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Characterization of CalE10, the *N*-oxidase involved in calicheamicin hydroxyaminosugar formation

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Calicheamicin (CLM) γ_1^I (Fig. 1, **5**) is a prominent member of the 10-membered enediyne family.[1] Like all enediynes, CLM-induced oxidative DNA strand scission is enabled by cycloaromatization of the enediyne core to form a highly reactive diradical species.[2] In CLM, this reactive intermediate is positioned in the DNA minor groove via the aryltetrasaccharide wherein the unique conformation of the CLM hydroxylamino glycosidic bond contributes to both DNA specificity and affinity.[3] The incredible potency of CLM has been harnessed for clinical use (Mylotarg®),[4] and CLM biosynthetic studies have unveiled a variety of unique features. For example, the recent elucidation of gene clusters encoding both 9-membered and 10-membered enediynes revealed a unified, divergent polyketide paradigm for enediyne core biosynthesis,[5] likely originating from a common polyene precursor.[6] Studies on CLM self-resistance also revealed the first 'self-sacrifice' resistance mechanism,[7] while CLM glycosyltransferase-catalyzed 'sugar exchange' and 'aglycon exchange' reactions enabled the production of >70 differentially glycosylated CLM variants.[8] Despite the prevalence of deoxy- and aminosugars in nature,[9] only a few naturally-occuring *N*-oxidized aminosugars, such as the one found in CLM, have been identified.[10] Putative *N*-oxidase genes for rubranitrose, kijanose, and the CLM/esperamicin hydroxylaminosugar biosynthesis have been put forth yet, the enzymes involved in aminosugar *N*-oxidation remain elusive.[5b,5d,10,11] Herein we describe the first reported *in vitro* characterization of an aminosugar *N*-oxidase, CalE10, responsible for CLM hydroxylaminosugar formation.

A comparison among the gene clusters encoding 10-membered enediynes[5b-5d] and indolocarbazoles[12] presented a genomic basis from which to propose the biosynthetic pathway for hydroxyaminosugar precursor TDP-4-hydroxyamino-6-deoxy-α-D-glucose (Scheme 1, **4**). Specifically, this comparative genomic analysis (Scheme S1) enabled the elimination from consideration genes for the biosynthesis of the 10-membered enediyne core (common to **5**, **14,** and **16**) and the CLM aminopentose (common to **5**, **14,** and **18,** but not **19**). Genes anticipated to be involved in the biosynthesis of orsellinic acid[13] and the terminal rhamnose precursor[9] were also excluded based upon well-established precedent for these pathways. The remaining genes were anticipated to be integral to CLM thiosugar or hydroxylaminosugar biosynthesis. In conjunction with the well-established routes to aminosugar biosynthesis,[9] and reminiscent of the P450 *N*-oxidase in β-lactam biosynthesis (NocL),[14] this information led to the proposed pathway highlighted in Scheme 1 wherein two P450s (CalO2 and CalE10) were identified as aminosugar oxidase candidates.

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Supporting Information **Available:** Assay procedures and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

