Enediyne Biosynthese

DOI: 10.1002/anie.201206462

Environmental Control of the Calicheamicin Polyketide Synthase Leads to Detection of a Programmed Octaketide and a Proposal for Enediyne Biosynthesis**

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The family of enediyne natural products exhibit potent antitumor and antibiotic activity, in addition to displaying structural features that are quite remarkable both as a synthetic challenge and a biosynthetic enigma.[1] Members of this group typically have a structural component designed for DNA recognition and a carbocyclic core containing an alkene (or masked alkene) flanked by triple bonds. It is this enediyne moiety that gives this class of compounds their name and their potent activity.

As was predicted by classical isotope-labeling studies,[2] biosynthetic gene-cluster sequences for both 9- and 10-membered ring subclasses of enediynes confirm that the carbon skeletons of the enediyne core structures are made by a family of type I iterative polyketide synthases (PKSs).[3] These multifunctional enzymes use a single set of domains to catalyze the repeated condensation and reductive processing of activated malonyl units, analogous to mammalian fatty acid synthesis. In polyketide biosynthesis, however, the extent of processing in each round of two-carbon extension is controlled by the PKS, allowing for incorporation of ketones, hydroxy groups, and double bonds into the growing polyketide chain. Intermediates remain covalently tethered to the PKS throughout the chain elongation process, complicating the investigation of these programmed events.[4] Furthermore, the repeated use of a single set of domains in iterative systems often makes it difficult to predict the direct PKS product, particularly if it is highly modified en route to the final natural product.

Despite the characterization of several polyketides that can be made by enediyne PKSs, their biosynthetically relevant products have yet to be determined. Likewise, the timing and mechanism of divergence to the three core structure families (Figure 1 A) remains unclear.[5] New findings reported herein reveal environmental factors that dramatically affect the chemistry of the enediyne PKS CalE8 and may shed light on why the earliest steps of enediyne biosynthesis have eluded characterization. Moreover, the discovery of a structurally unique metabolite provides insight that can be used to construct a plausible mechanism to account for enediyne core assembly.

Each enediyne biosynthetic cluster has a discrete thioesterase (TE), usually located just downstream of the enediyne PKS. Previously, heptaene **1**[6] and methylhexaenone **2**[7] were found to be products of 9- and 10-membered enediyne PKS + TE coexpression systems, respectively, leading others to speculate that differentiation to the distinct subclasses begins at this stage. Results from our in vitro experiments with the PKS and TE for the 10-membered enediyne calicheamicin not only confirmed production of **2** and identified its labile precursor **3**, but also revealed that heptaene **1** is a major product of both subclasses (Figure 1 B).[8] This observation led us to suggest that the enediyne PKSs alone are not responsible for determining the fate of immature polyketides. This deduction has since been strengthened by further experiments,[9] providing additional support for our hypothesis that one or more accessory enzymes act in concert with the PKS to dictate the cyclization patterns of polyketide precursors into the enediyne core structures.

Shen, Thorson, and co-workers assayed five enediyne PKS-TE pairs and found heptaene **1** as a major product from all combinations, indicating that the enediyne TEs are interchangeable even across subclasses.[9b] Earlier complementation experiments in PKS knockout strains of 9-membered enediyne producers demonstrated that PKSs from other 9-membered enediyne systems can effectively substitute for the native synthase, restoring antibiotic production.[6] Notably, however, the PKS that produces the 10-membered calicheamicin ring could not complement these 9-membered enediyne-producing knockout strains. From these experiments it can be inferred that the free heptaene is not a precursor of the enediyne natural products, strongly suggesting that heptaene **1** is a shunt product. Accordingly, we set out to establish the inherent chemistry of the calicheamicin enediyne PKS CalE8 in vivo, in the absence of its cognate TE. Herein, we report the first identification of fermentation products from the heterologous expression of an enediyne PKS alone, including a new octaketide polyene that has a β-hydroxy acid moiety. Our results highlight the marked effects of environmental factors on the chemistry of the enediyne PKS CalE8 and may provide mechanistic insight into the early steps of calicheamicin biosynthesis.

CalE8 was expressed heterologously in *Escherichia coli* for an extended period of time to allow for the accumulation

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[**] We are deeply grateful to K. L. Fiedler and Prof. R. J. Cotter of the Middle Atlantic Mass Spectrometry Laboratory at the Johns Hopkins School of Medicine and to Shimadzu Scientific Instruments, Inc. (Columbia, MD) for their technical expertise and access to a Shimadzu LC-IT-TOF. This work was supported by NIH Grant ES001670.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201206462.

