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Production of Octaketide Polyenes by the Calicheamicin Polyketide Synthase CalE8: Implications for the Biosynthesis of Enediyne Core Structures

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Enediyne antitumor antibiotics are notable for their distinctive molecular architecture and unique DNA cleavage mechanism.[1,2] Formation of an aryl diradical capable of abstracting two hydrogen atoms, one from each strand of DNA, ultimately results in double-stranded DNA scission. While total syntheses of several of these antibiotics have been achieved,[3−5] knowledge of their origins in nature had, until recently, been limited to classical labeling studies revealing their polyketide folding patterns.[6−8] Five biosynthetic gene clusters are now known for this class of natural products,[9−13] but the key steps of enediyne core assembly remain undefined.

A family of highly reducing iterative type I polyketide synthase (PKS) enzymes is responsible for synthesizing the carbon skeletons of the enediyne core structures.[9,10] These PKSs are highly conserved among the enediynes yet are quite distinct from other PKSs, especially in their domain organization (Figure 1A).[14] The enediyne natural products are classified as either 9- or 10-membered, according to the size of the smallest carbocyclic ring containing the triple bonds. The neocarzinostatin (NCS) chromophore and calicheamicin γ_1^I are prominent examples from each group (Figure 1B). Determining the point at which the biosynthetic pathways to these two families diverge is an important first step in understanding this biosynthetic problem.

Recent reports[15,16] of two different metabolites isolated from heterologous coexpression experiments of enediyne PKSs (one from each of the two structural categories) with their separately encoded thioesterases have led to the suggestion that the 9- and 10-membered enediynes diverge biosynthetically at the PKS stage (Figure 1C). We now report the observation of both of these products arising from a single biosynthetic system, calling into question both the assertion of divergence at this point and the legitimacy of either of these products as a true precursor on the path to calicheamicin.

Heptaene **1** was isolated by Shen and co-workers[15] from the heterologous coexpression of NcsE, the NCS enediyne PKS, and the putative thioesterase NcsE10, marking the first reported product of an enediyne PKS. This hydrocarbon polyene was also found when homologues SgcE and SgcE10, located in the biosynthetic gene cluster for the 9-membered enediyne C-1027, were coexpressed. As a consequence of these observations, **1** was purported to be a common intermediate in the biosynthetic pathways to the 9-membered enediynes.

More recently, the methyl hexaenone **2**, derived from the actions of the calicheamicin PKS CalE8 and its downstream thioesterase CalE7, was characterized.[16] Liang and co-workers argued that a divergence of the biosynthetic pathways to the 9- and 10-membered enediynes begins at the PKS stage based on the apparent control of oxidation states by each PKS during the last round of chain extension. They also noted differences in the UV/vis spectra of the purified SgcE and CalE8 proteins themselves as further proof of differentiation occurring at this point.

