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Role of the Cytochrome P450 NocL in Nocardicin A Biosynthesis

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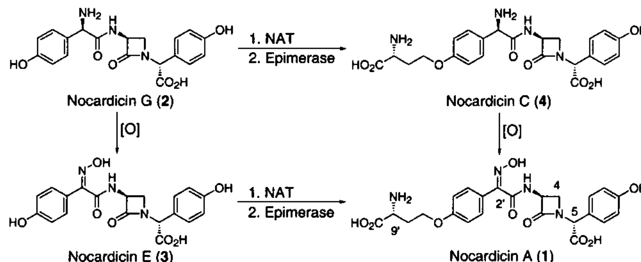
The actinomycete *Nocardia uniformis* subsp. *tsuyamanesis* (ATCC 21806) produces a class of monocyclic β-lactam antibiotics, the most potent of which is nocardicin A (**1**, Scheme 1).[1] The amino acid origins of this metabolite have been firmly established: L-tyrosine is incorporated into both aromatic residues via 4-hydroxyphenylglycine (HPG), and the β-lactam ring carbon atoms are derived from serine.[2] Attachment of the homoseryl side chain, derived from *S*-adenosylmethionine, is mediated by 3-amino-3-carboxypropyltransferase (NAT),[3] and epimerization at the resulting C-9′ center has been demonstrated in a cell-free extract prepared from *N. uniformis*.[4] The subsequent transformations to assemble the nocardicin structural framework have yet to be elucidated, including the preferred route taken from nocardicin G (**2**), the earliest identified β-lactam intermediate,[5] to nocardicin A, and oxidation of the C-2′ primary amine. Intact incorporation of paired isotopes from [2-^{13}C,^{15}N]-4-HPG established that the oxime in fact arises by way of amine oxidation.[6] The oxime notably present in nocardicin A is rare among secondary metabolites, and the means of its introduction is the focus of this investigation.

While the occurrence of *N*-oxidized natural products is uncommon, a variety of functionalities are known. A number of hydroxamate siderophores and azoxy antibiotics involve a hydroxylamine intermediate. For all cases thus far characterized, the enzymes implicated for these *N*-hydroxylations are flavin monooxygenases.[7,8] The nitro group of pyrrolnitrin, for example, is believed to be the product of a non-heme iron-dependent dioxygenase and a primary amine precursor.[9] In plants, glucosinolate and cyanogenic glucoside biosynthesis from both aromatic and aliphatic amino acids proceeds via aldoxime intermediates. Oxime formation for these systems is mediated by members of the CYP79 family of cytochrome P450 enzymes.[10] In addition, mammalian microsomal cytochrome P450s and flavin monoxygenases that take part in xenobiotic metabolism have demonstrated *N*-oxidation capabilities.[11] We have identified the protein responsible for oxime formation in nocardicin biosynthesis, the first prokaryotic enzyme determined to catalyze such a transformation.

A putative nocardicin A biosynthetic cluster was identified from a cosmid library of the *N. uniformis* genome.[12] One of the open reading frames, *nocL*, encodes a gene product with significant similarity to proteins of the cytochrome P450 superfamily, including the highly conserved regions attributed to molecular oxygen and heme binding.[13] An oxidative step[6] in nocardicin biosynthesis is mandated to account for the oxime moiety in nocardicins A and E (**3**), suggesting NocL as a likely candidate for this transformation.

NocL was heterologously expressed in *Escherichia coli* BL21(DE3). The gene was cloned into pET28b(+) under control of the T7 promoter, so that the translated protein would contain an amino terminal hexahistidine fusion. The *Nocardia* protein was found to be largely insoluble. However, by reducing the induction temperature from 37 °C to 22 °C and supplementing the growth medium with 1 mM δ-aminolevulinic acid, NocL was obtained at a level of 1.6 mg/L. The polyhistidine-tagged protein was purified to near homogeneity by nickel chelate chromatography. The protein produced the carbon monoxide difference spectrum diagnostic of the cytochrome P450 superfamily.[14]

Scheme 1



The ability of NocL to catalyze oxime formation in the presence of spinach ferredoxin, spinach ferredoxin-NADP$^+$ reductase, NADPH, and nocardicins C (**4**) and G (**2**) was examined by using a colorimetric assay specific to the presence of the oxime moiety.[15] The derivatization procedure produces the diazo dye 5-(*p*-sulfophenylazo)-8-quinolinol, which gives an absorption maximum between 490 and 510 nm.[16] The reaction containing NocL and nocardicin C clearly generated the pigment, consistent with oxime formation (Figure 1A). Additionally, the reaction of NocL and either nocardicin C or G was monitored by reverse-phase HPLC. No product formation was observed with nocardicin G. As the reaction with nocardicin C progressed, a new peak appeared in the reaction mixture with the same retention time (10.9 min) as an authentic specimen of nocardicin A (Figure 1B). This peak was purified and analyzed by electrospray ionization mass spectrometry. A $[M + H]^+$ ion peak at *m/z* 501.3 was identified, and an MS/MS spectrum of this parent ion produced fragment ions at *m/z* 351.1 and 322.1, a pattern identical with an authentic sample.

