versus 115 μM for Me_3-[9]aneN_3) because it can form the bis-chelate product[15c] much more readily.[16]

Metal binding to protein is the most likely explanation for the results, because free TERPY and coordinatively saturated [Cu(TERPY)$_2$](ClO$_4$)$_2$[15a] were inactive. The maximized effect at a copper to TERPY ratio of 1:1 and the reduced inhibitory effect in the absence of chloride ions (Figure 1) imply that the active blocker is [Cu(TERPY)Cl]$^+$,[15a] which coordinates free and *N*-acetylated amino acids readily.[17, 18] Accordingly, the IC_{50} values of both [Cu(TERPY)Cl](ClO$_4$)[15a] and [Cu(TERPY)Cl$_2$][15a] were similar to that of the inhibitor formed in situ (16 versus 20 μM). The difference is explained by slow inhibitor formation. Pre-incubation (40 minutes) of Cu^{2+} and TERPY (400 μM) prior to substrate (19 μM) and enzyme addition increased k_{obs} by 15%. This also suggests formation of the active inhibitor prior to interaction with the enzyme. In the absence of chloride, [Cu(TERPY)(solvent)]$^{2+}$ (solvent = H_2O[15a] or Bis-Tris) is the likely inhibitor. As Kex2 inhibition is competitive with substrate (Figure 3), the inhibitor probably binds at the active site, of which catalytic His213 seems to be the best copper-coordinating residue. A study of Hg^{2+} inhibition of proteinase K (a homologue of Kex2) implies that nearby, buried Cys217 may also participate in copper coordination after modification of the pocket.[19] Cationic [Cu(TERPY)Cl]$^+$ and [Cu(TERPY)(solvent)]$^{2+}$ should be favored to reach the active site, which is designed for polybasic substrates.[10, 11]

Our results suggest that metal complexes could be used in vivo to block enzymes that are inhibited by metal ions in vitro. But chelates showing the desired effect must be modified for specificity and higher target affinity. The combinatorial synthesis approach is modestly demonstrated by the TERPY derivative 4′-(4-methoxyphenyl)-2,2′:6′,2″-terpyridine (MPT), with an IC_{50} value (10 μM) that is half of that of TERPY (Figure 1). Experiments to achieve specificity by tethering target-selective peptides[20] (or antibodies) are currently underway. Extracellular targets such as cell-surface furin (which processes toxin precursors) seem well suited for the outlined approach due to the presence of metal-exchanging carriers such as serum albumin.[21]

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On the Origin of Deoxypentoses: Evidence to Support a Glucose Progenitor in the Biosynthesis of Calicheamicin

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The deoxysugars have long been recognized as a unique and essential class of naturally occurring carbohydrate. These deoxygenated and often functionalized sugars present unusual

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hydrophobic and hydrophilic domains essential to their roles in molecular recognition events. Arguably the most diverse group of naturally occurring deoxysugars are the deoxyhexoses, the biosynthetic characterization of which has received a great deal of attention in the last decade.[1] The deoxypentose deoxyribose of DNA equals, or exceeds, the natural deoxyhexoses in biomass. As with many functionalized deoxyhexoses, the biosynthesis of this vital molecule has been very well characterized.[2] Interestingly, a few examples of deoxypentoses have also been found as ligands of bioactive bacterial secondary metabolites.[3] The orthosomycin antibiotics avilamycin (**4**) and evernimicin (**5**) (Scheme 1) contain one (sugar F) and two (F and G) deoxypentoses, respectively. The enediyne antitumor antibiotics calicheamcin (**1**) and esperamicin (**3**) also carry a common aminodideoxypentose (sugar C), while the enediyne maduropeptin (**2**) bears a unique branched dideoxyaminopentose.[4] However, insights regarding the biosynthesis of these unique ligands is sparse. Classical labeling studies on esperamicin[5] suggest the aminodideoxypentose ligand may derive from glucose, presumably by loss of CO_2. In contrast, a recent BLAST analysis of the proteins encoded by the avilamycin biosynthetic gene locus was used to suggest that the corresponding deoxypentose is ribose derived.[6]