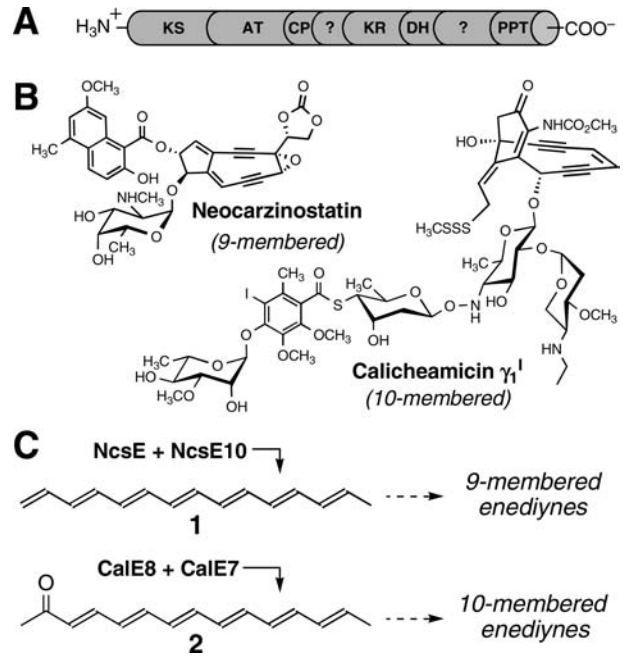

Figure 1. (A) Enediyne PKS domain organization. AT, acyl transferase; CP, acyl carrier protein; PPT, phosphopantetheinyl transferase. KS, KR, and DH are defined in the text. (B) Selected enediyne anticancer antibiotics. (C) Current literature proposals for enediyne biosynthesis. Polyenes **1** and **2**, isolated from coexpression experiments, have been advocated as precursors to their respective enediyne families. The olefin stereochemistry depicted has not been verified for all double bonds.

We have cloned the full length enediyne PKS *calE8* and its associated thioesterase *calE7* from the genomic DNA of *Micromonospora echinospora* ssp. *calichensis* for *in vitro* reconstitution experiments with purified enzymes. As has been noted by others, CalE7 assists in the efficient release of enzyme-bound polyketides.[16,17] Purification of CalE8 following heterologous expression in *Escherichia coli* yields a bright yellow solution of soluble, active protein. We found the UV/vis spectrum to reveal both a broad absorbance in the 350−500 nm range and the fine structure indicative of a hydrocarbon polyene system, similar to that seen with SgcE (Figure 2A). This feature is harder to discern at lower concentrations but is present nonetheless.

Cysteine 211, identified as a catalytic residue for the β-ketoacyl synthase (KS) domain of CalE8,[14] was replaced with alanine to give CalE8-C211A, a KS° mutant of CalE8 that is colorless when purified from *E. coli* (Figure 2B). The KS domain is responsible for catalyzing the decarboxylative condensations of malonate units necessary for polyketide chain extension. Accordingly, inactivation of this domain affords a PKS that is incapable of polyketide

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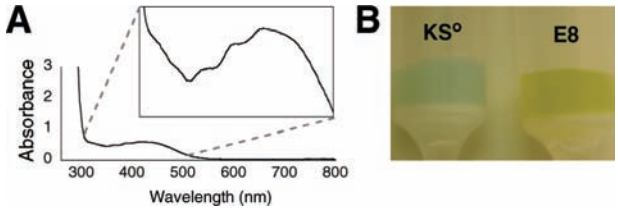


Figure 2. (A) UV/vis spectrum of the PKS CalE8 as purified from heterologous expression in *E. coli*. Inset shows expansion from 305 to 480 nm. (B) Colorless CalE8-C211A (KS°) and yellow CalE8 (E8) bound to Ni-NTA resin prior to elution.

synthesis, providing an ideal control for our investigations. This mutant also proved essential for optimization of sample preparation and LC/MS analysis of the frequently labile reaction products.

The methyl ketone **2** characterized by Liang and co-workers was observed early in our *in vitro* studies of calicheamicin biosynthetic enzymes as well (see Supporting Information). We have additionally been able to identify its anticipated precursor, 3-oxohexadeca-4,6,8,10,12,14-hexaenoic acid (**3**), in extracts from the same *in vitro* reaction of CalE8 and CalE7. A broad peak in the HPLC chromatogram was found to have an exact mass of $m/z = 259.1373$ (MH^+), providing the first direct evidence for this presumed hydrolysis product of CalE8. The decarboxylation of this labile compound to **2** over time can be monitored by HPLC, further supporting its assignment as the β-keto acid **3** (Figure 3). Although this observation does not establish whether CalE7 is responsible for catalyzing this decarboxylation as has been proposed,[17] it does prove that this transformation can proceed nonenzymatically.

In addition to corroborating the production of methyl ketone **2** and identifying its immediate precursor **3**, we have observed heptaene **1**, the proposed common progenitor to 9-membered enediynes, as a prominent product of the *in vitro* reaction of CalE8 and CalE7, the biosynthetic enzymes for the 10-membered enediyne calicheamicin. The distinctive UV/vis signature and HPLC retention behavior in combination with exact mass confirmation identify the peak at 24 min as heptaene **1** (Figure 4). The hydrocarbon heptaene is unstable to a variety of conditions including light, heat, and acid—behavior that has been noted for the carotenoids and other hydrocarbon polyenes.[18] Liang and co-workers quenched their *in vitro* reactions with trifluoroacetic acid; this is likely why they were not able to observe the sensitive heptaene.

