THE EFFECT OF METHYLATION INHIBITORS ON CITREAMICIN BIOSYNTHESIS IN *Micromonospora citrea*

CEDRIC J. PEARCE*, GUY T. CARTER*, JEANNE A. NIETSCHE, DONALD B. BORDERS,
MICHAEL GREENSTEIN and WILLIAM M. MAIESE

Medical Research Division, American Cyanamid Company, Lederle Laboratories,
Pearl River, New York 10965, U.S.A.

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When the citreamicin-producing organism *Micromonospora citrea* NRRL 189351 was incubated in the presence of the methylation inhibitors sinefungin or aminopterin, biosynthesis of the ζ component was stimulated approximately 20 to 200-fold above the level normally produced. Inhibition of a second methylation reaction, which is superficially very similar to the first, was not detected. Other known methylation inhibitors failed to yield any change in the natural pattern of citreamicins produced. This approach is an excellent route for preparing citreamicin ζ, which can be used as a substrate for semi-synthesis or for further biosynthetic studies.

The citreamicins (Fig. 1, 1~5)1,2 are polycyclic aromatic antibiotics that are produced by *Micromonospora citrea* and are active against a variety of Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. All of the citreamicins identified from cultures of *M. citrea* share a common xanthone nucleus but differ in the extent of further modifications; the ζ component (Fig. 1, 4), for example, contains only one methoxy group on the G-ring.

Xanthones in general comprise a variety of plant, fungal and bacterial secondary products, and compounds structurally related to the citreamicins include simaomicin3), lysolipin4), cervinomycin5), and actinoplanone6). The biosynthesis of xanthones has been shown to be an unpredictable process. Evidence suggests that the citreamicins are acetate-derived polyketides formed from an extensively modified single chain7). In contrast, the related antibiotic simaomicin is derived from acetate via a relatively straightforward mechanism, also involving a single polyketide chain8). The fungal metabolites taji-xanthone and shamixanthone9), sterigmatocystin10), and ravenelin11) have also been reported to be derived from single acetate-derived polyketide precursors. However, in the case of higher plant xanthones, e.g., the Gentianeceae-xanthones12), mangostin13) and mangiferin14), evidence has been obtained suggesting the involvement of a polyketide with a shikimate-derived starter unit.

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This communication addresses the methylation steps involved in the biosynthesis of citreomycin. Both O-methyl groups of the α component have been shown to be derived from methionine\textsuperscript{7).} Because we are interested in exploiting all semi-synthetic routes to novel products with improved activity, we have a requirement for as wide a variety of starting materials as possible, including the mono-methylated ζ and the dihydroxy component. A biosynthetic solution to this problem is to inhibit these methylation reactions using one of the known methylation inhibitors. Herein, we report the effects of sinefungin, aminopterin, amethopterin, DL-ethionine and L-methionine on the methylation reactions leading to the citreomycins.

**Materials and Methods**

**Culture of M. citrea**

*M. citrea* was grown in 100 ml of a medium containing (in g/liter): yeast extract 5, NZ Amine 5, dextrin 20, glucose 10 and CaCO\textsubscript{3} 1, in a 500-ml Erlenmeyer flask at 30°C and 200 rpm on a rotary shaker for 3 days, and was preserved in a frozen state at -75°C following the addition of an equal volume of glycerol. Approximately 1.5 ml of this frozen culture was used to inoculate 100 ml of a similar seed medium, which was then incubated at 30°C and 200 rpm on a rotary shaker. After 3 days, this culture was added at a rate of 5% to a production medium consisting of either (in g/liter): (A) dextrin 12.5, sucrose 12.5, soya peptone 10 and CaCO\textsubscript{3} 3.5, or (B) dextrin 25, Bacto-Peptone 5, CaCO\textsubscript{3} 1, sucrose 12.5 and ferric ammonium citrate 0.05. Following a further 5 days incubation on a rotary shaker at 140 rpm and 30°C, citreomycin analysis was carried out.

**Addition of Inhibitors**

Sinefungin, aminopterin, amethopterin, DL-ethionine and L-methionine were obtained from Sigma Chemical Company. Sinefungin was dissolved in sterile water, aminopterin and (+)-amethopterin (methotrexate) in sterile water containing sufficient 5 N NaOH for dissolution, and ethionine and methionine in sterile acidified water. Solutions of all inhibitors contained 5 mg per ml and were added to the cultures at the times indicated in the text.

**Analysis and Characterization of the Citreomycins**

Three volumes of 95% acetone containing 5% 2 N HCl were added to one volume of fermentation broth, and the mixture vortexed. After centrifugation, the clarified supernatant was analyzed by HPLC using a Waters model 590-based system, with a C-18 column, which was developed with 65% acetonitrile: 35% 0.05 N ammonium acetate buffer, pH 4.5. The citreomycins were detected by monitoring absorbance at 254 nm. In later experiments designed to compare the purified product from the methylation-inhibition experiment with a genuine ζ standard, a Hewlett Packard HP1090 HPLC with a photodiode array detector was employed.

