Enediyne Biosynthesis

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Identification of a Nonaketide Product for the Iterative Polyketide Synthase in Biosynthesis of the Nine-Membered Enediyne C-1027**

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Enediyne natural products are potent antitumor antibiotics characterized by an enediyne core containing two acetylenic groups conjugated to a double bond in a nine- or ten-membered carbocycle.[1] Their structural complexity poses a formidable hurdle to the elucidation of their biosynthetic origin. In recent years, studies from the Shen and Thorson research groups have revealed a unified paradigm for enediyne biosynthesis,[2–6] which involves a conserved iterative type I polyketide synthase (PKS) responsible for synthesis of the carbon skeleton of the enediyne cores.[7–9] However, most enzymatic steps involved in assembly of the enediyne cores remain unknown.

Characterization of the conserved iterative PKS enzymes has led to different perspectives on enediyne core biosynthesis. Shen et al. isolated an all-*trans* heptaene **1** (Figure 1 A) from heterologous cells expressing the PKS SgcE from the gene cluster of C-1027 and proposed it as a potential precursor for the nine-membered enediyne core.[10] Subsequently, Liang et al. identified a **1**-resembling hexaenone as the product of the PKS CalE8 from the gene cluster of the ten-membered calicheamicin,[11] but later suggested heptaene **1** as the common precursor of both nine- and ten-membered enediyne cores in a separate study.[12] However, recharacterization of CalE8 by Townsend et al. suggests that neither heptaene **1** nor the hexaenone is a true precursor of calicheamicin and that a *trans*-acting enzyme may be needed for proper functioning of the PKS.[13]

Similarly, a more recent in vivo functional investigation suggests a universal PKS-bound intermediate for modification by other enzymes en route to the enediyne cores.[14] In these studies, the polyene-forming activity of the PKSs was found to be strictly dependent on a conserved hot-dog thioesterase. Herein, we report that SgcE synthesizes a nonaketide product distinct from all the reported polyenes without reliance on the thioesterase, which suggests a possible new biosynthetic route to the enediyne cores.

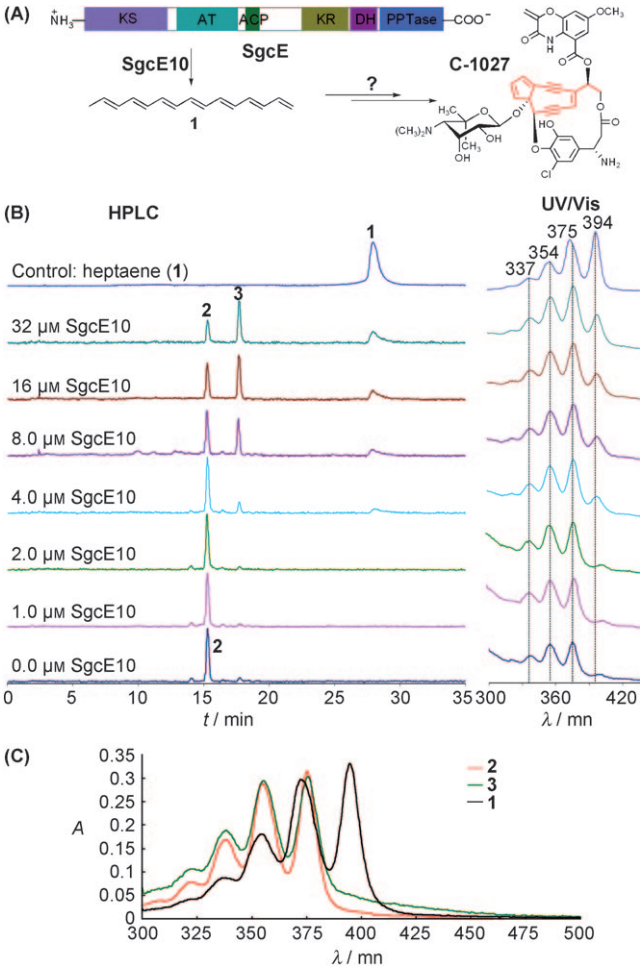

Figure 1. A) Domain organization of the enediyne PKS SgcE and its previously proposed involvement in the biosynthesis of C-1027. ACP, acyl carrier protein; PPTase, phosphopantetheinyl transferase; see text for definitions of KS, AT, KR, and DH. B) HPLC and UV/Vis analyses of the ethyl acetate extracts from enzymic reactions containing 2.5 μM SgcE at pH 7.0. The HPLC chromatograph and UV/Vis spectrum of the same extract are given in the same color. The UV/Vis detector was set at 355 nm in the HPLC analyses. C) UV/Vis spectra of the products **1**–**3**.