The discovery of heptaene **1**, previously seen only in 9-membered enediyne systems, as a major product of the calicheamicin enzymes CalE8 and CalE7 brings a sharper perspective to enediyne biogenetic theory. It is the first experimental evidence indicating that differentiation of immature polyketides to 9- or 10-membered enediyne precursors is not solely inherent to the enediyne PKSs. This observation vivifies the argument for a more convergent view

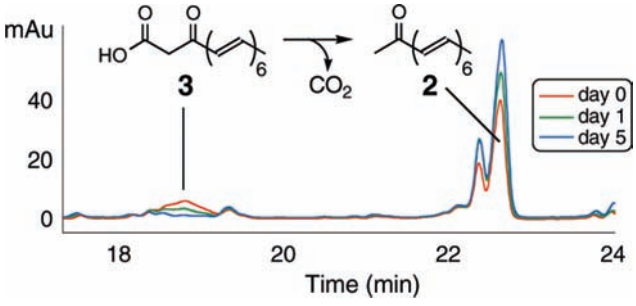


Figure 3. HPLC chromatogram of *in vitro* reaction products of CalE8 and CalE7, recorded at 375 nm. Decarboxylation of β-keto acid **3** to methyl ketone **2** occurs spontaneously over time in reaction extracts.

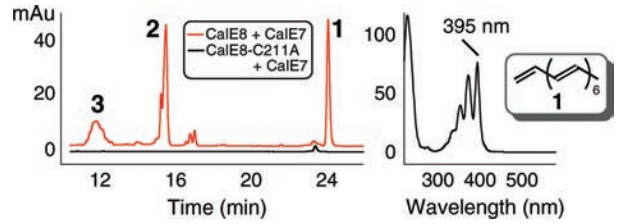


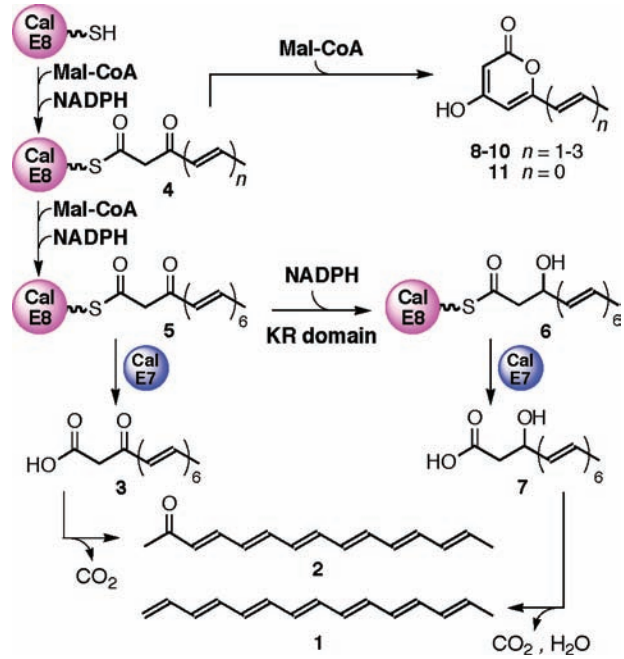
Figure 4. Heptaene **1** is synthesized from malonyl-CoA by CalE8 and CalE7, along with octaketides **2** and **3**. Left panel: HPLC chromatogram of a typical in vitro reaction, recorded at 375 nm. Right panel: Absorption spectrum of the HPLC peak at 24 min showing the signature fine structure of a hydrocarbon heptaene.

of enediyne biosynthesis in which the highly similar PKS enzymes from both 9- and 10-membered systems share a common blueprint for polyketide assembly.[19]

