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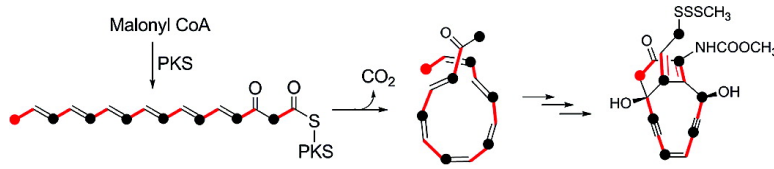

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Characterization of a Carbonyl-Conjugated Polyene Precursor in 10-Membered Enediyne Biosynthesis

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Enediyne natural products produced by soil and marine microorganisms are characterized by a structurally unique 9- or 10-membered enediyne moiety (Figure 1a). Intercalation of enediyne natural products into chromosomal DNA leads to double-stranded scission through an oxidative radical mechanism.[1] Largely due to the DNA cleavage activity, enediyne natural products can induce cell apoptosis and are some of the most potent natural antitumor agents discovered so far.[2] In contrast to the well-understood DNA cleavage mechanism, the biosynthetic mechanism for the enediyne moiety largely remains to be elucidated. The recent sequencing of the gene clusters for the biosynthesis of several enediyne natural products suggested that an iterative polyketide synthase (PKS) and a dozen auxiliary enzymes are involved in the biosynthesis of the enediyne moiety.[3–6] Further genomic screening uncovered a conserved "minimal enediyne PKS gene cassette" for enediyne biosynthesis.[7] When this paper was being prepared, a linear polyene precursor was reported for the 9-membered enediyne of C-1027.[8] On the basis of the study of the iterative PKS (CalE8) and two auxiliary enzymes involved in calicheamicin biosynthesis, here we report a novel carbonyl-conjugated polyene that is likely to be the precursor of the 10-membered enediyne moiety.

In the gene cluster for calicheamicin biosynthesis, the gene *CalE8* encodes a PKS (CalE8) that contains seven domains including a novel PPTase domain and a domain with unknown function (Figure 1b); whereas *CalE7* was predicted to encode a hot-dog fold thioesterase (TE).[3,9] CalE8 was expressed in *Escherichia coli* and purified as a dimeric protein with a bright yellow coloration (Figure 2 a, left panel). Extensive dialysis or extraction with organic solvent after protein denaturation could not separate the yellow pigment from the protein, suggesting that the yellow pigment is covalently linked to the protein. The yellow pigment could be gradually removed from CalE8 by incubation with purified TE. This observation suggested that the yellow pigment is covalently attached to the phosphopantetheinyl group of the ACP domain by a thioester linkage and likely to be synthesized by CalE8 in *E. coli*. This is reasonable because it is possible for CalE8 to activate itself by self-phosphopantetheinylation with the integrated PPTase domain.[9] Subsequently, we found that the coexpression of CalE8 and TE produced colorless PKS, suggesting that the release of the PKS product by TE occurs in *E. coli* as well. Interestingly, the purified TE from coexpression is colored, and the yellow pigment could be readily extracted into organic solvent. These observations indicated that some of the yellow pigments are bound noncovalently by TE after release from the PKS. In contrast to the polyene generated by the homologous SgcE in C-1027 biosynthesis,[8] which can be found in large quantities in the yellow cell debris, the cell debris from the coexpression of CalE8/TE was pale and most of the yellow pigment generated by CalE8 seemed to be associated with TE rather than cell debris. HPLC analysis of the pigment extracted from the

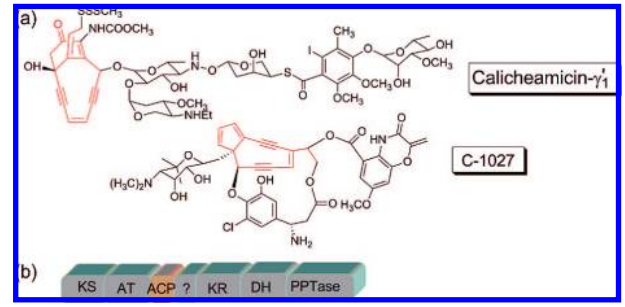

Figure 1. (a) Structures of two representative enediyne natural products with the 10- and 9-membered enediyne moieties highlighted. (b) Predicted domain composition of CalE8. (AT: acyl transferase, KS: ketoacyl synthase, ACP: acyl carrier protein, KR: ketoreductase, DH: dehydratase, PPTase: phosphopantetheinyl transferase).

