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Materials and Methods
SOM Text

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Biosynthesis of the Eneidyne Antitumor Antibiotic C-1027

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C-1027 is a potent antitumor agent with a previously undescribed molecular architecture and mode of action. Cloning and characterization of the 85-kilobase C-1027 biosynthesis gene cluster from *Streptomyces globisporus* revealed (i) an iterative type I polyketide synthase that is distinct from any bacterial polyketide synthases known to date, (ii) a general polyketide pathway for the biosynthesis of both the 9- and 10-membered enediyne antibiotics, and (iii) a convergent biosynthetic strategy for the C-1027 chromophore from four building blocks. Manipulation of genes governing C-1027 biosynthesis allowed us to produce an enediyne compound in a predicted manner.

pher the genetic and biochemical basis for the biosynthesis of structurally complex natural products and to explore ways to make antitumor agents by manipulating genes governing enediyne biosynthesis. Here, we report the cloning, sequencing, and characterization of the complete C-1027 biosynthesis gene cluster from *S. globisporus*, revealing an iterative type I polyketide synthase (PKS) with a previously undescribed domain organization and a convergent biosynthetic strategy for **1** from four biosynthetic building blocks. Manipulation of genes governing C-1027 biosynthesis allowed us to engineer enediyne compounds, as exemplified by deshydroxy-C-1027 (**2**).

We have previously cloned the *sgcAB* genes and demonstrated that they are essential for C-1027 production in *S. globisporus* (12). Because antibiotic production genes commonly occur as a cluster in actinomyces, we set out to identify the C-1027 biosynthesis gene cluster by chromosomal walking from the *sgcAB* locus. An 85-kb contiguous DNA from *S. globisporus* was sequenced and analyzed, revealing 67 open reading frames (ORFs) (see SOM) (Fig. 2A). To determine the boundaries of the C-1027 gene cluster, ORFs at both ends of the sequenced region

C-1027 is a chromoprotein antibiotic produced by *Streptomyces globisporus* and composed of an apoprotein and the C-1027 chromophore (**1**) (Fig. 1) (1). It belongs to the enediyne family of antibiotics, structurally characterized by a unit consisting of two acetylenic groups conjugated to a double bond or incipient double bond within a nine- or 10-membered ring, as exemplified by **1** or calicheamicin γ_1^I , respectively [see supporting online material (SOM) (fig. S1)]. The enediyne antibiotics are potent antitumor agents with a previously undescribed molecular architecture and mode of action (2–5). Although the natural enediynes have seen limited use as clinical drugs mainly because of substantial toxicity, various polymer-based delivery systems or enediyne-antibody conjugates have shown clinical promise and success in anticancer chemotherapy (4–7). A

great challenge will be to develop ways to make new enediynes for mechanistic and clinical studies.

Genetic manipulations of genes governing secondary metabolism offer a promising alternative to preparing complex natural products biosynthetically (8–11). The success of this approach depends on the cloning and genetic and biochemical characterization of the biosynthetic pathways of the metabolites. The enediynes offer the opportunity to deci-

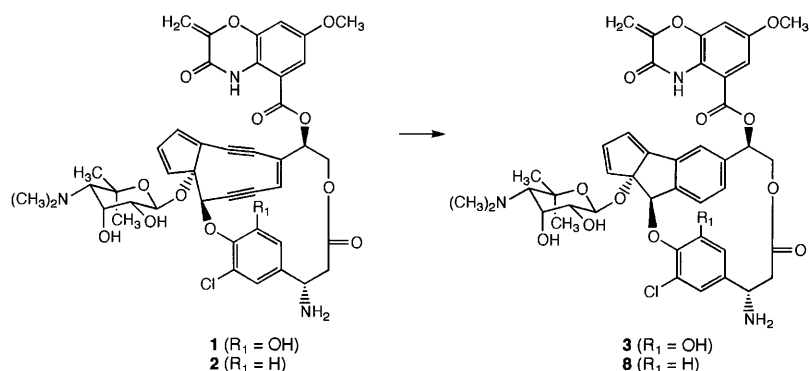


Fig. 1. Structures of the C-1027 (**1**) and deshydroxy-C-1027 (**2**) chromophores and their aromatized products (**3**) and (**8**).

