Methymycin Biosynthesis. Isolation of P450 Monooxygenase Activity in a Cell-Free System from *Streptomyces venezuelae*

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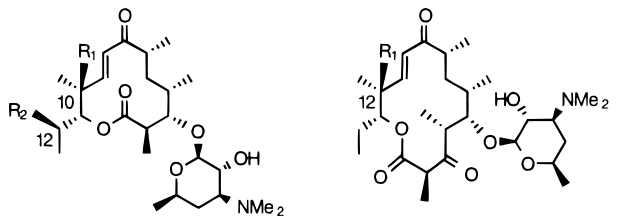
In contrast to the large number of known cytochromes P450 of microbial, plant, and animal origin that are involved in a myriad of metabolic and catabolic processes, only a handful of bacterial P450s that play a role in the biosynthesis of macrolide antibiotics have been identified.[1,2] Of the latter enzymes, only two have been studied in detail, erythromycin D 12-hydroxylase (EryK)[2a] and 6-deoxyerythronolide B 6-hydroxylase (EryF),[2b] both of which are involved in the late stages of erythromycin biosynthesis. The X-ray crystal structure of EryF has recently been reported.[3] Our interest in macrolide biosynthetic pathways has led us to examine the role of P450s in the final stages of methymycin biosynthesis.

The 12-membered ring macrolide antibiotic methymycin (**1**), and its congener neomethymycin (**2**) are produced by *Streptomyces venezuelae*, accompanied by varying levels of the 14-membered ring homologues narbomycin (**3**) and picromycin (**4**) (Figure 1).[4] Incorporation studies[5] with simple acetate and propionate precursors, as well as with advanced intermediates of polyketide chain elongation, have provided strong evidence that the macrolide aglycon of methymycin arises from the condensation of a propionyl starter unit with 1 malonyl-CoA and 4 methylmalonyl-CoA units by a processive mechanism, typical of a modular type I polyketide synthase (PKS). We also observed that addition of the monooxygenase inhibitor, xanthotoxin (8-methoxypsoralen) to cultures of *S. venezuelae* resulted in accumulation of the parent methymycin aglycon, 10-deoxymethynolide (10-dMet, **5**).[6]

On the basis of the latter results, we intially assumed that **5** might be the substrate for a hydroxylation leading to methynolide, which upon glycosylation would give methymycin. All attempts to detect the oxidation of the aglycon **5**, however, using cell-free extracts of *S. venezuelae* in the presence of appropriate redox cofactors were unsuccessful.[7] By analogy to the late stage oxidation catalyzed by EryK during erythromycin biosynthesis,[2a] we therefore considered the possibility that the actual substrate for the hydroxylation step in methymycin biosynthesis might be the glycosylated intermediate **6**, a metabolite known as YC-17 that had previously been reported in early culture filtrates of *S. venezuelae* MCRL 0376.[8]



1 R_1 = OH; R_2 = H
2 R_1 = H; R_2 = OH
3 R_1 = H
4 R_1 = OH

Figure 1. Structures of methymycin (**1**), neomethymycin (**2**), narbomycin (**3**), and picromycin (**4**).

Since YC-17 (**6**) was not readily available, we synthesized the required sample by glycosylation of 10-dMet (**5**) (Scheme 1). The protected fluorodesosamine **7** was prepared from desosamine hydrochloride by the method of Suzuki et al.[9] Coupling of (**7**) and 10-dMet (**5**) with bis(cyclopentadienyl)hafnium dichloride (Cp_2HfCl_2)/$AgClO_4$ gave YC-17 (**6**) and its α-anomer in a ratio of 12:1 in an overall yield of 45%.[10] Similarly, [^{14}C]YC-17 (**6**) was prepared using radiolabeled 10-dMet (**5**) obtained from cultures of *S. venezuelae* that had been grown in the presence of xanthotoxin and sodium [1-^{14}C]propionate.[11]

A cell-free extract of *S. venezuelae* was prepared from mycelia harvested from 34-h fermentation cultures.[5,6] The supernatant, obtained after passage of cells through a French press, was subjected to polyethyleneimine (PEI, 0.2%) treatment, and the supernatant was brought to 75% saturation with $(NH_4)_2SO_4$. The resultant precipitate was resuspended in buffer and desalted on Sephadex G-25.

The desalted ammonium sulfate fraction was incubated with YC-17 (**6**) in the presence of NADPH and P450 electron transport proteins, ferredoxin and ferredoxin-NADP$^+$ reductase.[12] Products isolated from preparative scale incubations were separated by preparative TLC[13] and compared by FABMS with authentic standards of **1** and **2**. These results clearly demonstrated the conversion of YC-17 (**6**) to two products which were identified as methymycin (**1**) and neomethymycin (**2**) (Scheme 2). Control experiments demonstrated (i) dependence of this conversion on the presence of protein, (ii) an absolute requirement for NADPH, and (iii) inhibition of product formation when partially purified protein was treated with CO.[14]

To characterize the enzymatic reaction further, a series of concentrations of [^{14}C]YC-17 (**6**) from 16.5 to 330 μM were incubated with the desalted ammonium sulfate fraction for 120 min. After extraction with EtOAc, the resulting methymycin (**1**)