Citreomycin ζ was isolated and characterized as discussed previously\textsuperscript{1).}

**Results and Discussion**

In preliminary experiments, fermentations of *M. citrea* were supplemented with various methylation inhibitors, and the broths analyzed for the citreomycins. When sinefungin or aminopterin were added, the biosynthesis of one product, later shown to be the ζ component, was stimulated in a dose dependent way (Figs. 2 and 3). A concomitant decrease in the production of the α component was observed. Optimal production of the ζ component was achieved by adding 167 μg/ml sinefungin to production medium B; yields as high as 220 μg/ml were attained in comparison to a control productivity of approximately 1 ~ 10 μg/ml. Aminopterin was less effective, yielding up to 50 μg/ml of the ζ component under similar conditions. In both cases, highest yields of the ζ component were observed when inhibitor was added at
Fig. 2. The HPLC traces shown illustrate the effect of sinefungin on the biosynthesis of citreamicins.

The upper trace is the analysis of a control culture grown in medium B, and the lower trace is from a similar culture to which has been added 250 µg/ml of sinefungin at the time of inoculation.

Fig. 3. The effect of methylation inhibitors on citreamicin ζ biosynthesis.

- Sinefungin, ○ aminopterin.

Inhibition of the methylation by sinefungin and aminopterin could be partially reversed by the addition of L-methionine to the fermentation media (Fig. 4), although this amino acid was toxic to the culture at relatively low levels, and inhibited overall citreamicin biosynthesis.

Adding d-methionine and DL-ethionine to citreamicin-producing cultures did not stimulate ζ biosynthesis, presumably because the citreamicin-biosynthetic methyltransferases are not sufficiently sensitive to these potential inhibitors to produce a detectable effect. Since both of these amino acids were toxic at high concentrations (660 µg/ml) it is assumed they are taken up by the cells. The production media used in our experiments contain approximately 50~100 µg/ml methionine (data on media components from Difco Technical Information Service) which may be sufficient to mask the inhibitory effect of these compounds on citreamicin biosynthesis. Although it has previously been shown\textsuperscript{15,16} that tetracycline methylation can be inhibited by ethionine (50~100 µg/ml) and this effect could be reversed by methionine,
concentrations required for this reversal were 5~10-fold higher than those of the antimetabolite. These data suggest that if citreamicin biosynthetic enzymes were sensitive to ethionine our experiments should lead to the production of the \( \zeta \) component. Amethopterin (up to 2 mg/ml) had no detectable effect on antibiotic production; however, impermeability to amethopterin cannot be excluded.

Methylation inhibitors have been reported previously to direct the biosynthesis of demethylated fermentation products. For example, it has been shown that monensin biosynthesis by \textit{Streptomyces cinnamomonesis} can be inhibited by the addition to producing cultures of aminopterin, a folate reductase inhibitor, and sulfonamides, which inhibit the formation of folates. The metabolic pattern is changed so that demethyl monensin is produced, although a substantial effect is observed upon the overall production of antibiotic as well\(^7\). Sinefungin, an antibiotic analog of \textit{S}-adenosyl homocysteine, a natural inhibitor of several methylyases, is also a known potent inhibitor of methylation reactions involved in primary\(^8\) and secondary\(^9\) metabolism in a variety of organisms. Further examples of methylation inhibitors are DL-ethionine which can act both as a methylation inhibitor\(^{15,16}\) and as a substrate for biosynthetic ethylation\(^{20}\), and \(\text{D}-\text{methionine}\) which has been reported to be a weak methylation inhibitor\(^{21}\).

Biological methylation reactions ubiquitously employ the methyl group of methionine, and our data confirm the methoxy groups of citreamicin are derived \textit{via} this route\(^7\). It is perhaps somewhat enigmatic that only the methylation occurring at position 18 is sensitive to inhibition by sinefungin or aminopterin. While the reason for this specificity of inhibition is unclear, there are a number of possible explanations. The methylation which is not inhibited could occur early in the biosynthesis and may be a prerequisite for complete transformation of a key polyketide-intermediate into the final product. This type of argument is proposed for the effect of methylation inhibitors on monensin biosynthesis\(^17\), and early methylation steps have been postulated for the biosynthesis of other polyketide antibiotics, \textit{e.g.} tetracyclines\(^{22}\). A further explanation is that there may be two very specific methyltransferases present, one of which is much more sensitive to sinefungin and aminopterin than the other. Presumably methyltransferases involved with primary metabolism are not as sensitive to sinefungin or aminopterin as are the citreamicin biosynthetic enzymes since no detectable effect was observed on cell growth. From our data, we cannot distinguish between the possibilities given.

References