The conversion of a primary amine to an oxime presumably requires two oxidative steps, perhaps in a mechanism similar to that proposed for aldoxime formation by the CYP79 enzymes (Scheme 2).[10b] Successive hydroxylation of nocardicin C to 2′-*N*-hydroxy-nocardicin C and ultimately to 2′-*N*,*N*-dihydroxy-nocardicin C could be followed by dehydration to produce a nitroso species, and then tautomerization to the oxime. Nitrogen oxygenation reactions are not commonly catalyzed by cytochrome P450 enzymes, and, as a result, the mechanism of these transformations is poorly understood. There is strong support for an amminium radical involved in P450-catalyzed amine dealkylations, and it has been proposed *N*-oxygenation proceeds in a similar fashion.[17] However, lack of a similar effect on rates of dealkylation and *N*-oxygenation for a series of para-substituted *N*,*N*-dimethylanilines argues the two reactions proceed with either different mechanisms or a different rate determining step.[18] For the case of oxime

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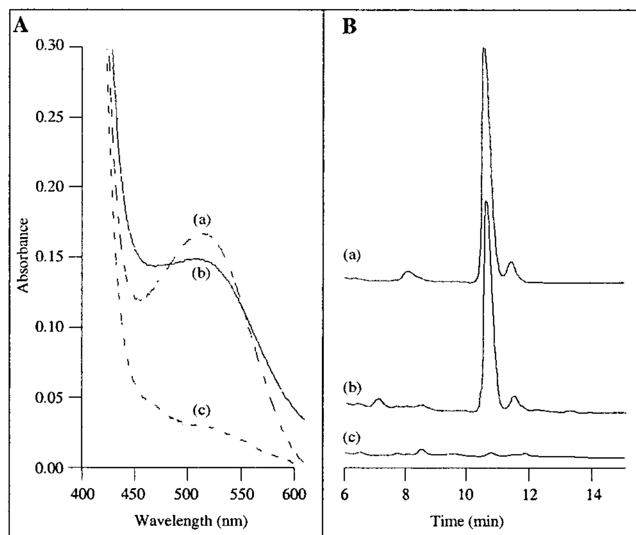
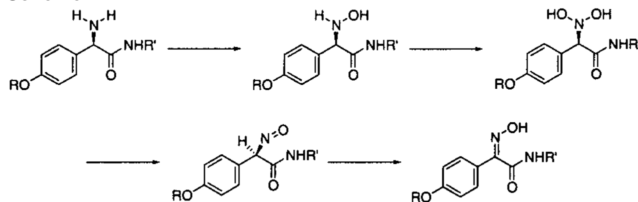


Figure 1. Absorption spectrum of the derivatized reaction mixtures (A) and HPLC analysis of the NocL reaction mixture with nocardicin C (B): (a) reaction mixture with nocardicin A internal standard; (b) reaction mixture; and (c) reaction mixture with heat-inactivated NocL. The HPLC chromatogram was monitored at 270 nm in 10% acetonitrile and 0.1% trifluoroacetic acid.

Scheme 2



formation, an alternative mechanism may involve, for example, dehydrogenation of a hydroxylamine.

There is a high level of protein sequence similarity between NocL and cytochromes P450 involved in the elaboration of polyketide, particularly macrolide, antibiotics. NocL displayed the highest sequence homology (53% identity, 66% similarity) to MycG, a P450 responsible for a hydroxylation and an epoxidation in the biosynthesis of mycinamicin in *Micromonospora griseorubida*.[19] No significant similarity was detected with the known oxime-forming enzymes from the CYP79 family, nitric oxide synthase, or the mammalian microsomal P450s known to effect *N*-hydroxylation.[11a–c] It could be argued, however, that this lack of similarity is at least partially due to the differences between eukaryotic and prokaryotic cytochrome P450s. One type of P450 capable of acting upon a heteroatom substrate that does show limited similarity to NocL is nitric oxide reductase from the fungus *Fusarium oxysporum* (35% identity, 50% similarity).[20] This enzyme catalyzes an uncommon reaction for a P450: reduction of two molecules of nitric oxide to nitrous oxide and water. If the function of NocL were predicted based solely on its similarity to *C*-hydroxylases and epoxidases, *N*-oxygenation would not have been anticipated. It would be of interest to determine whether other *N*-oxidizing prokaryotic P450s emerge, and if they show similarity to NocL.

From the evidence presented, it can be concluded that NocL is a cytochrome P450 that acts on the 2′-amine of nocardicin C to produce the oxime contained in nocardicin A. Due to the occurrence of nocardicin E in *N. uniformis* fermentation broth, it is surprising that the ability of NocL to oxidize nocardicin G is not also observed. It is possible nocardicin G is indeed a substrate for NocL, but is utilized at least 50-fold less efficiently and escapes detection under the limits of the derivatization and HPLC assays. The bias of NocL to effect oxime formation from nocardicin C over nocardicin G, complements the in vitro function of NAT, for which nocardicin G is the preferred substrate for 3-amino-3-carboxypropyl group transfer.[3] It now appears that the biosynthetic route taken from nocardicin G to nocardicin A first requires the attachment of the homoseryl side chain and is followed by inversion at the C-9′ stereocenter and oxime formation. NocL is the first example of a prokaryotic cytochrome P450 capable of effecting the oxidation of an amine to an oxime. The mechanism of this unusual reaction and further characterization of NocL are currently being investigated.

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