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were subjected to inactivation by gene disruptions (see SOM). Inactivation of genes within the C-1027 gene cluster, as exemplified by *sgcA*, *sgcC*, *sgcC1*, *sgcD6*, and *sgcE*, abolished C-1027 production (Fig. 3), whereas that of genes outside the C-1027 gene cluster, such as ORF(-5), ORF(-3), and ORF54, had no effect on C-1027 production, leading to the assignment of the cluster boundaries at *sgcB1* and *sgcR3*, respectively. C-1027 production was monitored by bioassay against *Micrococcus luteus* and high performance liquid chromatography (HPLC) analysis of **1** (SOM), which undergoes facile Bergman cyclization to yield the aromatized

product (**3**) (Figs. 1 and 3A) (13, 14). The identities of **1** and **3** were confirmed by electrospray ionization-mass spectrometry (ESI-MS) analyses. **1** showed (M + H)⁺ and (M + Na)⁺ ions at *m/z* = 844 and 866, consistent with the molecular formula C₄₃H₄₂N₃O₁₃Cl, and **3** showed an (M + H)⁺ ion at *m/z* = 846, consistent with the molecular formula C₄₃H₄₄N₃O₁₃Cl. Consistent with the structure of **1**, those identified within the C-1027 cluster include 13 genes, *sgcE* to *sgcE11* and *sgcF*, encoding the enediyne core (**4**) biosynthesis; seven genes, *sgcA* to *sgcA6*, encoding deoxy aminosugar (**5**) biosynthesis; six genes, *sgcC* to *sgcC5*, encoding β-amino acid

(**6**) biosynthesis; and seven genes, *sgcD* to *sgcD6*, encoding benzoxazolinat (**7**) biosynthesis (Fig. 2).

Three types of PKSs are known for polyketide biosynthesis in bacteria. Type I and type II systems are known for aliphatic (10) and aromatic polyketides (11), respectively, and type III system is largely known for monocyclic aromatic polyketides (15, 16). Because the enediyne cores bear no structural resemblance to any characterized polyketides, we cannot predict what type of PKS is responsible for their biosynthesis. In fact, it remains controversial whether the enediyne cores are assembled by de novo