The observed octaketide products **1**−**3** can be rationalized using only the expected functions of CalE8 and CalE7, or any enediyne PKS/thioesterase pair (Scheme 1). CalE8 and the enediyne PKSs are predicted to have β-ketoreductase (KR) and dehydrase (DH) domains, which catalyze the NADPH-dependent reduction of the β-ketothioester intermediates and dehydration of the resulting hydroxyl groups, respectively. Execution of these two processing steps on successive rounds of ketide extension would lead to polyene-conjugated β-ketothioesters represented by **4**. If continued until the full programmed chain length was reached, the product of the final condensation would be enzyme-bound octaketide **5**, which contains a hexaene moiety. Using the thioesterase activity of CalE7, β-ketothioester **5** could be released from CalE8 directly, leading to detected products β-keto acid **3** and methyl ketone **2** (*via* decarboxylation of **3**). Alternatively, if the KR domain of CalE8 catalyzes one final reduction during this last round of chain extension to give β-hydroxythioester **6**, hydrolysis by CalE7 would release hypothetical free acid **7**. Dehydration and decarboxylation would lead to heptaene **1**, which has now been observed as another major component of the *in vitro* reaction of CalE8 and CalE7.

In accord with our own observations, CalE8 has also recently been reported to produce a series of compounds having the

Scheme 1. Biosynthetic Rationale for the Observed Products



molecular formula $C_XH_XO_3$ during *in vitro* reactions.[17] These have been suggested to correspond to tetra-, penta-, and hexaketide pyrone derailment products (**8**−**10**). These pyrones could arise from offloading of PKS-bound intermediates similar to **4**, whereby one more round of chain extension and a simple cyclization would release the 2-pyrones. In addition to these products, we also see the simple triacetic acid lactone (**11**).

Iterative PKSs are generally regarded as highly processive, releasing truncated or incorrectly processed polyketides in only trace amounts unless denied an essential component of the synthetic process.[20,21] The simultaneous production of two different 15-carbon linear polyenes by CalE8 with the assistance of thioesterase CalE7 indicates that the calicheamicin PKS is not selectively stopping at the ketone oxidation state in the final round of chain extension. The synthesis of truncated polyketides **8**−**11** alongside octaketides **1** and **2** leads us to suspect that CalE8 may be missing some necessary biosynthetic element. Canonical nonribosomal peptide synthetase (NRPS) and multidomainal PKS systems typically have all of the required modifying domains already encoded in their sequences. There is, however, a growing body of literature elucidating biosynthetic enzymes that act *in trans* on carrier protein-tethered intermediates, often prior to full chain extension.[22−24] The absence of such accessory enzymes frequently results in biosynthetic errors, which can manifest in a variety of ways.

In the landmark case of lovastatin biosynthesis, Hutchinson, Vederas, and co-workers were able to show that without LovC, a *trans*-acting enoyl reductase (ER), the lovastatin nonaketide synthase LovB does not synthesize a full-length product. Instead, LovB releases conjugated hexa- and heptaketide pyrone derailment products,[25] strikingly similar to the probable structures for CalE8 truncation products **8**−**10**. A recent case from tenellin biosynthesis describes the characterization of aberrant polyketide products that reach the full programmed chain length but have incorrect oxidation patterns when the mixed NRPS-PKS TenS is deprived of its interaction with a *trans*-acting ER.[26] The octaketide polyenes **1**, **2**, and **3** produced by the calicheamicin biosynthetic enzymes may also represent an instance of errant PKS behavior in the absence of required auxiliary enzymes, despite the fact that they reflect the full polyketide chain length expected for the calicheamicin aglycone.

We take the observations of both the methyl ketone **2** and the heptaene **1** from *in vitro* reactions of CalE8 and CalE7 as evidence that neither is the branchpoint metabolite to 10-membered and 9-membered enediynes, respectively. The similar production of the methyl ketone **2** from a 9-membered system, in addition to already-observed heptaene **1**, would bolster the argument for a more convergent model of enediyne biosynthesis and support our contention that divergence to 9- or 10-membered products results from the action of one or more accessory enzymes acting in concert with the enediyne PKS.

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Supporting Information Available: Detailed experimental procedures, HPLC conditions, and MS data for **1**−**3** and **8**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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