Our recent elucidation of the calicheamicin gene locus from *M. echinospora* ssp. *calichensis* revealed an elusive enediyne polyketide synthase (PKS) gene (*calE8*) near the center of the approximately 100-Kb calicheamicin locus.[7] Less than 10 Kb from this hallmark enediyne PKS gene resides a gene (*calS8*) identified by BLAST analysis of the encoded protein CalS8 as encoding an NDP-mannoaminouronic acid/NDP-glucuronic acid synthase (NDP = nucleoside diphosphate; Figure 1). We now report that the overexpression of *calS8* in *Escherischia coli* and subsequent CalS8 characterization reveals this protein to be a

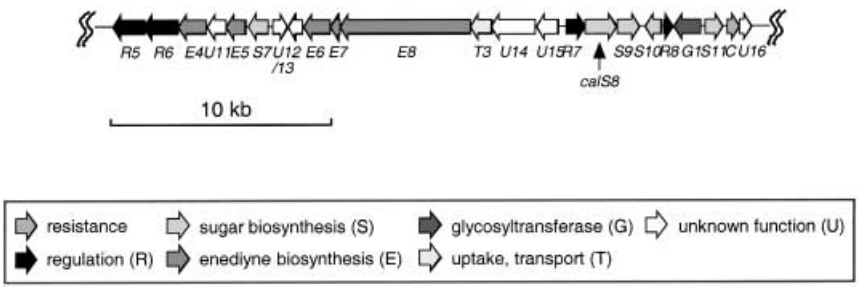
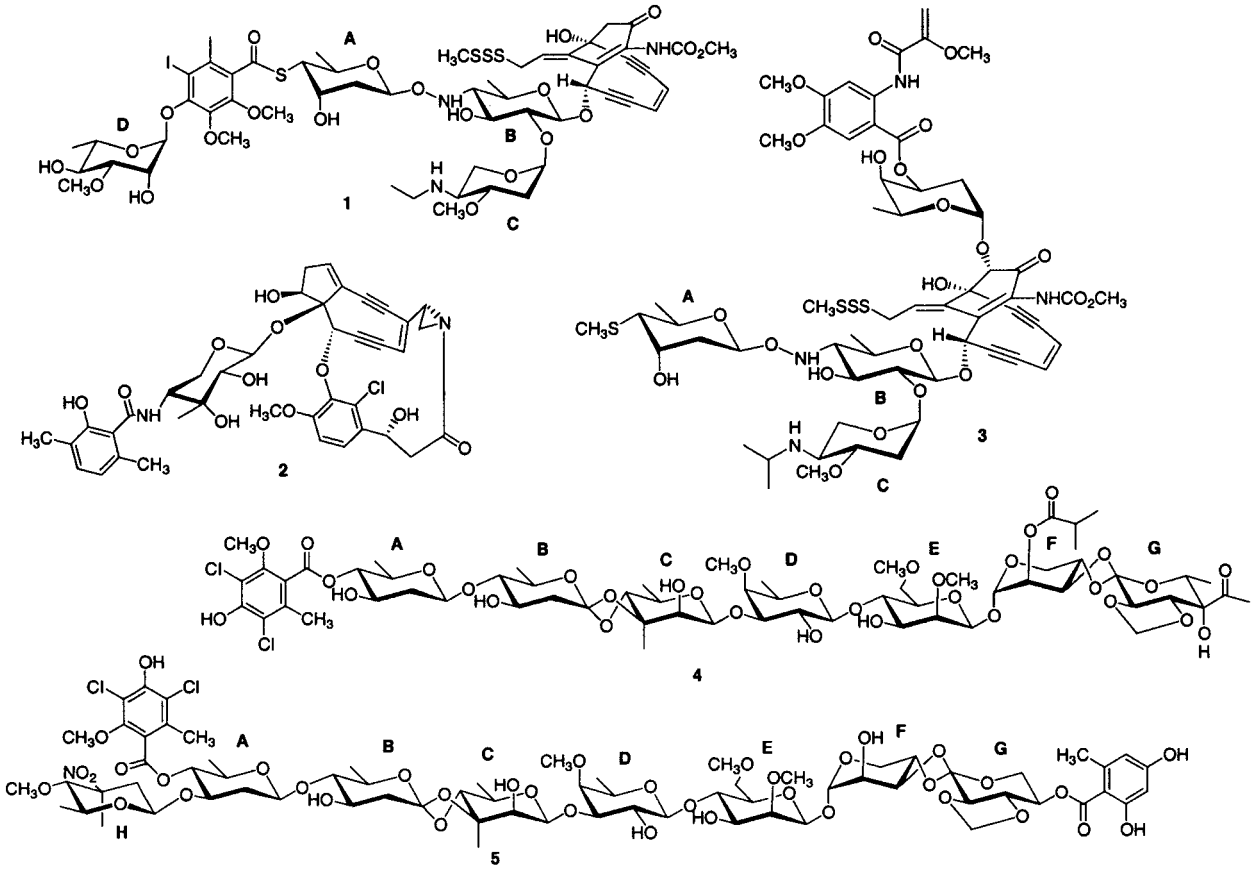