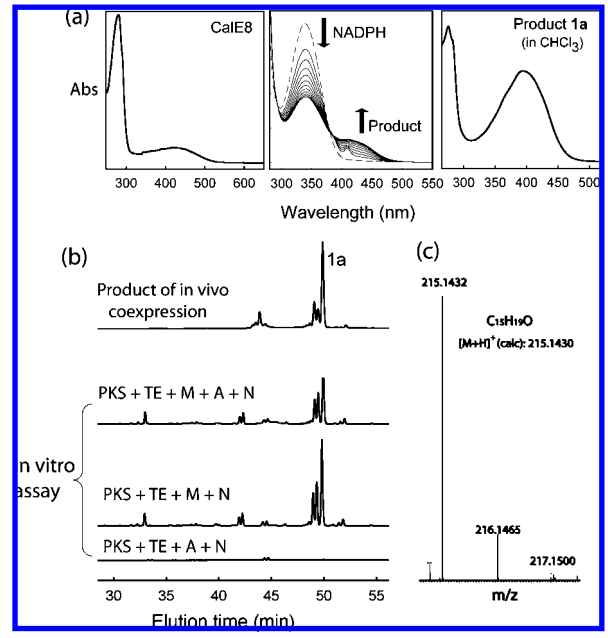

Figure 2. (a) Absorption spectra of CalE8 (left) and **1a** (right) as well as activity assay for CalE8 by absorption spectroscopy (middle panel, see text). (b) HPLC analysis of in vivo and in vitro products (M: malonyl CoA; A: acetyl CoA; N: NADPH). (c) Mass spectrometry analysis of **1a**.

colored TE showed that it consists of a major component (**1a**) and several other minor components (Figure 2b, trace 1).

The activities of CalE8 and TE were further examined by in vitro activity assay. The incubation of the colorless CalE8 and TE with acetyl CoA, malonyl CoA, and NADPH readily generated products that absorbed in the range of 400−450 nm (Figure 2a, middle panel). HPLC analysis of the reaction mixture revealed that

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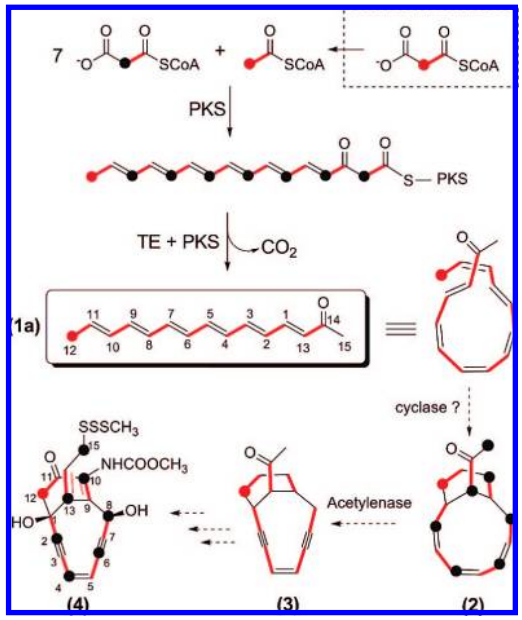

Figure 3. A possible biosynthetic mechanism for the 10-membered enediyne of calicheamicin. The incorporated acetate units are highlighted.

the major product of the in vitro reaction shares the same elution time and absorption spectrum with the in vivo product (**1a**) extracted from the coexpressed TE (Figure 2b). The reaction can proceed without the addition of acetyl CoA, indicating that CalE8 is capable of generating the "starter" acetyl CoA from malonyl CoA by an intrinsic decarboxylation mechanism.

