Published in final edited form as:

Chembiochem. 2014 July 7; 15(10): 1418–1421. doi:10.1002/cbic.201402119.

Characterization of the calicheamicin orsellinate C2-O-methyltransferase CalO6

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Abstract

While bacterial iterative type I polyketide synthases (iPKSs) are now known to participate in the biosynthesis of a small set of diverse natural products, the subsequent downstream modification of the resulting polyketide products remains poorly understood. Toward this goal, we report functional characterization of the putative orsellinic acid C2-O-methyltransferase (CalO6) involved in calicheamicin biosynthesis. This study suggests C2-O-methylation precedes C3-hydroxylation/methylation and C5-iodination and to require a coenzyme A- (CoA) or acyl carrier protein- (ACP) bound substrate.

Keywords

S-adenosylmethionine; iterative PKS; polyketide; acyl carrier protein; orsellinic acid; AdoMet; SAM

Bacterial iterative type I PKSs (iPKSs) are single polypeptides comprised of a series of fused distinctly functional domains that operate in a progressive, iterative fashion. These multifunctional enzymes assemble structurally diverse mono- or bicyclic- aromatic polyketides (**Scheme 1**)^[1] from acyl Co-enzyme A (CoA) precursors where subsequent modification/utilization of the aromatic core has been proposed or demonstrated to be dependent upon a dedicated acyltransferase (AT) (chlorothricin,^[2] pactamycin,^[3] avilamycin,^[4] tiacumicin B,^[5] and calicheamicin^[6]) or via adenylated intermediates (maduropeptin,^[7] polyketomycin,^[8] azinomycin B,^[9] and neocarzinostatin,^[10]). A primary point of structural divergence of iPKS-derived aromatic polyketides derives from downstream ‘tailoring’ modifications (hydroxylation, epoxidation, acylation, glycosylation, transamination, halogenation and/or methylation) of the resulting polyketide scaffold. Published work to date suggests the fundamental timing and/or biochemical basis of these subsequent modifications to also be widely variant and poorly understood. For example, the naphthoic acid *O*-methyltransferase (*O*-MT) NcsB1 in neocarzinostatin biosynthesis operates on the free naphthoic acid moiety prior to adenylation^[10c] while MdpB1-catalyzed C-

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methylation during the biosynthesis of maduropeptin utilizes a CoA-tethered 6-methylsalicylic acid substrate.^[7b] In contrast, TiaM-catalyzed halogenation occurs as the last step in tiacumicin B maturation, after the iPKS-derived aromatic component has been attached to nearly mature natural product.^[5] Thus, additional fundamental studies are needed to provide a general understanding of the factors that dictate the order and substrate preference of post-iPKS events.

A total of six enzymes (CalO1-CalO6) are postulated to participate in constructing the iPKS-derived modified orsellinic acid (OSA, 2,4-dihydroxy-6-methylbenzoic acid) component of the prototype naturally-occurring enediyne calicheamicin (**Scheme 2**). These include: CalO5, the iPKS OSA synthase; CalO2, the P450 hydroxylase; CalO3, the putative flavin-dependent halogenase; and CalO1/O6, putative AdoMet-dependent aromatic *O*-MTs. While CalO1 and CalO2 ligand-binding and crystallography studies implicated the requirement of thioester-conjugated (CoA- or ACP-) substrates for CalO1-catalyzed methylation and CalO2-catalyzed hydroxylation reactions,^[11] additional biochemical characterization of these, or the other, OSA-related enzymes in this pathway has been restricted by a lack of suitable substrates or knowledge regarding the timing of each enzymatic step. Herein we report the biochemical characterization of the calicheamicin OSA C2-*O*-MT CalO6 using *S*-*N*-acetylcysteaminy OSA (SNAC-OSA) as a substrate. This study confirms the regiospecificity of CalO6 and, in conjunction with the prior work on CalO1/O2, offers additional insights regarding the possible sequence of biosynthetic events of the key aromatic component of the calicheamicin aryltetrasaccharide.