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(10) Matsumoto, T.; Maeta, H.; Suzuki, K.; Tsuchihashi, G. *Tetrahedron Lett.* **1988**, *29*, 3575−3578. Product identity was confirmed by ^{1}H NMR, ^{13}C NMR, and FABMS.
(11) Cultures of *S. venezuelae* ATCC 15439 in SCM media[6] (6 × 100 mL) were incubated with 106.1 μCi sodium [1-^{14}C]propionate (52 mCi/mmol) and 21.6 mg/flask (1 mM) of xanthotoxin to yield 0.67 μCi of 10dMet (8.5 μCi/mmol). The derived [^{14}C]YC-17 had a specific activity of 7.7 μCi/mmol.
(12) The desalted protein was equilibrated with assay buffer (100 mM KH_2PO_4 (pH 7.3), 1 mM EDTA, 0.2 mM PMSF, 0.2 mM DTT, 10% glycerol). A typical incubation consisted of 1.90 mL protein, 3.5 μM spinach ferredoxin (Sigma), 0.1 units ferredoxin-NADP$^+$ reductase (Sigma), 1 mM NADPH (Sigma), and 2.0 mM YC-17 in a total assay volume of 2.0 mL. Incubations were carried out at 30 °C for 2 h.
(13) Assay mixtures were extracted with 3 × 1 mL EtOAc. The EtOAc soluble materials were applied to a 4 × 10 cm SiO_2 plate and developed three times with 90:10:1 CH_2Cl_2:MeOH:25% NH_4OH. The bands were excised from the plates and eluted with 10% MeOH/$CHCl_3$. Compound identity was established by FABMS and by coelution with unlabeled methymycin, neomethymycin, and YC-17 standards.
(14) Carbon monoxide (10% in helium) was bubbled through the protein solution for 60 s with exclusion of visible light. [CAUTION: CO is a toxic, odorless gas. Use only in a properly ventilated fume hood.]

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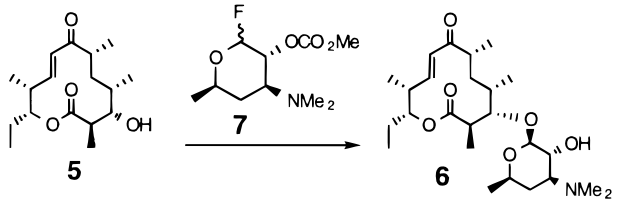
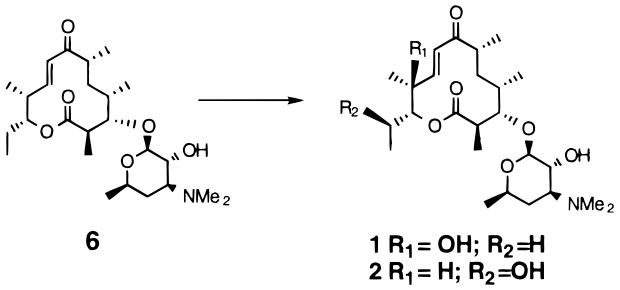
Scheme 1. Synthesis of YC-17 (**6**)[a]



[a] (a) 1.8 equiv **7**, 5 equiv Cp_2HfCl_2, 5 equiv $AgClO_4$, 4 Å molecular sieves, DCM, −20 °C (30 min) to 25 °C (36 h); (b) MeOH, Et_3N, H_2O (5:1:1), 70 °C, 4 h.

Scheme 2. Conversion of YC-17 (**6**) to **1** and **2** by *S. venezuelae* P450, O_2, NADPH, Ferredoxin, Ferredoxin-$NADP^+$ Reductase



and neomethymycin (**2**) were purified by TLC and individually counted. Direct fitting of the data to the Michaelis−Menten equation gave K_m and V_{max} values of 95 ± 10 μM, 2.8 ± 0.1 × 10^{-2} nmol s^{-1} mg $protein^{-1}$ and 255 ± 70 μM, 4.4 ± 0.7 × 10^{-2} nmol s^{-1} mg $protein^{-1}$ for methymycin (**1**) and neomethymycin (**2**) production, respectively.

The isolation of monooxygenase activities that can convert YC-17 to **1** and **2** represents only the third example of the characterization at the protein level of a P450 involved in macrolide biosynthesis and the first instance in which such a P450 has been isolated from the native producing organism. Both recombinant EryF and EryK, each of which catalyzes a hydroxylation in the formation of erythromycin, have been cloned and overexpressed.[2a,b] In addition, several other P450 sequences that are clustered with macrolide PKS genes have been reported, and the inferred function confirmed by complementation of mutants or by the generation of deletion mutants.[15]

In summary, we have demonstrated that oxidation of YC-17 at C-10 and at C-12 is P450-dependent and is the final step in the formation of methymycin (**1**) and neomethymycin (**2**), respectively. The fact that the observed K_m values for each hydroxylation differ by a factor of 2−4 suggests, but does not prove, that the formation of each metabolite is mediated by a distinct P450 monooxygenase. Confirmation of this suggestion must await purification of the individual hydroxylase activities and isolation of the relevant gene or genes. It should also be noted that an analogous hydroxylation at C-12 is required for the known conversion of narbomycin (**3**) to picromycin (**4**).[16]

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Supporting Information Available: Physical data and procedures for YC-17 and kinetic data (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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