A NEW FAMILY OF TETRACYCLINE PRECURSORS: N-DEMETHYLANHYDROTETRACYCLINES

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Although considerable progress has been made in the elucidation of the pathway of tetracycline biosynthesis using tracer methods or by the demonstration of precursor activity for a variety of tetracycline degradation products (McCormick et al., 1962, 1963), previously only one intermediate, 7-chloro-5a(11a)-dehydrotetracycline (McCormick et al., 1958), has been isolated from either tetracycline-producing strains or blocked mutants of Streptomyces aureofaciens or S. rimosus.

We wish to report that a group of N-demethylanhydrotetracyclines have been isolated from a variety of S. aureofaciens and S. rimosus strains grown in media containing antimetabolites of compounds involved in biological methylation reactions. These precursors were detected in the soluble fraction of sonic extracts obtained from a 7-chlorotetracycline (CTC)-producing strain, S. aureofaciens BC-41* grown in a corn steep liquor-sucrose medium containing L-ethionine. It was found that incubation of such a cell-free extract with C^{14}-methylmethionine, ATP and Mg^{++}, resulted in the incorporation of label into CTC. A similar incorporation was observed in a cell-free extract from a blocked mutant, S. aureofaciens S-2242**, but only upon addition of a solvent extract of S. aureofaciens BC-41 mycelium (Fig. 1).

*This strain was described by Doerschuk et al., 1959.

**This mutant was described by McCormick et al., 1960.

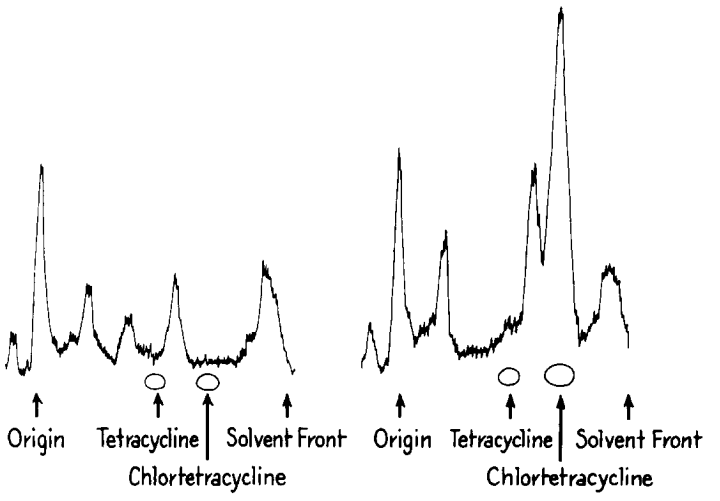


Figure 1. Synthesis of CTC-C^{14} from demethyl precursor in a sonic extract of S. aureofaciens S-2242. The incubation mixtures contained per ml.: 6 mg lyophilized S-2242 extract; (in μ moles), pH 7.0 Tris, 100; ATP, 1.0; Mg^{++}, 7.4; NADP, 0.10; G-6-P, 1.5; L-methionine methyl-C^{14}, 0.05. In addition, a fraction obtained by solvent extraction of BC-41 mycelium was included in the incubation mixture corresponding to the radioscan shown on the right. After incubation at 28°C for 2 hours, the proteins were precipitated with perchloric acid. The resulting supernatants were extracted with o-chlorophenol, the solvent removed by air evaporation and the residue paper chromatographed in a n-butanol/EDTA system buffered at pH 7.0. The chromatograms were scanned for radioactivity.

The BC-41 factor required for incorporation of label into CTC by S-2242 cell-free extracts appeared to be a precursor rather than a cofactor inasmuch as the incorporation could be directed into other tetracycline antibiotics when solvent extracts from other strains were substituted for the BC-41 factor. For example, using the same S-2242 cell-free extract, solvent extracts from tetracycline (TC), 7-chloro-6-demethyltetracycline (6-DMCTC) and CTC-producing strains directed the incorporation of label into TC, 6-DMCTC and CTC respectively.

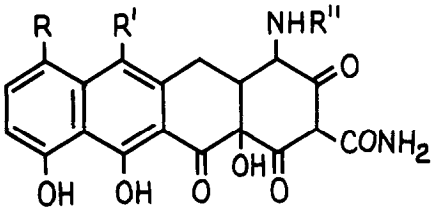
The label incorporated into CTC was found by alkaline degradation studies (Pasternack et al., 1952) to be located exclusively in the dimethylamino carbons. The synthesis of C^{14}-CTC was not affected by 6×10^{-5}M 2-(2-furyl)-5-mercapto-1,3,5-oxadiazole, a chlorination inhibitor (Goodman et al., 1959). These facts, together with the

observed synthesis of TC, 6-DMCTC and CTC in the same cell-free extract, permitted several conclusions to be drawn regarding the structure of the CTC precursor. This precursor, like CTC, contains chlorine and C-methyl. However, at least one N-methyl group is lacking.