For biochemical characterization, *N*-His₆-CalO6 was overproduced in *E. coli* BL21(DE3) and purified by affinity chromatography. In initial attempts, the free acids 2,4-dihydroxy benzoic acid (**6**), salicylic acid (**7**), 5-iodo-OSA (**8**), OSA (**9**), 3,4-dihydroxy benzoic acid (**10**) or 5-iodosalicylic acid (**6**) failed to turnover in presence of CalO6. Based on prior ligand binding studies that revealed the P450 hydroxylase CalO2 to bind SNAC-linked substrate mimics,^[11a] the corresponding SNAC-linked substrates (**1-5**, respectively; **Figure 1A**) were subsequently synthesized and assessed as substrates for CalO6. From this putative substrate set, only SNAC-OSA (**4** in **Figure 1A**) resulted in methylated products in the presence of CalO6. Specifically, two products (**Figure 1B**, **P1** and **P2** with retention times of 15.3 min and 20.0 min, respectively) were observed, both confirmed as mono-methylated SNAC-OSA analogs by high-resolution mass spectrometry (HRMS *m/z* 306.07603 [*M* + Na]⁺; calcd for C₁₃H₁₇NO₄S, 306.07705 [*M* + Na]⁺). For full characterization of products, the CalO6 reaction was scaled to provide ~900 μg (30%) and ~400 μg (13%) of **P1** and **P2**, respectively, after purification. Subsequent NMR spectroscopy revealed **P1** and **P2** as SNAC-2-methoxy-OSA and SNAC-4-methoxy-OSA, respectively (**Figure 1C**). The inability of CalO6 to utilize SNAC-2,4-dihydroxy-benzoic acid (**1** in **Figure 1A**) or SNAC-5-iodo-OSA (**3** in **Figure 1A**), implicates CalO6 the first step among the putative OSA tailoring reactions en route to calicheamicin. As ligand-binding studies previously revealed iodination to be critical for CalO2 binding, we put forth the putative sequence of events as illustrated in **Scheme 2**.

The CalO6 steady-state kinetic parameters in the context of **P1** and **P2** formation were determined based upon product formation monitored by HPLC (**Table 1**). Importantly,

compared to typical kinetic values reported for aromatic *O*-MTs,^[10c, 7b] the CalO6 K_M values reflected in this study are >10-fold higher while the corresponding k_{cat} values are ~1000-fold lower, suggesting the SNAC conjugates to be rather poor mimics of the putative native (CoA- or ACP-) substrate conjugate. However, this analysis revealed a ~3-fold kinetic bias toward **P1** formation, consistent with C2-OH methylation as the preferred CalO6 regiospecificity.

A CalO6 BLAST search revealed 39% sequence identity with the mitomycin 7-*O*-MT MmcR,^[12] 37% with the neocarzinostatin *O*-MT NcsB1^[10d] and 34% with the calicheamicin sugar 4'-*O*-MT, CalO1^[11b] (see supporting information **Figure S1**). To further explore the basis of CalO6 regiospecificity, a CalO6 homology model was constructed based upon MmcR using Swiss-Model (**Figure 2**).^[13] In most MTs, general acid/base catalysis contributes to rate acceleration wherein an active-site His participates as a catalytic base, as exemplified by the rebeccamycin 4'-*O*-MT, RebM.^[14] Sequence alignment (see supporting information **Figure S1**) implicates His252-Glu311 as the CalO6 catalytic diad and, consistent with this, the putative active-site base His252 is within hydrogen-bonding distance (3.1 Å) of Glu311 in the homology model. Manual docking of **4** and SAH to position the favored C2-OH within appropriate conformation for methylation (**4** C2-OH-His252 side chain imidazole, 3.1 Å; **4** C2-OH- SAH thiol 3.1 Å) invokes an unfavorable distance for C4 methylation (**4** C4-OH-His252 side chain imidazole, 6.6 Å; **Figure 2**). While this is consistent with a bias toward C2-*O*-methylation, the perceived suboptimal binding and slow turnover of **4** by CalO6 may allow for 'slipping' within the active and corresponding misalkylation of the substrate mimetic **4** (*i.e.*, C4-*O*-methylation rather than C2-*O*-methylation).