Figure 1. *The central portion of the calicheamicin locus, which contains* calS. *The* calS8 *sequence is deposited under Genbank Accession number AF505622.*



Scheme 1. *The structures of some bacterial natural products that bear deoxypentoses: calicheamicin (**1**) from* Micromonospora echinospora, *maduropeptin (**2**) from* Actinomadura madurea, *esperamicin (**3**) from* Actinomadura verrucosospora, *avilamycin (**4**) from* Streptomyces viridochromogenes, *and evernimicin (**5**) from* Micromonospora carbonacea.

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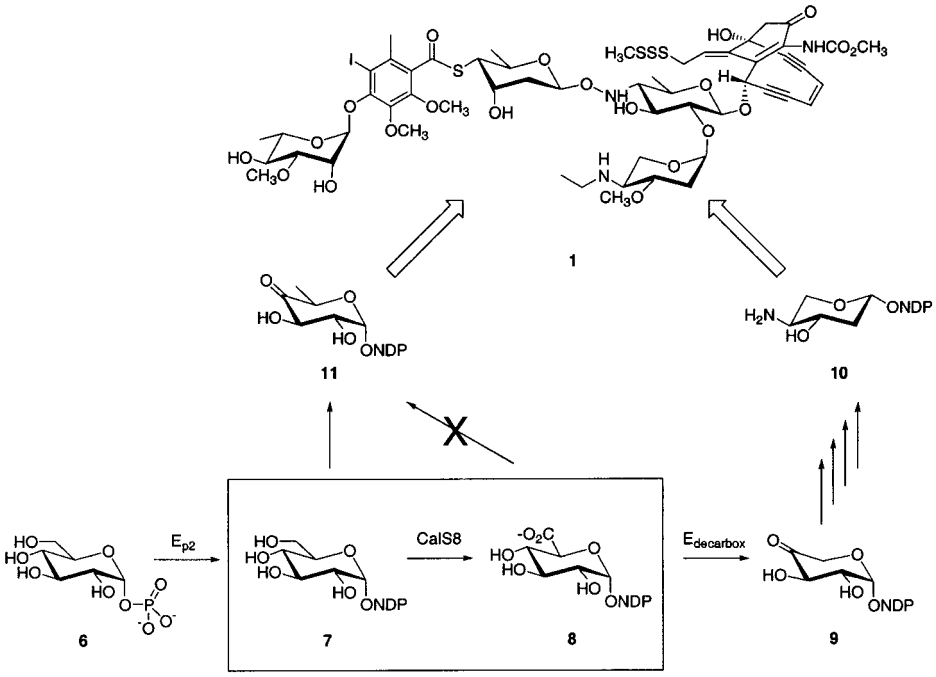
UDP-α-D-glucose dehydrogenase (UDPGlcDH; UDP = uridine diphosphate). While our analysis reveals that the CalS8 steady-state kinetic mechanism is consistent with that of other bacterial UDPGlcDHs,[8] CalS8 is surprisingly the first characterized UDPGlcDH involved in secondary metabolism and the first reported UDPGlcDH able to efficiently accept both UDP-α-D-glucose (UDP-Glc) and dTDP-α-D-glucose (dTDP-Glc; TDP = thymidine diphosphate) as substrates. More importantly, given that three of the four calicheamicin sugar ligands are 6-deoxyhexoses (and thus derive from a common 6-deoxy-4-keto-sugar nucleotide),[1] the reported CalS8 must participate in a unique pathway leading to the remaining aminodideoxypentose.