The pigment extracted from the coexpressed TE and in vitro reaction mixture was analyzed using LC-HRMS. The observed *m*/*z* of 215.1432 (MH^+) for **1a**, and the two minor components eluted immediately before **1a** suggested that they are most likely geometrical isomers sharing the same molecular formula of $C_{15}H_{18}O$ (calcd *m*/*z* 215.1430 (MH^+), Figure 2c). The fraction that contains mainly **1a** was separated and purified by HPLC for structure determination using NMR spectroscopy. $^1H-^1H$ COSY and TOCSY NMR established **1a** to be a linear carbonyl-conjugated polyene (3,5,7,9,11,13-pentadecen-2-one), in contrast to the simple polyene pentadecaheptaene ($C_{15}H_{18}$) precursor reported for 9-membered enediynes[8] (Figure 3 and Figure S4). The presence of a conjugated carbonyl group is in agreement with the absence of the signature fine structure in the absorption spectrum for simple polyenes (Figure 2a, right panel) and the presence of a strong 1676 cm^{-1} band in IR spectrum.[10] The 2D NMR spectra also revealed that the sample contains at least another geometrical isomer (**1b**) in addition to the major component **1a** (see Supporting Information).

The characterization of **1a** lends support to the hypothesis that the precursor of the 10-membered enediyne is a 15 carbon-containing linear molecule.[11,12] The proposed biosynthetic mechanism with **1a** as the precursor for 10-membered enediyne is consistent with the observations from the isotope labeling of esperamicin; that is, C_2, C_4, C_6, C_8, C_{10}, C_{12}, C_{13}, and C_{15} originated from the C_2 of the acetate unit, and the two carbons of the same yne group (C_2 and C_3, C_6, and C_7) originated from different acetate units[11,12] (Figure 3). Our results also indicated that C_{15} is likely to be part of the chain termination unit but not the starter unit. In addition, the absorption spectrum of CalE8 lacks the fine structure observed for SgcE,[8] suggesting that the CalE8-tethered intermediate differs from that of SgcE. We propose that the biosynthetic intermediate tethered to CalE8 is a carbonyl-conjugated polyene with a carbonyl group located at the C_{14} site, in contrast to the proposed 3-hydroxyl hexadecahexaene intermediate for SgcE (Figure 3 and Figure S4). Thus, these results suggest that the divergence of the biosynthetic pathways for 9- and 10-membered enediynes begins at the PKS stage. It remains to be elucidated how the homologous CalE8 and SgcE generate different products by controlling the oxidation state in the last round of chain extension. A decarboxylation reaction is likely to be involved in the generation of the 15-carbon products for both PKSs. It is not clear whether the decarboxylation is catalyzed by the PKS or TE.

The linear polyene **1a** needs to undergo oxidization and cyclization to generate the 10-membered enediyne. To further explore the biosynthetic mechanism, we expressed a putative acetylenase encoded by the gene *CalU15*.[3] The protein was purified as a monomeric protein that exhibits a broad shoulder peak around 330 nm, resembling the diiron-oxo cluster-containing desaturases (Figure S2).[13] The coexpression of the putative acetylenase together with CalE8 and TE did not generate any new product. Likewise, the inclusion of the putative acetylenase in the in vitro activity assay in the presence of protein or small-molecule electron donors did not affect the formation of **1a**. Although it remains to be confirmed, we speculate that the oxidation may occur after the cyclization of the linear intermediate (Figure 3). The next challenge would be the identification of the protein responsible for the cyclization of the linear polyene.

In summary, we have characterized a linear carbonyl-conjugated polyene that could be the common precursor for the 10-membered enediynes. The different precursors for 9- and 10-membered enediynes exemplify nature's ingenuity in tailoring the iterative PKSs for building the unique enediyne structures.

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Supporting Information Available: Experimental details and NMR and absorption spectroscopic data are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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