In summary, we have characterized CalO6 as the calicheamicin OSA C2-*O*-MT. This work implicates a dependence upon a thioester-conjugated substrate, the nature of which, while anticipated to be either ACP-based or CoA-based, cannot be gleaned from the current study. Re-examination of the calicheamicin gene cluster reveals no apparent gene encoding for a dedicated ACP or CoA ligase, suggesting the ACP domain within the orsellinate synthase CalO5 (**Scheme 2**) may serve as the putative substrate carrier for some or all subsequent tailoring reactions. In conjunction with the ligand-binding studies conducted with CalO2, the current study suggests C2-*O*-methylation to be an initiating tailoring event. From a more global perspective, prior studies to elucidate the aryltetrasaccharide glycosylation sequence of events failed to demonstrate **4** C4-*O*-rhamnosylation.^[15] While not confirmatory, when considered in the context of the current CalO6 study and previous CalO1/CalO2 work, the prior glycosylation studies are consistent with the currently favored hypothesis that orsellinate halogenation and *O*-methylation occur prior to aryltetrasaccharide assembly in the context of calicheamicin biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the School of Pharmacy Analytical Instrumentation Centre (University of Wisconsin-Madison) for analytical support. This work was supported by NIH RO1 CA84374 (JST) and the National Center for Advancing Translational Sciences (UL1TR000117).

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Scheme 1.

Representative natural products that contain aromatic polyketides moieties (highlighted in dark color) synthesized by bacterial iterative type I PKS.

Scheme 2.

The proposed pathway for OSA biosynthesis and modification *en route* to calicheamicin. The architecture and domain organization of the iPKS (CalO5) is illustrated at the top and the key CalO6-catalyzed C2-OH methylation reaction is highlighted within the box. Based upon the CalO6 biochemical studies described, 'R' is anticipated to be an ACP or CoA thioester.

Figure 1.

Range of substrates assessed in this study with the CalO6 substrate SNAC-OSA (**4**) and CalO6 reaction products **P1** and **P2** highlighted within the box. **B.** HPLC chromatogram of a representative CalO6 reactions with **4** at 37 °C for 1 h (i: SAM; ii: **4**; iii-v: CalO6 reactions containing 8, 16 and 24 µg of enzyme, respectively). Peaks labeled with **S**, **P1** and **P2** are substrate, SNAC-OSA, SNAC-2-methoxy-OSA and SNAC-4-methoxy OSA, respectively and SAH and/or SAM degradation products are denoted by stars (*) **C.** Sections of the **P1** and **P2** NOESY spectra (mixing time = 300 ms) where arrows in **A** indicate key NOE connections.

Figure 2. CalO6 homology model based upon mitomycin-7-*O*-methyltransferase, MmcR (PDB: 3GWZ) with the distances between the putative catalytic histidine (His252) and hydroxyl groups of C2- and C4- of SNAC-OSA indicated. In the stick model, SAH and SNAC-OSA are shaded dark, and CalO6 residues proposed to take part in the catalysis are in light grey.

Table 1

Kinetic parameters for CalO6 using variable SNAC-OSA (0.1 - 5 mM) and SAM (0.1 - 4 mM) in 25 mM Tris, 5 mM MgCl₂, pH 8.0 at 35 °C.

Product	Constant Substrate	Varied Substrate	$k_{\text{cat}} \times 10^{-3}$ (min ⁻¹)	K_M (mM)	$k_{\text{cat}} / K_M \times 10^{-3}$ (mM ⁻¹ min ⁻¹)
P1	SAM	SNAC-OSA	1.03 ± 0.03	1.27 ± 0.08	0.81
	SNAC-OSA	SAM	0.94 ± 0.03	0.30 ± 0.03	3.13
P2	SAM	SNAC-OSA	0.33 ± 0.01	1.31 ± 0.11	0.25
	SNAC-OSA	SAM	0.36 ± 0.01	0.56 ± 0.06	0.64
Total	SAM	SNAC-OSA	1.35 ± 0.03	1.27 ± 0.07	1.06
	SNAC-OSA	SAM	1.29 ± 0.03	0.35 ± 0.04	3.69