High-level expression of the maltose-binding-protein fusion molecule (mbp–CalS8) followed by a one-step affinity purification provided 13.5 mg L^{-1} near-homogenous preparation of mbp–CalQ with a specific activity of 5.0×10^{-2} U mg^{-1}.[9] CalS8 is nicotinamide adenine dinucleotide $(NAD)^+$ dependent and attempts to replace this cofactor with $NADP^+$ rendered the enzyme completely inactive. Several nucleotide diphosphosugar surrogates for UDP-Glc were also tested, of which only one, dTDP-Glc, was accepted to an appreciable extent (40% conversion).[10, 11] This notable dTDP-Glc conversion is unique in comparison to previously examined UDPGlcDHs[12] and may suggest either that CalS8 contributes to the production of other cellular reagents or that portions of the calicheamicin deoxysugar pathways may be pyrimidine indiscriminate. The CalS8 steady-state kinetic analysis is indicative of a ping-pong mechanism and gave Michaelis constants, K_m ($K_{m\text{-}UDP\text{-}Glc} = 0.180 \pm 0.012$ mM and $K_{m\text{-}NAD} = 0.50 \pm 0.023$ mM) consistent with previously reported UDPGlcDHs.[8] The observed CalS8 product inhibition profiles[13] are also consistent with an ordered bi-, uni-, uni-, bimolecular ping-pong mechanism first proposed for the bovine UDPGlcDH.[8e] As further confirmation, an alignment between CalS8 and the structurally defined *Streptococcus pyogenes* UDPGlcDH reveals that all 23 residues previously identified as critical for UDPGlcDH structure and function are conserved in CalS8.[8i] Campbell et al. also postulated that Arg 244 in the *S. pyogenes* UDPGlcDH defines substrate specificity as this residue is conserved in all UDPGlcDHs but is replaced by lysine in GDPManDHs (GDP = guanosine diphosphate). Interestingly, the residue in CalS8 in the position corresponding to Arg 244 in the *S. pyogenes* UDPGlcDH is Lys 340, which follows the GDPManDH paradigm. CalS8 lacks GDPManDH activity, therfore we conclude that this position has little influence on general sugar dehydrogenase substrate specificity.

In the context of calicheamicin, the *calS8* functional assignment provides information directly relevant to understanding the biosynthesis of deoxypentose ligands of secondary metabolites. Three of the four calicheamicin sugar ligands (Scheme 1, **1**, sugars A, B and D) are 6-deoxy derivatives, which are expected based on well-established precedent to derive from a common committed 6-deoxy-4-keto-sugar nucleotide intermediate **11**, as presented in Scheme 2.[1] Since there is no known route of interconversion between **11** and the determined product of CalS8, **8**, we are left to conclude that CalS8 must commit UDP- and/or dTDP-Glc to a unique pathway that leads to the remaining aminodideoxypentose of calicheamicin. Thus, like the 4,6-dehydratases in 6-deoxyhexose formation, GlcDHs may be the key enzyme necessary to commit sugar nucleotides to deoxypentoses in these pathways. Known chemistry can be invoked to postulate the pathway from **8** to **10**, which requires five additional enzymes: a glucuronate carboxylyase,[14] a 2,3-dehydratase and 2,3-enolyl reductase,[15] a pyranosyl aminotransferase,[16] and an *O*-methyltransferase.[17] Since sugar biosynthesis throughout secondary metabolite producers often follows common themes, we propose that this may be a general model for understanding the biosynthesis of other functionalized deoxypentose-bearing natural products, which include, but are not limited to, those illustrated in Scheme 1.



Scheme 2. *Proposed biogenesis of the four deoxysugars within the calicheamicin aryltetrasaccharide. The reaction catalyzed by CalS8 is highlighted in the box.*

Experimental Section

Materials: All culture media, assay, and biochemical reagents were purchased from Difco (Detroit, MI), Fisher Scientific (Pittsburgh, PA), or Sigma (St. Louis. MO). Fast performance liquid chromatography (FPLC) was performed on an automated Amersham-Pharmacia Biotech LCC-501-plus FPLC instrument (Piscataway, NJ). HPLC was performed on a RAININ Dynamax SD-200 instrument controlled with

Dynamax HPLC software. Routine mass spectra were recorded on a PE SCIEX API 100 LCMS mass spectrometer and HRMS was accomplished by the University of California, Riverside Mass Spectrometry Facility. Protein concentrations were determined by using the Bio-Rad (Hercules, CA) Protein Assay, with bovine serum albumin as the standard.