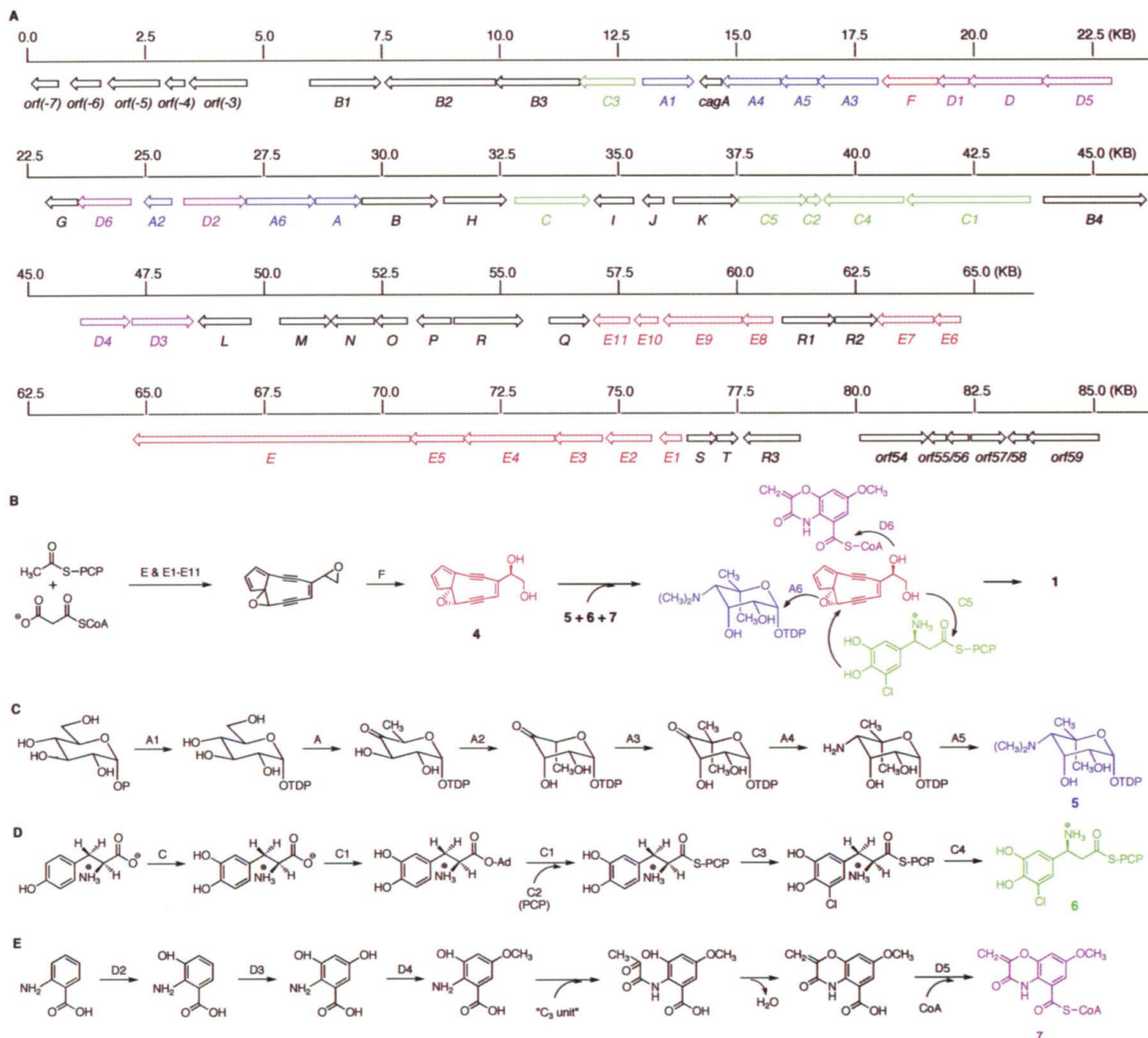


Fig. 2. Organization of the C-1027 biosynthesis gene cluster (A) and biosynthetic hypothesis for the enediyne core and a convergent assembly strategy (B), deoxy aminosugar (C), β-amino acid (D), and benzoxazolinat (E). Proposed functions for individual ORFs are summarized in GenBank

under accession number AY048920. ORFs outside the *sgcB1* to *sgcR3* region are not essential for C-1027 production. Color coding indicates the biosynthesis genes for the enediyne core (red), deoxy aminosugar (blue), β-amino acid (green), benzoxazolinat (purple), and all other genes (black).

polyketide biosynthesis or degradation from a fatty acid precursor, although feeding experiments with ^{13}C -labeled precursors unambiguously established that both the nine- (17) and 10-membered (18, 19) enediyne cores were derived from (minimally) eight head-to-tail acetate units. Strikingly, of the genes identified within the C-1027 cluster, there is only one, *sgcE*, that encodes a PKS. SgcE contains five domains—the ketoacyl synthase (KS), acyltransferase (AT), ketoreductase (KR), and dehydratase domains that are characteristic of known PKSs, and a domain at the COOH-terminus that is unique to enediyne PKSs (20, 21). In addition, a region between AT and KR might contain a putative acyl carrier protein domain (Fig. 4). SgcE could be envisaged to catalyze the assembly of a nascent linear polyunsaturated intermediate from acetyl and malonyl coenzyme A (CoA) in an iterative process, which, upon action of other enzyme activities, is subsequently desaturated to furnish the two yne groups and cyclized to afford the enediyne core. An acetylenase has been reported from the plant *Crepis alpina* and characterized as a non-

heme di-iron protein (22). Although no such homolog was found within the C-1027 cluster, close comparison of the C-1027 gene cluster with that of neocarzinostatin, another nine-membered enediyne antibiotic (21), revealed a group of ORFs (*sgcE1* to *sgcE11*), in addition to *sgcE*, which are highly conserved. SgcE6, SgcE7, and SgcE9 resemble various oxidoreductases; SgcE1, SgcE2, SgcE3, SgcE4, SgcE5, SgcE8, and SgcE11 show either no sequence homology or homology only to proteins of unknown function; and SgcE10 is highly homologous to a family of thioesterases. These enzymes, together with the SgcF epoxide hydrolase, are candidates for processing the nascent linear polyunsaturated intermediate into an enediyne intermediate such as **4** (Fig. 2A). To validate this hypothesis, we inactivated *sgcE* by replacing it with a mutant copy in which the KS domain was replaced with the erythromycin resistance gene, *ermE*. The resultant *S. globisporus* SB1005 mutant strain completely lost its ability to produce **1** (Fig. 3B). Introduction of pBS1019, in which the expression of *sgcE* is under the control of the constitutive *ermE** promoter, into SB1005 restored production of **1** to a level comparable to that of a wild-type organism (Fig. 3C) (see SOM). These findings unambiguously established that C-1027 enediyne core biosynthesis proceeds via a polyketide pathway. Remarkably, the SgcE enediyne PKS exhibits head-to-tail sequence homology (56 identity and 67% similarity) and has an identical domain organization to the CalE8 enediyne PKS that catalyzes the biosynthesis of the 10-membered enediyne core of calicheamicin in *Micromonospora echinospora* (20) (Fig. 4). These results suggest that the nine- and 10-membered enediyne cores share a common polyketide pathway. Type I PKSs that act iteratively to synthesize polyunsaturated polyketides from acetyl and malonyl CoAs have been reported (23, 24). However, the enediyne PKSs as a family are apparently distinct in both structure and mechanism from any bacterial PKSs known to date.