SgcE was both expressed alone and co-expressed with the hot-dog-fold thioesterase SgcE10 in *Escherichia coli*. Heptaene **1** was readily isolated from cells co-expressing SgcE and SgcE10 (see the Supporting Information), but was not detected in cells expressing SgcE only. Moreover, the isolated

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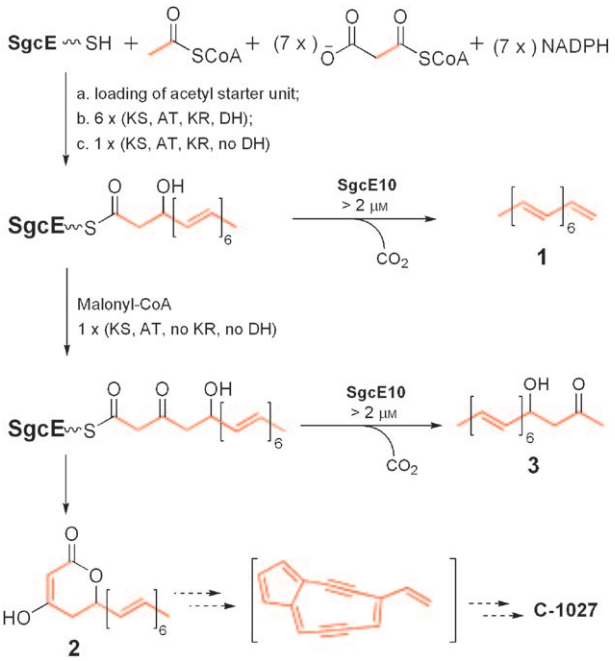
SgcE was yellowish with a broad absorption from 350 to 500 nm when it was expressed alone, but was essentially colorless when it was co-expressed with the thioesterase. These observations are consistent with the results in the previous in vivo functional characterization of SgcE.[10]

Both the yellowish and colorless forms of SgcE are active in assays at pH 7.0 using acetyl-coenzyme A (CoA), malonyl-CoA, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as substrates. Extraction of the reaction mixture by ethyl acetate affords a yellowish solution exhibiting multipeak UV/Vis absorptions, which overlap well with most parts of the absorption spectrum of heptaene **1** except for the most intense peak at 394 nm (Figure 1B). Only one product (**2**) with essentially the same optical absorptions is detected in the extract by HPLC methods (Figure 1C), thus indicating that it is the only extractable, chromogenic product of the enzymatic reaction.

This outcome is not changed by inclusion of the thioesterase SgcE10 in the enzymatic reaction at a concentration up to 2 μM. However, further increase of the SgcE10 concentration in the enzymatic reaction steadily decreases the yield of product **2** and leads to the production with increasing yields of two additional products, **3** and a compound with a retention time of 27 minutes (Figure 1B); the latter was confirmed to be identical to heptaene **1** by HPLC co-injection and their identical UV/Vis spectra. Similar results were obtained for reactions at pH 8.0, in which the products **3** and **4** were formed with higher yields at lower SgcE10 concentrations (see the Supporting Information, Figure S3). Products **1** and **3** cannot be formed from product **2** in the presence of SgcE10.