Sulfadiazine, L- and D-ethionine and a variety of methionine and homocysteine analogs were found to cause the accumulation of precursors. The choice of inhibitor and the level and time of addition for maximum precursor yields varies according to the particular culture used. For purposes of illustration we can consider the accumulation of precursor by S. rimosus T-1686B.* A synthetic medium similar to that described by McCormick et al. (1959) but containing 0.1 g/l l-histidine, 0.24 g/l phosphoric acid and no methionine was inoculated and then incubated on a rotary shaker for 48 hours at 28°C. After the addition of L-ethionine (80 μg/ml) the fermentation was continued for an additional 48 hours. The total precursor concentration was estimated at 500 μg/ml by absorbancy measurements at 430 mμ using 5a,6-anhydrotetracycline as a standard. The precursors were isolated by procedures involving solvent extraction, precipitation at pH 6.5, silica gel and cellulose column chromatography, and countercurrent distribution.

Using these procedures four demethyl precursors (III-VI) have been isolated in analytical purity from strain T-1686B. The ultraviolet spectra of precursors III and IV are closely related to that of 5a,6-anhydrotetracycline while those of V and VI closely parallel that of 6-demethyl-5a,6-anhydrotetracycline. The pairs of 4-amino (III and V) and 4-methylamino (IV and VI) precursors could be distinguished by microanalyses, N-methyl determinations and characterization of the volatile amines liberated by alkaline degradation (Pasternack et al., 1952).

*A Lederle isolate identified by Dr. H. D. Tresner.



(I) R = Cl, R' = CH_3, R'' = H
(II) R = Cl, R' = R'' = CH_3
(III) R = R'' = H, R' = CH_3
(IV) R = H, R' = R'' = CH_3
(V) R = R' = R'' = H
(VI) R = R' = H, R'' = CH_3
(VII) R = Cl, R' = R'' = H
(VIII) R = Cl, R' = H, R'' = CH_3

Figure 2. Tetracycline precursors elaborated in the presence of L-ethionine.

Precursors III and IV are converted to 5a,6-anhydrotetracycline in an S-2242 cell-free system containing S-adenosylmethionine (AMe) or by reaction with methyl iodide in tetrahydrofuran. Using the same techniques, precursors V and VI gave rise to 6-demethyl-5a,6-anhydrotetracycline.

Precursors V-VIII were isolated from S. aureofaciens ED-3636*, a strain which elaborates tetracycline antibiotics lacking C_6-methyl groups. Precursors V and VI were identified by direct comparison with the materials isolated from S. rimosus strain T-1686B. Precursors VII and VIII were spectroscopically related to 7-chloro-6-demethyl-5a,6-anhydrotetracycline to which they were converted by reaction with methyl iodide. The nature of the C_4 substituent was revealed by N-methyl analyses and volatile amine determinations.

Thus the structures of these precursors have been established from their physicochemical properties, suitable degradation reactions, and by biological and chemical conversion to compounds whose structures are unambiguously known.

*Obtained from the Chemical Production Section, Lederle Laboratories.

The biological reactions involved in the conversion of these precursors to the corresponding tetracycline antibiotics can be illustrated by the biosynthesis of tetracycline from 4-dedimethylamino-4-amino-5a,6-anhydrotetracycline (III). The first reactions, stepwise methylation of III, lead to 5a,6-anhydrotetracycline (IX) via 4-dedimethylamino-4-methylamino-5a,6-anhydrotetracycline (IV). These reactions require only AMe as cofactor. Interestingly, ethionine levels up to 2.7 mM have no effect on either the activation of methionine or the transfer of methyl from S-adenosylmethionine to either III or IV by the S-2242 extract. The action of ethionine in causing precursor accumulation may therefore involve interference with methionine synthesis.

The oxidation of IX at C_6 requires NADPH and oxygen. No evidence for a free hydroperoxide intermediate (Scott and Bedford, 1962) was obtained. The final reaction, reduction of the 5a,11a double bond, is an NADPH-dependent reaction. No requirement for Cosynthetic Factor I (Miller et al., 1960) could be demonstrated in this reaction.

