIRLIE, P. KNITTEL, T. MONEY, AND G. T. PHILLIPS, *Chem. Commun.*, 323 (1971).
31. H. TAGUCHI, U. SANKAWA, AND S. SHIBATA, *Chem. Pharm. Bull. (Tokyo)*, **19**, 2054 (1969).
32. S. GATENBECK AND U. BRUNSBERG, *Acta Chem. Scand.*, **20**, 2334 (1966).
33. R. BENTLEY, in "Lipid Metabolism" (S. J. WAKIL, Ed.), Academic Press, New York, p. 481 (1970).
34. R. BENTLEY AND I. M. CAMPBELL, in "Comprehensive Biochemistry" (M. Florkin and E. H. Stotz, Eds.), Elsevier, Amsterdam, p. 415 (1968).
35. G. PETTERSSON, *Acta Chem. Scand.*, **19**, 1724 (1965).
36. R. F. CURTIS, P. C. HARRIES, C. H. HASSALL, AND J. D. LEVI, *Biochem. J.*, **90**, 43 (1964).
37. S. TAKENAKA, N. OJIMA, AND S. SETO, *J. Chem. Soc. Chem. Commun.*, 391 (1972).
38. G. PETTERSSON, *Acta Chem. Scand.*, **19**, 414 (1965).
39. M. W. STEWARD AND N. M. PACKTER, *Biochem. J.*, **109**, 1 (1968).
40. J. R. D. MCCORMICK, E. R. JENSEN, N. H. ARNOLD, H. S. COREY, U. H. JOACHIM, S. JOHNSON, P. A. MILLER, AND N. O. SJOLANDER, *J. Amer. Chem. Soc.*, **90**, 7127 (1968).