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MACROLIDE BIOSYNTHESIS: A SINGLE CYTOCHROME P450, PicK, IS RESPONSIBLE FOR THE HYDROXYLATIONS THAT GENERATE METHYMYCIN, NEOMETHYMYCIN, AND PICROMYCIN IN *STREPTOMYCES VENEZUELAE*

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Abstract: The final step in the biosynthesis of methymycin, neomethymycin, and picromycin is an hydroxylation, shown to be carried out by the cytochrome P-450 monooxygenase, PicK. Direct comparison of the relative k_{cat}/K_m values for the two substrates, YC-17 and narbomycin, showed a threefold rate preference of picK for narbomycin.

The actinomycete, *Streptomyces venezuelae*, is unusual in that it produces both 12- and 14-membered ring macrolide antibiotics, methymycin (**1**) and neomethymycin (**2**) in the first case, and picromycin (**3**) in the other.[1] All three metabolites are closely related biosynthetically, **1** and **2** being derived from 1 acetate and 5 propionate building blocks, with the biosynthesis of **3** involving addition of one more propionate unit to the polyketide chain.[2] Recent evidence has established that a single biosynthetic gene cluster encodes the polyketide synthase or synthases responsible for the biosynthesis of both methymycin and picromycin.[3,4]

The last step in the formation of both the 12-membered and 14-membered ring macrolides is a regiospecific hydroxylation of the respective glycosylated intermediates YC-17 (**4**)[5] and narbomycin (**5**).[6] We have shown that cell-free extracts of *S. venezuelae* can convert **4** to a mixture of methymycin (**1**) and neomethymycin (**2**).[5] These studies identified YC-17 as the substrate for the native hydroxylase, to the exclusion of 10-deoxymethynolide (**6**),[7] indicating that addition of desosamine precedes oxidation. The crude monooxygenase from *S. venezuelae* was further characterized as a cytochrome P-450, showing an absolute requirement for NADPH and a dependence on ferredoxin and NADPH oxidoreductase. On the other hand, it could not be determined whether the hydroxylations leading to the formation of **1** and **2** were catalyzed by a single P-450 or two distinct proteins.

Recently, sequencing of the methymycin/picromycin gene cluster has identified a single open-reading frame, designated *picK*, that encodes a cytochrome P-450.[3,4] The corresponding 46-kDa gene product, PicK, was expressed and purified from *Escherichia coli* with a His-tag at the C-terminus of the protein.[3] Product and steady

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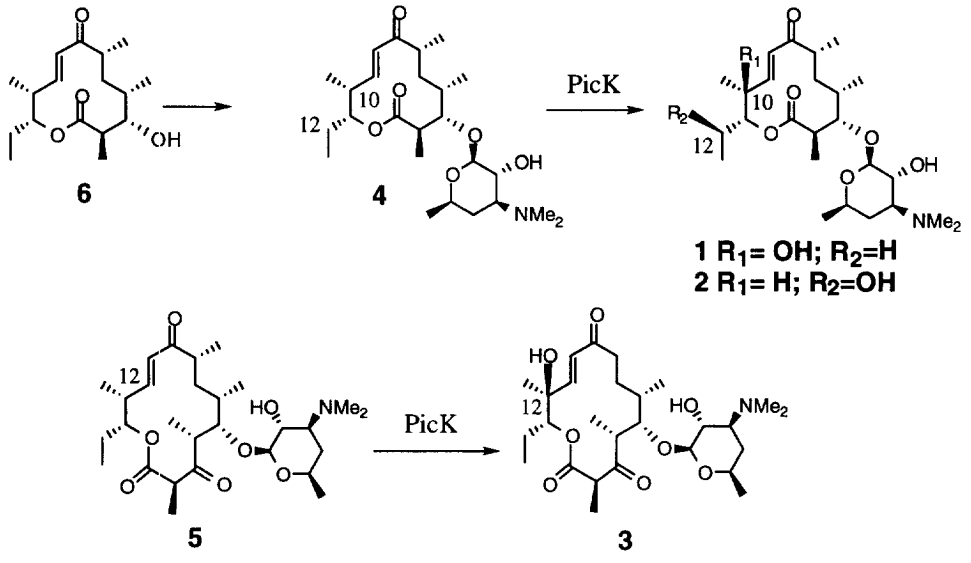
state kinetic studies demonstrated that picK/6-His converted narbomycin (**5**) to picromycin (**3**). The question then arose whether PicK also corresponded to the previously characterized *S. venezuelae* monooxygenase and could thus mediate the formation of both methymycin and neomethymycin as well. We now report that picK/6-His does indeed convert YC-17 (**4**) to both methymycin (**1**) and neomethymycin (**2**) and compare the kinetic behavior of the enzyme with respect to the two substrates.