Product **2** was isotope-labeled using ^{13}C-malonic acid and determined to be a nonaketide, 5,6-dihydro-4-hydroxy-6-(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*-tridecahexaen-1-yl)-2*H*-pyran-2-one, by various NMR techniques in combination with mass spectrometric analysis of the turnover product before and after palladium-catalyzed hydrogenation. Product **2** exists in equilibrium between a major enol tautomer (Scheme 1) and a minor keto tautomer. Meanwhile, product **3** was determined to be 2-oxo-5*E*,7*E*,9*E*,11*E*,13*E*,15*E*-heptadecahexaen-4-ol by mass spectrometry and NMR techniques. It contains the same π-conjugation system as product **2**, consistent with the observation of identical UV/Vis spectra for these two compounds (Figure 1C). Notably, the six conjugated double bonds in both products are all in the *trans* configuration, since *J*-resolved 2D NMR experiments show that the splitting of the olefinic proton signals is closely similar to that of heptaene **1**, the hexaenone congener of which has been confirmed to contain only *trans*-configured double bonds.[12]

The structure of product **2** allows us to deduce the biosynthetic logic of the iterative type I PKS SgcE. After being primed with an acetyl group as the starter unit, the PKS catalyzes eight consecutive decarboxylative Claisen condensations of malonyl CoA thioester. The β-ketoacyl synthase (KS), acyl transferase (AT), ketoreductase (KR), and dehydratase (DH) domains of the synthase function sequentially in each of the first six repetitive polyketide chain elongation cycles to form the six conjugated double bonds. Subsequently, the DH domain loses its catalytic activity in the last two elongation cycles and the KR domain is inactive in the last



Scheme 1. Proposed catalytic functions for SgcE and SgcE10 in biosynthesis of the enediyne antibiotic C-1027.

cycle, probably because of the unique chain-length control mechanism of the PKS, thus leading to the formation of 3-oxo-5-hydroxy-6*E*,8*E*,10*E*,12*E*,14*E*,16*E*-octadecahexaenoyl-*S*-SgcE. Finally, a thermodynamically favorable δ lactonization affords the nonaketide product **2** (Scheme 1). This SgcE catalytic process does not require the assistance of a second enzyme and is not affected by SgcE10 at a low to moderate concentration, contrary to the suggestion that SgcE10 is indispensable to SgcE activities.[10,12]

The origins of products **1** and **3** are also implicated in their structures. These products are formed from hydrolysis of the SgcE-bound intermediates by SgcE10 and subsequent decarboxylation of the β-oxo-carboxylates (Scheme 1). As such, both compounds are aberrant products formed only in the presence of the thioesterase at high concentrations. It is not clear why the intermediates giving rise to **1** and **3** are more susceptible than others to hydrolysis by SgcE10 at pH 7.0. However, other intermediates in the polyketide synthesis appear to be also accessible by SgcE10 to form more side products at pH 8.0 (see the Supporting Information, Figure S3).

The behavior of SgcE10 in the synthesis of nonaketide **2** is typical of a type II thioesterase commonly found in polyketide and nonribosomal peptide syntheses.[15,16] The thioesterase is thus likely to be a proofreading enzyme to regenerate the mis-acylated PKS under physiological conditions. Its aberrant activity to hydrolyze the normal biosynthetic intermediates at high concentrations is consistent with the finding of heptaene **1** in cells co-expressing both SgcE and SgcE10 but not in cells expressing SgcE only.[10] The absence of products **2** and **3** in SgcE-expressing cells may be because of their degradation by host enzymes. Moreover, the failure to detect products **2** and **3** in a previous HPLC analysis of the

SgcE products may result from the setting of the UV/Vis detector at 400 nm,[12] at which neither of these products shows absorption.

Product **2** is structurally distinct from the minor pyrone side products formed from the short-chain biosynthetic intermediates en route to the mature polyketide product.[13,17] As the only molecule synthesized efficiently at a physiological pH value, it is a potential precursor in the biosynthesis of the C-1027 enediyne core. This finding suggests a probable new biosynthetic route to the nine-membered enediyne core of C-1027, although it is not immediately clear how the four extra carbon atoms in the nonaketide are shed in the process. Since the enediyne PKSs are interchangeable within the nine-membered family,[10] this newly found nonaketide is also a potential precursor for other nine-membered enediyne cores. This likely role of the nonaketide does not contradict the isotope-labeling pattern of neocarzinostatin.[18] To provide direct evidence for this potential role, product **2** was isotope-labeled for incorporation into the final enediyne natural product. However, this attempt failed probably because of instability of the nonaketide under cell-culturing conditions, consistent with the inability to find this compound in cells expressing the PKS.