To test the ability of CalO2 and CalE10 to catalyze aminosugar *N*-oxidation, the corresponding enzymes were overproduced in *Streptomyces lividans* as *N*-His_6-fusions. While *N*-His_6-CalO2 displayed a typical P450 Soret peak (418 nm),[15] *N*-His_6-CalE10 exhibited two distinct maxima (418 nm and 386 nm) of equal intensity indicative of a heme iron mixed spin state. The reduced CO bound spectra for both enzymes displayed a typical P450 Soret peak (450 nm) (Fig. 1A). Subsequent *in vitro* assays employed a series of putative TDP-sugar substrates (Fig. 1B, **1**, **3**, **6**-**13**; 10 mM),[8,16] 0.5 mg mL^{-1} P450 (CalO2 or CalE10) and a standard spinach ferredoxin/reductase system.[17] *N*-His_6-CalE10-catalyzed transformation of **3** afforded two new products (Fig. 1C) with mass and IR consistent with hydroxyaminosugar **4a** (Scheme 1, major) and nitrosugar **4b** (minor), while aminosugar **8** with the same enzyme led to the corresponding hydroxylamino derivative in trace amounts. Steady state kinetic analysis of the CalE10-catalyzed oxidation of **3** revealed kinetic parameters (k_{cat} = 0.04 ± 0.01 sec^{-1}; K_m = 7.6 ± 1.2 μM) similar to other natural product P450s.[17] Consistent with the stringent aminosugar regiospecificity observed, subsequent ligand-binding studies revealed a reverse type I difference spectrum[17e,18] with determined K_d values of 9.1 ± 1.1 μM, 17.3 ± 1.8 μM, 165 ± 27 μM, and >150 μM for **3**, **8**, **1,** and **10**, respectively, while TDP or 4-amino-4-deoxy-α-D-Glc-1-phosphate led to no heme perturbation. No apparent sugar nucleotide binding or oxidation was observed with CalO2, consistent with the ability of CalO2 to bind substituted aromatic acids (as possible orsellinic acid surrogates).[15]

In summary, this study establishes, for the first time, CalE10 as the requisite CLM NDP-aminosugar *N*-oxidase and confirms that oxidation occurs at the sugar nucleotide stage prior to glycosyltransfer. Furthermore, substrate specificity studies revealed CalE10-catalyzed oxidation to be regiospecific with limited over-oxidation *in vitro*. As the first characterization of an aminosugar *N*-oxidase, this study also presents a foundation for the future study of other *N*-oxidases involved in hydroxylamino-, nitroso-, and/or nitrosugar formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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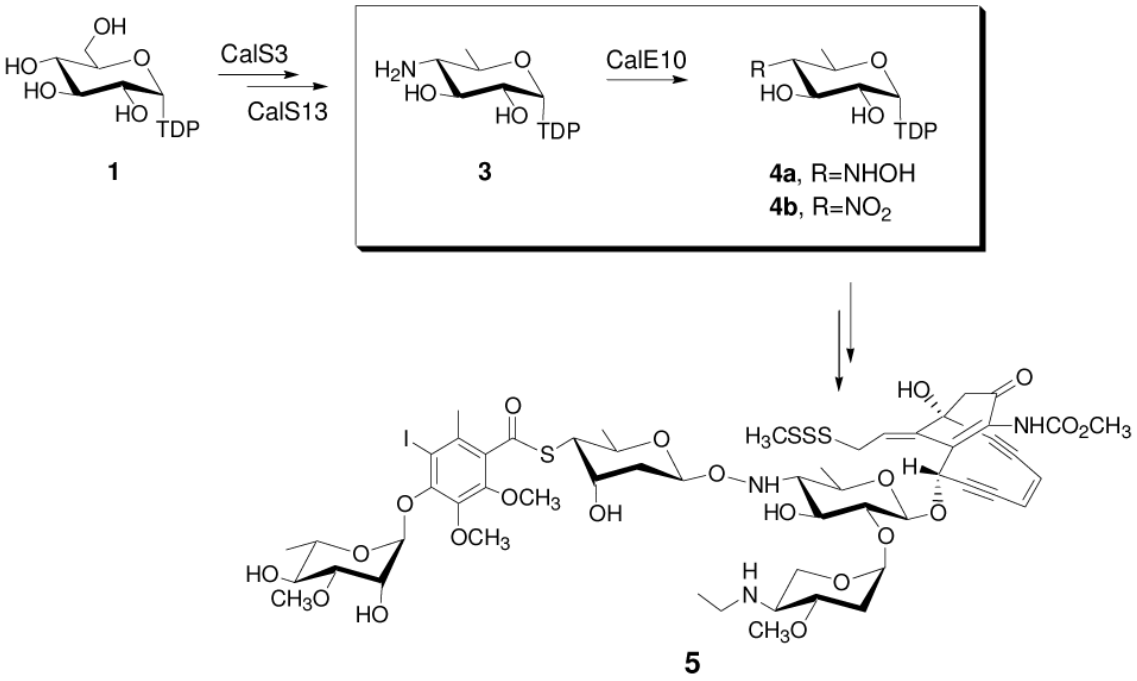

Scheme 1.
Proposed biosynthesis of 4-hydroxyamino-6-deoxy-α-Dglucose common to CLM (**5**) and esperamicin.