PicK/6-His, purified as previously described,[3] was incubated with semisynthetic [^{14}C]YC-17 (**4**).[5] The identities of the products formed from YC-17 were confirmed by co-elution on TLC with known standards of **1** and **2**, and counting the purified radiolabeled samples of **1** and **2** thus obtained, as previously described.[5] The kinetics of the conversion of YC-17 to **1** and **2** were monitored using a continuous spectrophotometric assay measuring NADPH consumption.[8,9] The conversion of narbomycin (**5**) to **3** was also assayed under identical conditions. Double reciprocal plots of velocity vs. substrate concentration gave clear evidence of substrate inhibition in both cases. The data were therefore fit directly to the equation $v = V_{max}/[1 + (K_m/[S]) + ([S]/K_i)]$. The calculated steady-state parameters are given in Table 1. The K_m for YC–17 measured with purified PicK/6-His is in good agreement with the value previously obtained using the crude *S. venezuelae* extract;[5] similarly the *k*cat and K_m for narbomycin are in excellent agreement with the values of 1.3 ± 0.2 s^{-1} and 100 ± 40 μM previously determined using Tris buffer and a discontinuous LC-MS assay.[3] Substrate inhibition has previously been observed in the analogous hydroxylation catalyzed by erythromycin D 12-hydroxylase (EryK).[8] Analysis of the results of radiochemical assays run at a variety of YC-17 concentrations established a relative k_{cat}/K_m for methymycin and neomethymycin formation of 1:1. Control experiments demonstrated an absolute requirement

for NADPH and both electron transport proteins. Finally, the UV/VIS spectrum of a sample of PicK/6-His that had been treated with $Na_2S_2O_4$ and then treated with CO showed the characteristic reduced P-450 absorbance at 450 nm.

Table 1. Steady-state kinetic parameters for PicK/6-His

substrate	K_m (μM)	K_i (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
YC-17 (**4**)	130 ± 20	2.2 ±0.4	1.1 ± 0.08	0.008 ± 0.004
narbomycin (**5**)	88 ± 24	0.44 ± 0.1	2.0 ± 0.3	0.023 ± 0.01

The relative k_{cat}/K_m for narbomycin vs. YC-17 is 3:1, indicating a slight preference for the 14-membered ring substrate. The cytochrome P-450 strikes an intriguing balance between strict substrate specificity (PicK will not process the corresponding macrolide aglycones) and a permissiveness that allows for oxidation of two distinct substrates of differing ring size, one of which, **4**, undergoes competing hydroxylation at two different positions, C-10 and C-12. Thus a single enzyme, PicK, is responsible for the final reaction in the biosynthesis of both 12-membered and 14-membered ring macrolide antibiotics in *S. venezuelae*, converting YC-17 into both **1** and **2**, and narbomycin to picromycin (**3**).[10]

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References

1. Maezawa, I.; Kinumaki, A.; Suzuki, M. *J. Antibiot.* **1974**, *27*, 84. Donin, M. N.; Pagano, J.; Dutcher, J. D.; McKee, C. M. *Antibiot. Ann.* **1953-4**, *1*, 179. Pearlman, D.; O'Brien, E. *Antibiot. Chemother.* **1954**, *4*, 894. Rickards, R. W.; Smith, R. M.; Majer, J. *J. Chem. Soc., Chem. Commun.* **1968**, 1049. Prelog, V.; Gold, A. M.; Talbot, G.; Zamojski, A. *Helv. Chim. Acta* **1962**, *45*, 4.

2. Cane, D. E.; Lambalot, R. H.; Prabhakaran, P. C.; Ott, W. R. *J. Am. Chem. Soc.* **1993**, *115*, 522. Birch, A. J.; Pride, E.; Rickards, R. W.; Thompson, P. J.; Dutcher, J. D.; Perlman, D.; Djerassi, C. *Chem. and Ind.*, **Oct. 1 1960**, 1245. Omura, S.; Takeshima, H.; Nakagawa, A.; Miyazawa, J. *J. Antibiot.* **1976**, *29*, 316.

3. Betlach, M.; Kealey, J. T.; Betlach, M.; Ashley, G.; McDaniel, R. *Biochemistry* **1998**, in press.

4. Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.-W.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, in press.

5. Cane, D. E.; Graziani, E. I. *J. Am. Chem. Soc.* **1998**, *120*, 2682.

6. Hori, T.; Maezawa, I.; Nagahama, N.; Suzuki, M. *Chem. Commun.* **1971**, 304.

7. Lambalot, R. H.; Cane, D. E. *J. Antibiot.* **1992**, *45*, 1981.

8. Lambalot, R. H.; Cane, D. E.; Aparicio, J. J.; Katz, L. *Biochemistry* **1995**, *34*, 1858.

9. Typical assay conditions: 500 nM PicK, 9 μM spinach ferredoxin, 0.1 units ferredoxin-NADP+ reductase, 170 μM NADPH, and substrate (50 μM to 2 mM) in buffer (100 mM KH_2PO_4 (pH 7.3), 1 mM EDTA).

10. Sherman et al. have recently shown that a *picK* (*pikC* using their nomenclature) deletion mutant of *S. venezuelae* accumulates both **4** and **5**. See Xue, Y.; Wilson, D.; Zhao, L.; Liu, N.-W.; Sherman, D. H. *Chem. & Biol.* **1998**, in press, and ref 4.