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Rationally Engineered Total Biosynthesis of a Synthetic Analog of a Natural Quinomycin Depsipeptide in *Escherichia coli*

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Quinomycin antibiotics, such as echinomycin **1** and triostin A **2**^[1] (Scheme 1), are nonribosomally synthesized peptides characterized by the C₂ symmetric cyclic depsipeptide core to which bicyclic quinoxaline or quinoline chromophores are attached.^[2] These natural products are biosynthesized by nonribosomal peptide (NRP) synthetases (NRPSs) and their associated enzymes in a variety of microorganisms, primarily Streptomyces, and constitute a pharmaceutically important class of compounds.^[3–5] This group of antibiotics shows activity against Gram-positive bacteria^[6] and a variety of tumor cells^[7,8] through bis-intercalating DNA at a nanomolar level,^[9–12] and is also a potent HIV reverse transcriptase inhibitor.^[13] Because of the wide range of biological activities of NRPs, there is a significant interest in elucidating the biosynthetic mechanisms of various NRPs and engineering the biosynthetic enzymes for preparing analogs with novel and useful biological activities.

We have recently reported the discovery of the entire echinomycin biosynthetic gene cluster and the first successful demonstration of *de novo* biosynthesis of biologically active forms of heterologous NRPs **1** and **2** in *Escherichia coli*.^[14] We introduced the echinomycin biosynthetic pathway, composed of fifteen genes including eight for the quinoxaline-2-carboxylic acid (QXC) biosynthesis, into *E. coli* over three plasmids. The great advantage of

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using *E. coli* as a heterologous host is the availability of the wealth and the techniques for its genetic manipulations, its robustness toward heterologous protein production and its ease of handling. Also, since scaling up of *E. coli* culture is relatively simple and economical, rational re-designing of a given biosynthetic pathway affords an efficient and environmentally benign approach for large-scale productions of target natural products. In addition, the accumulation of knowledge on the catalytic mechanisms and the structures^[15] of various NRPS domains has made rational manipulations of individual domains for the production of natural product analogs increasingly feasible. Here, we report the first *in vivo* total synthesis of an unnatural NRP, des-*N*-tetramethyl triostin A called TANDEM **3**, from glucose and simple salts via rational engineering of the plasmid-borne echinomycin biosynthetic pathway in *E. coli*.

To demonstrate the versatility and ease of our plasmid-based approach for establishing and engineering a heterologous biosynthetic pathway in *E. coli*, we have chosen to biosynthesize **3**, a synthetically prepared derivative of **2**.^[16] It has been known that **2** bis-intercalates into DNA and exhibits a distinct preference for GC-rich sequences. However, **3** shows a dramatically different DNA sequence preference and binds selectively into alternating AT sequences.^[17–19] For the *E. coli* production of **3**, we hypothesized that by 1) inactivating the two methylation (M) domains of the 340-kDa bimodular NRPS Ecm7, responsible for the *N*-methylation of the cysteine and the valine residues in the core peptide backbone, and 2) omitting Ecm18, responsible for the thioacetal formation,^[14] we can redesign the echinomycin biosynthetic pathway into a TANDEM biosynthetic pathway. We reported recently the identification of the biosynthetic gene cluster for **2**. However, we chose to use the echinomycin biosynthetic pathway for production of **3**, because sequence analysis of the two pathways indicated that they are virtually identical.^[20]

In NRPS, the first of the three conserved motifs of the M domain, **GXGXG** (Figure 1),^[21–23] is highly similar to the glycine-rich *S*-adenosyl-L-methionine-binding motif commonly found in other types of methyltransferases.^[22] Also, a previous study has shown that mutating the central glycine of the motif (underlined **G**) in the pyochelin NRPS M domain^[21] led to the formation of des-*N*-methylated pyochelin. Therefore, we constructed a G1000S/G1002S/G1004S/G2439S/G2441S/ G2443S hexaplet mutant of Ecm7 termed Ecm7* that carries GXGXG to SXSXS mutations in each of the two modules. We verified its production in the *E. coli* strain BL21 (DE3) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.^[24]

The exchange of the wild-type *ecm7* with the mutant *ecm7** and the deletion of *ecm18* in the original echinomycin biosynthetic pathway was accomplished readily using routine molecular biological techniques.^[14,24] The resulting new BL21 (DE3) strain was grown in M9 minimal medium using a fermentor. Protein production was induced with isopropylthio- β -D-galactoside (IPTG) at 15 °C, and the culture was incubated for another 14 days with a supply of a feed medium containing salts and glucose. At the end, the cell pellet was harvested and extracted with acetone, while the culture supernatant was extracted with ethyl acetate.