The availability of the gene cluster provides

a basis for investigating the mechanism of C-1027 biosynthesis and for producing new enediyne compounds by manipulating C-1027 biosynthesis genes. The seven deoxy aminoglycoside biosynthesis genes encode a thymine diphosphate (TDP)-glucose synthetase (SgcA1), a TDP-glucose 4,6-dehydratase (SgcA), a TDP-4-keto-6-deoxyglucose epimerase (SgcA2), a C-methyl transferase (SgcA3), an amino transferase (SgcA4), an N-methyl transferase (SgcA5), and a glycosyl transferase (SgcA6). Together, they agree with the enzyme functions that would be required for the biosynthesis of **5** from glucose-1-phosphate (Fig. 2C) and the attachment of **5** to **4** (Fig. 2B). We validated this hypothesis by inactivating *sgcA* (12) (see SOM). The resultant *S. globisporus* SB1001 mutant strain completely lost its ability to produce **1** (Fig. 3D). The six β -amino acid biosynthesis genes encode a phenol hydroxylase (SgcC), a nonribosomal peptide synthetase (NRPS)-adenylation enzyme (SgcC1), an NRPS peptidyl-carrier protein (PCP) (SgcC2), a halogenase (SgcC3), an aminomutase (SgcC4), and an NRPS-condensation enzyme (SgcC5). These enzyme functions agree well with the proposed biosynthetic pathway for **6** from tyrosine (Fig. 2D), which is apparently activated as an aminoacyl-S-PCP for attachment to **4** by SgcC5 (Fig. 2B). Although the precise timing of each reaction remains unknown, sequence analysis of SgcC1 suggests that it activates an α -amino acid (25, 26). The latter prediction is consistent with the finding that covalent tethering of an amino acid as an aminoacyl-S-PCP is a general strategy to sequester, and thus divert, a fraction of the amino acid into secondary metabolism (27). Indeed, inactivation of *sgcC1* resulted in the isolation of an *S. globisporus* SB1003 mutant strain (see SOM) that completely lost its ability to produce **1** (Fig. 3E). The seven benzoxazolinone biosynthesis genes encode the anthranilate synthase I and II subunits (SgcD and SgcD1), a monooxygenase (SgcD2), a P-450 hydroxylase (SgcD3), an O-methyl transferase (SgcD4), a CoA ligase (SgcD5), and an acyltransferase (SgcD6). These enzyme functions support the hypothesis that the biosynthesis of **7** starts from

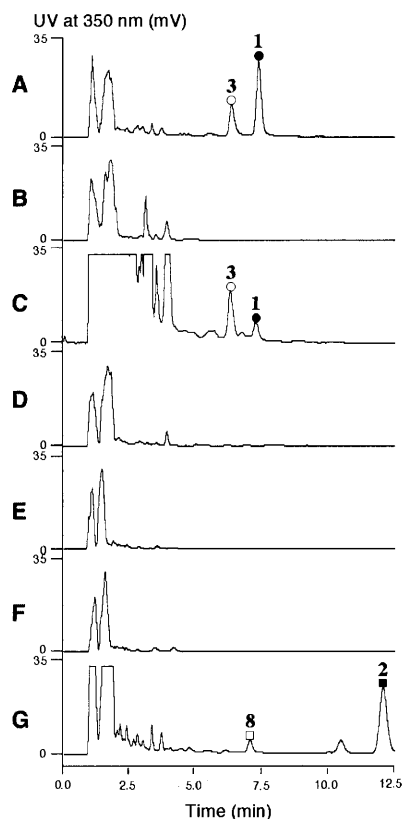


Fig. 3. HPLC analysis of the C-1027 chromophores isolated from *S. globisporus* strains. (A) wild-type, (B) *sgcE* replacement mutant SB1005, (C) SB1005 complemented by pBS1019 that overexpresses *sgcE*, (D) *sgcA* disruption mutant SB1001, (E) *sgcC1* disruption mutant SB1003, (F) *sgcD6* disruption mutant SB1004, and (G) *sgcC* disruption mutant SB1006. Structures for **1**, **2**, **3**, and **8** are shown in Fig. 1.