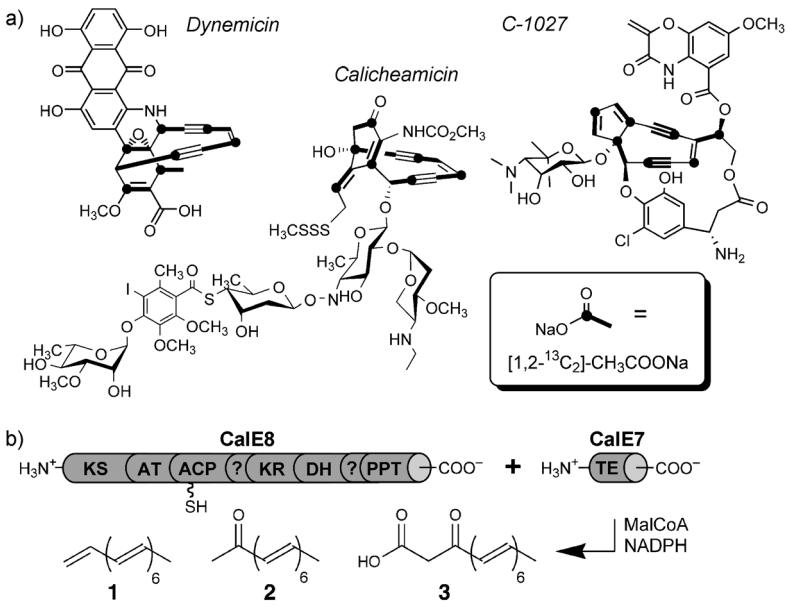


Figure 1. a) Selected enediyne antibiotics, with polyketide chains highlighted for each of the three core-structure families. Enrichment patterns from sodium [1,2-$^{13}C_2$] acetate feeding experiments[2] are shown. b) Octaketides produced by the calicheamicin enediyne PKS CalE8 and thioesterase CalE7. KS, β-ketoacyl synthase; AT, acyl transferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydrase; PPT, phosphopantetheinyl transferase; MalCoA = malonyl coenzyme A; NADPH = nicotinamide adenine dinucleotide phosphate.

of fermentation products. Organic extracts of cell pellets were partitioned, and the base-soluble fraction was subjected to high-resolution liquid chromatography–mass spectrometry (HR-LCMS) analysis. Cells expressing CalE8-C211A, a point mutant incapable of polyketide chain extension,[8] were cultured and processed in parallel with cells expressing wild-type protein in order to provide a negative control sample.

Cells expressing CalE8 were found to contain a number of new metabolites, the majority of which fall into a series differing by "C_2H_2" increments based on exact mass analysis. These data suggest a set of polyketides that vary by individual extensions of the growing chain, each processed to the alkene state. Based on the chemical behavior, relative retention times, and UV/Vis spectra of these compounds, we have identified this family of products as the carboxylic acid-conjugated polyenes **4a–e** (Figure 2). These metabolites are consistent with the expected capabilities and restrictions of CalE8, which has ketoreductase (KR) and dehydrase (DH) domains but lacks an enoyl reductase (ER), required for saturation of the polyketide backbone (see Figure 1 B for the PKS domain organization). The collection of chain lengths observed may reflect interception of the growing polyketide chain by endogenous *E. coli* metabolites or enzymes, suggesting that enediyne PKSs are not as highly processive as, for example, non-reducing iterative PKSs.[10] This conclusion is further supported by reports of truncated pyrone products released from the enediyne PKSs during in vitro reactions.[7,8] The intermediate chain-length products reported herein could also be indicative of a relatively low rate of malonyl-CoA regeneration, in comparison to the rate of consumption by CalE8 under fermentation conditions.