In summary, we have redefined SgcE as a specific producer of nonaketide **2**, thus demonstrating that it is an iterative PKS with a distinct chain-termination mechanism in which its functional domains are sequentially disengaged from the polyketide synthesis. In the meantime, we have also shown that the hot-dog protein SgcE10 is most likely a type II thioesterase in the polyketide synthesis. These findings may facilitate elucidation of the intriguing biosynthetic steps leading to the nine-membered enediyne cores. In addition, the clear distinction between this nonaketide and the octaketide polyene products reported for PKSs from the ten-membered enediyne family raises the question again of whether biosynthesis of nine- and ten-membered enediynes diverges at the PKS stage.

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[1] A. L. Smith, K. C. Nicolaou, *J. Med. Chem.* **1996**, *39*, 2103 – 2117.
[2] W. Liu, S. D. Christenson, S. Standage, B. Shen, *Science* **2002**, *297*, 1170 – 1173.
[3] J. Ahlert, E. Shepard, N. Lomovskaya, E. Zazopoulos, A. Staffa, B. O. Bachmann, K. Huang, L. Fonstein, A. Czisny, R. E. Whitwam, C. M. Farnet, J. S. Thorson, *Science* **2002**, *297*, 1173 – 1176.
[4] W. Liu, K. Nonaka, L. Nie, J. Zhang, S. D. Christenson, J. Bae, S. G. Van Lanen, E. Zazopoulos, C. M. Farnet, C. F. Yang, B. Shen, *Chem. Biol.* **2005**, *12*, 293 – 302.
[5] S. G. Van Lanen, T. Oh, W. Liu, E. Wendt-Pienkowski, B. Shen, *J. Am. Chem. Soc.* **2007**, *129*, 13082 – 13094.
[6] Q. Gao, J. S. Thorson, *FEMS Microbiol. Lett.* **2008**, *282*, 105 – 114.
[7] W. Liu, J. Ahlert, Q. Gao, E. Wendt-Pienkowski, B. Shen, J. S. Thorson, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 11959 – 11963.
[8] E. Zazopoulos, K. Huang, A. Staffa, W. Liu, B. O. Bachmann, K. Nonaka, J. Ahlert, J. S. Thorson, B. Shen, C. M. Farnet, *Nat. Biotechnol.* **2003**, *21*, 187 – 190.
[9] G. P. Horsman, S. G. Van Lanen, B. Shen, *Methods Enzymol.* **2009**, *459*, 97 – 112.
[10] J. Zhang, S. G. Van Lanen, J. Ju, W. Liu, P. C. Dorrestein, W. Li, N. L. Kelleher, B. Shen, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 1460 – 1465.
[11] R. Kong, L. P. Goh, C. W. Liew, Q. S. Ho, E. Murugan, B. Li, K. Tang, Z.-X. Liang, *J. Am. Chem. Soc.* **2008**, *130*, 8142 – 8143.
[12] H. Sun, R. Kong, D. Zhu, M. Lu, Q. Ji, C. W. Liew, J. Lescar, G. Zhong, Z.-X. Liang, *Chem. Commun.* **2009**, 7399 – 7401.
[13] K. Belecki, J. M. Crawford, C. A. Townsend, *J. Am. Chem. Soc.* **2009**, *131*, 12564 – 12566.
[14] G. P. Horsman, Y. Chen, J. S. Thorson, B. Shen, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11331 – 11335.
[15] B. S. Kim, T. A. Cropp, B. J. Beck, D. H. Sherman, K. A. Reynolds, *J. Biol. Chem.* **2002**, *277*, 48028 – 48034.
[16] Z.-F. Guo, Y. Sun, S. Zheng, Z. Guo, *Biochemistry* **2009**, *48*, 1712 – 1722.
[17] Z.-X. Liang, *Nat. Prod. Rep.* **2010**, *27*, 499 – 528.
[18] O. D. Hensens, J. L. Giner, I. H. Goldberg, *J. Am. Chem. Soc.* **1989**, *111*, 3295 – 3299.