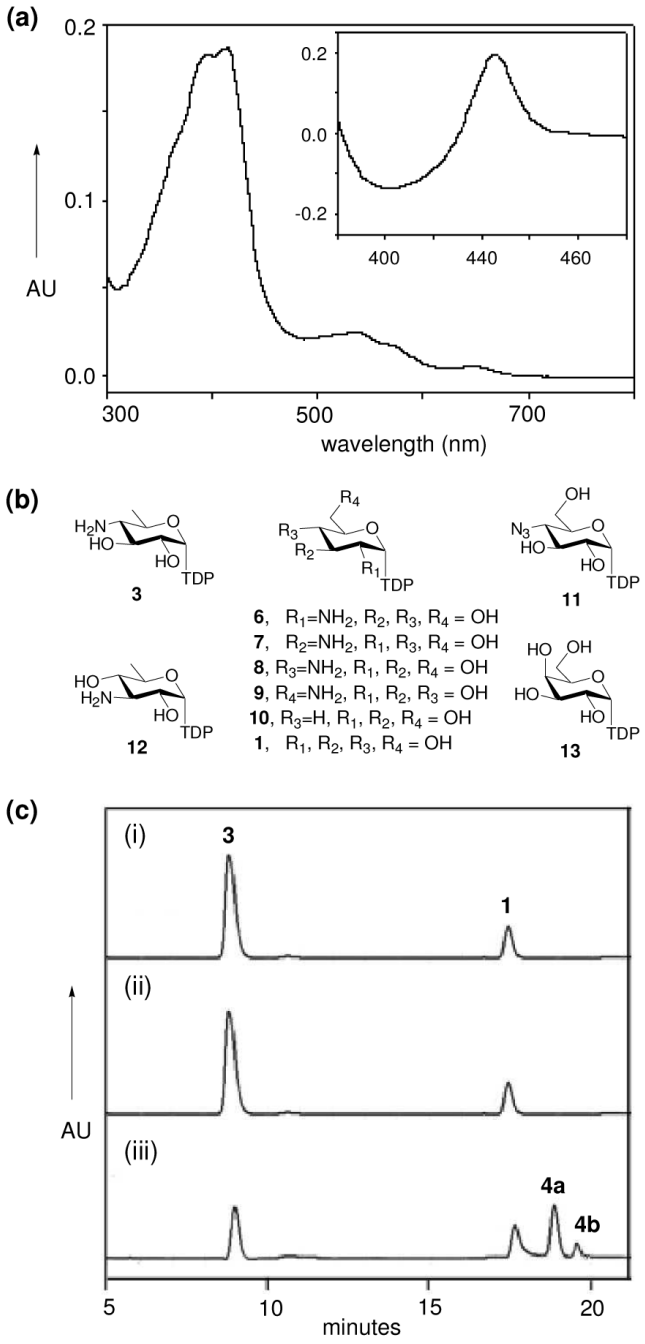

Figure 1.

(a) Oxidized spectrum of CalE10 and difference spectra of reduced CO-bound species (inset). **(b)** Putative sugar nucleotide substrates used in this study. **(c)** HPLC analyses of assays with **3** as the substrate: (i) no P450 (control); (ii) CalO2; (iii) CalE10. A trace amount of **1** remains from chemoenzymatic synthesis of **3**. See supporting information for experimental details.