Expression, purification, and properties of CalS8: The *Micromonospora calS8* gene was PCR amplified from cosmid clone JT-13a (34,35), subcloned into the vector pMAL-c2, and overexpressed as a maltose-binding-protein fusion species (mbp – CalS8) in *E. coli* (XL-1-Blue). The cells (8.0 g per 2 L culture broth) were harvested 2 h after induction with isopropyl-β-D-thiogalactopyranoside, resuspended in buffer A (80 mL; 50 mM tris(hydroxymethyl)aminomethane (Tris) – HCl, 200 mM NaCl, 1 mM ethylenediaminetetraacetate, pH 7.5), and disrupted by on-ice sonication in short pulses of 30 seconds ($\times$ 4). After centrifugation (10000 $\times$ g, 15 min), the resulting supernatant was resolved on amylose resin pre-equilibrated with buffer A (2.0 $\times$ 10 cm, 1 mL min^{-1}). The column was washed with 12 column volumes buffer A and then eluted with buffer A that contained maltose (10 mM). Four 9-mL fractions containing mbp – CalQ were combined and concentrated to a final volume of 7 mL.

Enzyme assay, kinetics, and product inhibition: CalS8 activity was assayed by following the corresponding conversion of NAD^+ to NADH at 340 nm ($\varepsilon = 6220\,M^{-1}$) on a Beckman DU 7400 UV/Vis spectrophotometer as previously described.[8g] All assays were performed at 30 °C in Tris – HCl (50 mM, pH 7.5) containing dithiothreitol (2 mM, 1 mL total volume). Initial velocities were measured during the first three minutes after initiation with CalS8 (2.4 $\times$ 10^{-3} U). One unit (U) is defined as the amount of enzyme required to produce 1 µmol NADH at 30 °C in 3 minutes. The substrate concentrations for given initial velocity studies were 0.3 – 1.3 mM UDP-Glc and 0.1 – 0.5 mM NAD^+. Product inhibition studies were performed by inclusion of a range of fixed concentrations of either UDP-GlcA (0.02 – 0.15 mM) or NADH (6.0 µM – 20 µM) in the assay buffer and variation of the concentration of either UDP-Glc or NAD^+ in the presence of saturating concentrations of the other substrate (2 mM). Initial kinetic and inhibition data was analyzed by the best fit to the Michaelis – Menton equation calculated with KaleidaGraph software.

Reaction product analysis: The supernatant from representative assays was also analyzed by HPLC. Samples (20 µL) were resolved on a Sphereclone 5µm SAX column (150 $\times$ 4.6 mm) fitted with a guard column, with a linear gradient (50 – 200 mM phosphate buffer, pH 5.0, 1.5 mL min^{-1}, A_{275nm}). Under these conditions, the retention times for commercially available UDP-Glc and UDP-GlcA were 6.7 min and 11.3 min, respectively. The CalS8 product from UDP-Glc co-eluted with commercially available UDP-GlcA and the identity of the product was confirmed by HRMS.

Substrate and cofactor specificity: To evaluate substrate and cofactor specificity, saturating levels of other sugar nucleotides (1 mM)[10, 11] or $NADP^+$ (1 mM) were assayed as previously described for wild-type substrates. For $NADP^+$ assays, CalQ activity was assayed by following the corresponding conversion of $NADP^+$ to NADPH at 340 nm ($\varepsilon_{340} = 6220\,M^{-1}\,cm^{-1}$).

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[9] This specific activity is on the same order as that of Sigma liver UDPGlcDH, determined with our assay to be 7.0×10^{-2} U mg^{-1}.

[10] These nucleotide diphosphosugar surrogates include commercially available dTDP-Glc, CDP-Glc, GDP-Glc, ADP-Glc, UDP-Man, UDP-Gal, and a variety of "unnatural" sugar nucleotides synthesized by chemoenzymatic routes[11].

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[12] The best of which report a 16% conversion of dTDP-α-D-glucose[8g].

[13] Analysis with UDP-α-D-GlcA as the inhibitor and UDP-α-D-Glc as the variable substrate revealed competitive inhibition ($K_{I\text{-}UGlcA} = 0.054 \pm (8.8 \times 10^{-4})$ mM). Alternatively, when NAD^+ was the variable substrate or NADH was used as the inhibitor and UDP-α-D-glucose as the variable substrate, similar noncompetitive patterns were observed. NADH was found to be a competitive inhibitor for NAD^+ ($K_{I\text{-}NADH} = 2.7 \pm 0.030$ µM).

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