Through our efforts to improve titers, we found that protecting the cultures from ambient light substantially altered the polyketide output of CalE8. A previously unknown metabolite, the β-hydroxy acid **5**, was found as a major product of cultures grown in the dark (Figure 3). Initial analysis of this compound by LC–MS gave an apparent molecular formula of $C_{16}H_{18}O_2$, suggesting that it is a structural isomer of the octaketide **4e**, which contains a heptaene moiety conjugated to the carboxylic acid headgroup. However, because of the drastic polarity difference between the two polyketides, shown by their retention times on HPLC, we were suspicious of this assignment. Furthermore, the UV/Vis spectrum of the new product bore the signature absorption and fine structure of an isolated hexaene moiety.[11] We reasoned that an allylic alcohol such as the β-hydroxy acid **5** might easily undergo in-source dehydration during MS analysis. Indeed, HR-LCMS analysis in negative-ion mode provided an exact mass for a parent compound with a molecular formula $C_{16}H_{20}O_3$. Upon closer inspection, this β-hydroxy acid was also observed in extracts from cultures grown in

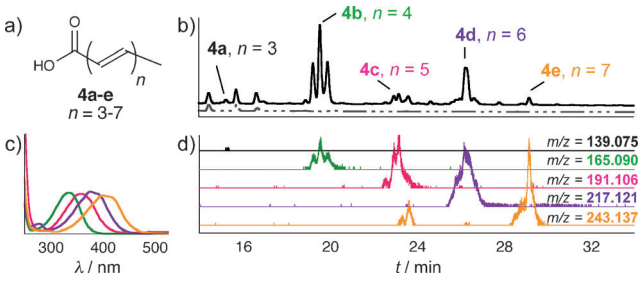


Figure 2. a) Chemical formula for the acid-conjugated polyenes **4a–e**. b) HPLC chromatograms of fermentation extracts at 325 nm (black: CalE8; dashed gray: CalE8-C211A (negative control)). c) UV/Vis spectra for **4b–e**, extracted from the HPLC diode array; normalized based on λ_{max}. Spectrum for **4a** unavailable owing to overlapping signals at lower wavelengths. d) Extracted ion chromatograms of observed masses corresponding to MH^+ for **4a–e**.

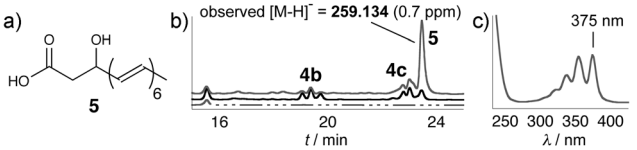
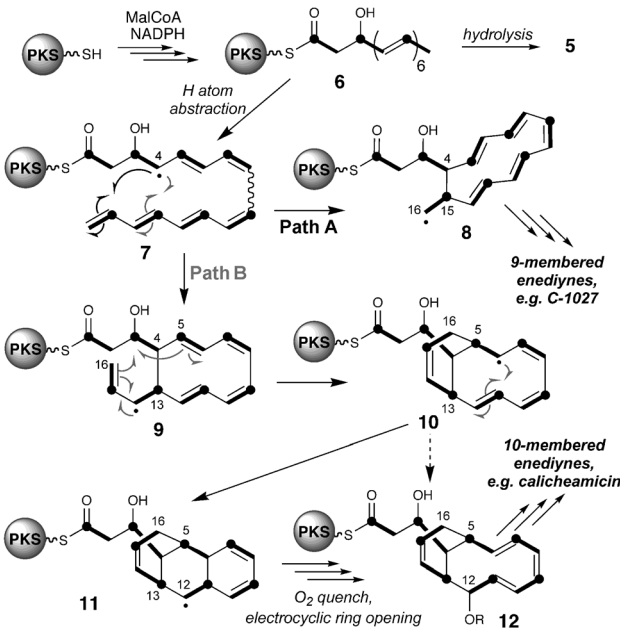


Figure 3. a) Chemical formula of β-hydroxy acid **5**. b) HPLC chromatograms recorded at 370 nm comparing fermentation extracts from cultures protected from or exposed to light (gray: CalE8, dark induction; black: CalE8, light induction; dashed gray: CalE8-C211A (negative control)). c) UV/Vis spectrum for **5**, extracted from the HPLC diode array.

ambient light conditions, although only in trace amounts (Figure 2d, 23 min; observed $[M-H_2O+H]^+ = 243.137$).