The strict order in which these reactions occur suggests that neither 6-deoxy- nor N-demethyltetracycline antibiotics are likely to be found as fermentation products. The synthesis of 6-demethyl intermediates by S. rimosus T-1686B shows that the inability of S. rimosus to convert 6-demethyl-5a,6-anhydrotetracycline to 6-demethyl-5-hydroxytetracycline (McCormick et al., 1962) is responsible for the nonsynthesis of 6-demethyltetracycline antibiotics by S. rimosus (Zygmunt, 1962) under conditions favoring their synthesis by S. aureofaciens (Goodman and Miller, 1962; Hendlin et al., 1962; Neidleman et al., 1963).

The preparation of C^{14}-labeled tetracyclines at high specific activities has heretofore not been possible. Specifically labeled tetracyclines could be synthesized from the N-demethylanhydrotetracyclines at activities limited only by the activity of the methyl iodide available.

The N-demethylanhydrotetracyclines may also prove valuable as chemical intermediates in the synthesis of new tetracycline antibiotics.

It is now clear that the C_4-nitrogen and the N-methyls of the tetracyclines are incorporated in separate reactions rather than as a preformed dimethylamino unit. Methylation of the anhydrotetracycline amine is similar to other biological methylations. A summary of the terminal reactions in tetracycline biosynthesis is shown in Figure 3.

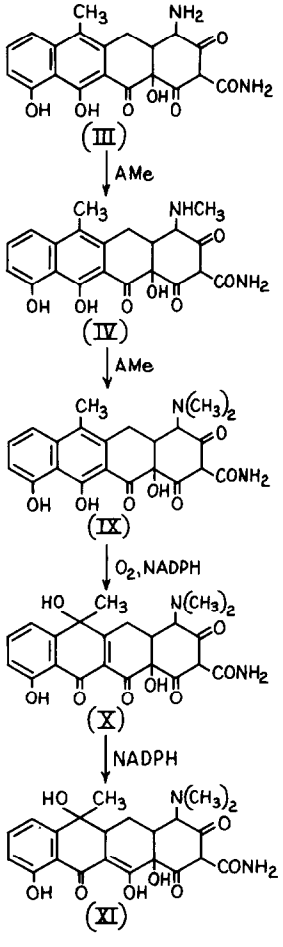


Figure 3. Biological conversion of 4-dedimethylamino-4-amino-5a,6-anhydrotetracycline (III) to tetracycline (XI).

Acknowledgements

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References

Doerschuk, A. P., McCormick, J. R. D., Goodman, J., Szumski, S. A., Growich, J. A., Miller, P. A., Bitler, B. A., Jensen, E. R., Matrishin, M., Petty, M. A. and Phelps, A. S., J. Am. Chem. Soc. 81, 3069 (1959).

Goodman, J. J., Matrishin, M., Young, R. W. and McCormick, J. R. D., J. Bact. 78, 492 (1959).

Goodman, J. J. and Miller, P. A., Biotechnol. Bioeng. 4, 391 (1962).

Hendlin, D., Dulaney, E. L., Dresher, D., Cook, T. and Chaiet, L., Biochim. Biophys. Acta 58, 635 (1962).

McCormick, J. R. D., Johnson, S., Sjolander, N. O., J. Am. Chem. Soc. 85, 1692 (1963).

McCormick, J. R. D., Miller, P. A., Johnson, S., Arnold, N., Sjolander, N. O., J. Am. Chem. Soc. 80, 5572 (1958).

McCormick, J. R. D., Hirsch, U., Sjolander, N. O. and Doerschuk, A. P., J. Am. Chem. Soc. 82, 5006 (1960).

McCormick, J. R. D., Sjolander, N. O., Johnson, S. and Doerschuk, A. P., J. Bact. 77, 475 (1959).

Miller, P. A., Sjolander, N. O., Nalesnyk, S., Arnold, N., Johnson, S., Doerschuk, A. P. and McCormick, J. R. D., J. Am. Chem. Soc. 82, 5002 (1960).

Neidleman, S. L., Bienstock, E. and Bennett, R. E., Biochim. Biophys. Acta 71, 199 (1963).

Pasternack, R., Bavley, A., Wagner, R. L., Hochstein, F. A., Regna, P. P., and Brunings, K. J., J. Am. Chem. Soc. 74, 1926 (1952).

Scott, A. I. and Bedford, C. T., J. Am. Chem. Soc. 84, 2271 (1962).

Zygmunt, W., J. Bact. 84, 1126 (1962).