Fig. 4. Comparison between the SgcE PKS catalyzing the nine-membered enediyne core in C-1027 biosynthesis and the CalE8 PKS catalyzing the 10-membered enediyne core in calicheamicin biosynthesis (20). aa, amino acid; KS, ketoacyl synthase; AT, acyltransferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; and TD, COOH-terminal domain.

anthranilate, a common intermediate from the shikimate pathway (Fig. 2E). The colocalization of SgcD and SgcD1 along with the other C-1027 production genes assures the availability of anthranilate for secondary metabolite biosynthesis. Although it remains unclear what the origin of the C₃ unit is and how it is fused to the anthranilate intermediate to form the morpholinone moiety of **7**, the latter is apparently activated as an acyl-S-CoA for attachment to **4** by SgcD6 (Fig. 2B). To support this hypothesis, we inactivated *sgcD6* (see SOM). The resultant *S. globisporus* SB1004 mutant strain completely lost its ability to produce **1** (Fig. 3F). The fact that the biosynthetic building blocks are activated as aminoacyl-S-PCP, acyl-S-CoA, and nucleotide diphosphosugar; and are attached to the enediyne core by an NRPS condensation enzyme, an acyltransferase, and a glycosyl transferase, respectively, highlights nature's efficiency and versatility in synthesizing complex molecules.

Finally, we inactivated the *sgcC* hydroxylase gene to demonstrate the production of enediyne metabolites by manipulating genes governing C-1027 biosynthesis (see SOM). The resulting *S. globisporus* SB1006 mutant strain still produces a chromoprotein complex that is biologically active, as judged by bioassay against *M. luteus*, but is distinct from **1** upon HPLC analysis (Fig. 3G). The resulting enediyne chromophores were isolated (see SOM) and subjected to ESI-MS analysis. **2** exhibited an (M + H)⁺ ion at *m/z* = 828, consistent with the molecular formula C₄₃H₄₂N₃O₁₂Cl, and **8** showed an (M + H)⁺ ion at *m/z* = 830, consistent with the molecular formula C₄₃H₄₄N₃O₁₂Cl. By comparison with **1** and **3**, **2** and **8** were deduced to be deshydroxy-C-1027 (**2**) and its aromatized product (**8**) (Fig. 1), as would be predicted according to Fig. 2D. Intriguingly, **2** is at least fivefold more stable than **1** at 25°C, in respect to undergoing the Bergman cyclization (see SOM), a property that could be potentially explored in developing C-1027 into a clinically useful drug. We envisage applying methods of combinatorial biosynthesis to the enediyne system for the production of polyketides.

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Supporting Online Material

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Materials and Methods
Fig. S1

21 March 2002; accepted 10 June 2002

The Calicheamicin Gene Cluster and Its Iterative Type I Enediyne PKS

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The enediynes exemplify nature's ingenuity. We have cloned and characterized the biosynthetic locus coding for perhaps the most notorious member of the nonchromoprotein enediyne family, calicheamicin. This gene cluster contains an unusual polyketide synthase (PKS) that is demonstrated to be essential for enediyne biosynthesis. Comparison of the calicheamicin locus with the locus encoding the chromoprotein enediyne C-1027 reveals that the enediyne PKS is highly conserved among these distinct enediyne families. Contrary to previous hypotheses, this suggests that the chromoprotein and nonchromoprotein enediynes are generated by similar biosynthetic pathways.

Calicheamicin γ₁¹ (Fig. 1) from *Micromonospora echinospora* ssp. *calichensis* is the most prominent member of the nonchromoprotein enediyne family and, as an antitumor agent, is > 5000 times as potent as adriamycin (*1*). Calicheamicin has two distinct structural regions: The aryltetrasaccharide is composed of a set of carbohydrate and aromatic units, which delivers the metabolite specifically into the minor groove of DNA (*2*); the aglycone, or "warhead," consists of a highly functionalized bicyclo[7.3.1]tridecadiene core structure

with an allylic trisulfide serving as the initial trigger for warhead cycloaromatization (*3*). Once the aryltetrasaccharide is docked to DNA, aromatization of the bicyclo[7.3.1]tridecadiene core structure, by means of a 1,4-dehydrobenzene-diradical, results in the site-specific oxidative double-strand scission of the targeted DNA (*4*). In vitro and in vivo studies confirm the role of calicheamicin as a DNA-damaging agent and even suggest that calicheamicin may favor cleavage at certain chromosomal sites (*5*). Although this extraordinary reactivity has sparked considerable interest in the pharmaceutical industry, calicheamicin lacks tumor specificity. To circumvent this problem, conjugation of calicheamicin to tumor-specific monoclonal antibodies has been used as a targeting strategy. The recent success of calicheamicin-CD33 antibody conjugates (Mylotarg) to treat acute myelogenous leukemia

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