The β-hydroxy acid **5**, which is the full chain length expected for the calicheamicin core structure, provides the first definitive example of programmed control of polyketide processing by CalE8. Its production cannot be ascribed to aberrant activity owing to limited substrate availability (like β-keto acid **3** and methylhexaenone **2**) or to potential over-processing (like heptaene **1**). Guo and co-workers reported the characterization of a nonaketide pyrone obtained from in vitro reconstitution of SgcE, the PKS producing the 9-membered enediyne involved in the biosynthesis of C-1027.[12] This pyrone also displays evidence of PKS processing control, maintaining a hydroxy group at the equivalent position to that of **5**, suggesting that this programming may be common to enediyne PKSs from both subfamilies. Accordingly, we wish to speculate how a β-hydroxy acid, such as **5**, might provide a previously unrecognized starting point from which to access cyclic enediyne core structures: selective maintenance of the β-hydroxy group guarantees that the conjugated polyene system terminates at the γ-position, which is the single common carbon involved in cyclization for all enediyne families, based on isotope-labeling studies.[2] Furthermore, the hydroxy group may contribute to substrate recognition by downstream biosynthetic enzymes in the pathway, distinguishing **5** from the polyene acid series **4a**–**e**.



Scheme 1. A proposed biosynthetic mechanism inspired by detection of β-hydroxy acid **5**. R = H, OH, or aryltetrasaccharide (see Figure 1A).

Although more research is necessary to construct a definitive biosynthetic pathway for the enediyne core structures, we propose a mechanism that uses the intrinsic propensity of conjugated polyenes to facilitate radical cyclizations. Because **5** has not been observed in extracts from in vitro reconstitution reactions, further biosynthetic reactions are likely to occur from thioester **6**, its PKS-bound precursor (Scheme 1). Another biosynthetic enzyme(s) in the cluster could abstract a hydrogen atom from the terminus of **6**, yielding resonance equivalent **7**, which can cyclize to **8** or **9** with regiospecificity directed by the accessory enzyme(s). In Path A (C4–C15), the resulting radical in **8** is likely quenched; downstream events would be responsible for further cyclization and maturation to the core structures of the 9-membered enediynes. Although dynemicin is a 10-membered enediyne, its cyclization pattern[2b] indicates that it shares this connectivity with the 9-membered enediynes and may also proceed down Path A. For Path B (C4–C13), continuing the radical cascade from **9** to **10**/**11** facilitates the second cyclization reaction (C5–C16) necessary to form the bicyclic ring system in calicheamicin. Quenching with O_2 at the C12 position (which originates from the C2 atom of acetate) offers a compelling mechanistic rationale for the observed oxygenation at the corresponding position of calicheamicin. A downstream electrocyclic ring-opening reaction would alternatively reveal a 10-membered triene ring substrate for desaturation to the enediyne motif.

This mechanistic proposal results in cyclized intermediate **12** that bears a strong resemblance to the core of calicheamicin. We have suggested radical chemistry to achieve this, although there are a number of other options that would take advantage of the reactivity of the conjugated polyene in **6**, including cationic and electrocyclic processes. Arriving at a much lower oxidation state than the natural product is unavoidable, given that we are restricted by the partially reduced state of the observed polyene. Early predictions favoring aldol chemistry to cyclize linear enediyne precursors, a common strategy in polyketide biosynthesis, would have produced intermediates much closer to the final oxidation state. This would avoid the need to re-oxidize at sites that were reduced by the PKS. Although more straightforward, a biogenetic proposal that relies on programmed retention of carbonyl groups in the early rounds of polyketide chain extension is not compatible with the experimental evidence at hand. All of the reported products of the enediyne PKSs contain terminal polyenes. This suggests that a biosynthetic strategy is in place to raise the oxidation level later in the pathway, in addition to the rest of the modification steps required to reach the final enediyne core structures. Further experimental work is necessary to determine the nature and timing of these events.

The striking increase in the levels of the β-hydroxy acid **5** when fermented in the dark leads us to speculate that light exposure may inhibit the early steps of calicheamicin biosynthesis. One possible rationale is that the PKS-bound polyene intermediates are photoisomerized and are subsequently non-productive or inefficient substrates for further chain elongation. Under this theory, protection from light may assist in directing flux to the full chain length expected for the calicheamicin enediyne core. We cannot, at this point, rule out the converse: that the true biosynthetic pathway of calicheamicin relies on a specific photochemical reaction, perhaps producing a substrate for an accessory enzyme that would act upon the growing chain. Regardless, the dramatic effect of light on the fermentation output has direct ramifications for experimental design, at the very least. This technical observation may be the lynchpin that has prevented

the identification of one or more accessory enzymes that interact with the enediyne PKSs.

Received: August 10, 2012
Published online: October 8, 2012

Keywords: biosynthesis · enediynes · fermentation products · natural products · polyketides

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