Production of the expected unnatural natural product **3** was confirmed by observing the presence of $[M+Na]^+$ and $[M+H]^+$ ions at $m/z = 1053.31$ and 1031.20 , respectively, in the peak eluted over 18.3–18.6 min (Figure 2A) using LC–ESI–MS (Figure 2B). The results were consistent with the molecular formula, $C_{46}H_{54}N_{12}O_{12}S_2$, and the MS spectra obtained with the authentic reference of **3**^[25] (Figure 2C). The smaller peak at 16.7 min appears to be one of the solution conformers of TANDEM previously reported.^[28] Although the isolated yield of approximately 200 μ g of **3** per liter of the fermentation culture was lower than that achieved in *E. coli* for the unmodified natural products **1** and **2** with the wild-type enzymes,^[14] a few fold decrease in isolated yield for this unnatural natural product biosynthesized by a mutagenized enzyme in *E. coli* seemed quite reasonable. The fact that we were able to obtain TANDEM from the engineered biosynthetic system suggests that the mutated enzyme Ecm7* exhibited the expected activity for the formation of the des-*N*-methylated compound as efficiently as its parent enzyme. Moreover, our results also support the idea that unnatural straight-chain peptide was accepted as a substrate for elongation by the NRPS modules, and for homodimerization and cyclorelease by the thioesterase domain. In addition, the cyclic des-*N*-methylated peptide intermediate is likely converted into **3** by Ecm17, a predicted disulfide bond-forming enzyme (Scheme 2).

While detailed characterizations of the biosynthetic mechanism are currently ongoing, these observations underscore the versatility and the robustness of the NRPS enzymes and the associated auxiliary enzymes, and the amenability of the system toward further engineering. Use of the *E. coli* plasmid-based system allows fast and simple transfer of the biosynthetic pathways of interest from the original production hosts, such as the streptomycetes, into the easy-to-handle and readily optimizable^[29] heterologous host, as well as engineering of such pathways for the production of unnatural natural products. Specifically, we have demonstrated that the rational engineering of the NRPS scaffold in *E. coli* can be a fast and powerful approach for the synthesis of unnatural natural peptidyl products. While we must expand the scope of compounds that can be biosynthesized in *E. coli* through engineering of natural product biosynthetic machineries to demonstrate the usefulness of the approach, the result shown here provides a further support for the use of *E. coli* as a flexible, sturdy platform for a fast, large-scale production of pharmaceutically useful molecules and their analogs in an environmentally friendly fashion from simple carbon and nitrogen sources.

Experimental Section

Bacterial strains and DNA manipulation

DNA manipulations were performed in *Escherichia coli* (*E. coli*) DH5 α (Invitrogen) using standard procedures.^[30] Overproduction of recombinant proteins was carried out in *E. coli* BL21 (DE3) (Invitrogen). Restriction enzymes were from New England Biolabs and Fermentas.

Site-directed mutagenesis and construction of the expression vectors

Plasmid pKW470, based on pET28b (Novagen), was prepared to overexpress *ecm6* using the T7 expression system. Plasmid pKW479, based on pRSFDuet-1 (Novagen), was prepared to overexpress *ecm7*. The pRSFDuet-1 vector was modified by deleting the unique

Sph I restriction endonuclease site. A 7.7-kbp *Sph* I-restricted fragment from pKW479 containing the *ecm7* gene was ligated into LITMUS38 (New England Biolabs) to yield pKW481. Plasmid pKW481 served as the template for site-directed mutagenesis. Mutations were introduced into *ecm7* using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) following the protocol recommended by the manufacturer. The oligonucleotide primers used for the mutagenesis are listed in Supporting Information Table S1. A 7.7-kbp *Sph* I-restricted fragment from the two resulting pKW481-based plasmids containing the mutated *ecm7* genes were put back into pKW479 to yield pKW606 for the production of the mutated enzyme Ecm7* with six mutations, G1000S, G1002S, G1004S, G2439S, G2441S and G2443S. The *Xba* I/*Spe* I double-digested fragment from pKW470 containing *ecm6* was cloned into pKW606 to yield pKW607, a pRSFDuet-1 carrying *ecm6* and *ecm7**. The accuracy of the DNA sequences of the coding regions of the plasmids prepared here, including the mutagenized positions, was confirmed by DNA sequencing (Supporting Information Figure S1).

***E. coli* production of compound 3**

pKW607, along with pKW532 and pKW539 carrying the rest of the echinomycin biosynthetic genes^[14] were introduced into BL21 (DE3) for the *de novo* production of **3** in *E. coli*. BL21 (DE3) transformed with pKW532/pKW539/pKW607 was incubated at 37 °C overnight in 2 mL LB medium supplemented with carbenicillin (100 µg/mL), spectinomycin (50 µg/mL), and kanamycin (50 µg/mL). Subsequently, the culture was transferred into 200 mL M9 minimal medium with the same antibiotics at the same concentration as described above and incubated for overnight at 37 °C. The entire culture was used to inoculate four liters of M9 minimal medium kept at 37 °C and pH 7.0 by the BioFlo 110 fermentor system (New Brunswick Scientific). When the culture reached the O.D.₆₀₀ of 0.6, the temperature was reduced to 15 °C. Subsequently, the culture was supplemented with IPTG at the final concentration of 200 µM, and feeding of the feeding media^[31] was initiated. After 14 days of incubation, the culture was centrifuged to separate the supernatant and the cells. Four liters of the supernatant were extracted with ethyl acetate (2×4 liters), while the cell pellet was extracted with acetone (600 mL) followed by extraction with chloroform (600 mL).

Isolation and characterization of compound 3 from *E. coli* culture

Silica gel 60 (particle size 0.040–0.063 mm; Merck) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254) and inspected under the UV light (254 and 365 nm). To isolate **3** from *E. coli* culture, the acetone-chloroform extracts of the cells and the ethyl acetate extracts of the culture filtrate were combined and concentrated *in vacuo* to give an oily material. This material was extracted with cyclohexane/CH₃CN and concentrated *in vacuo* to give a brown residue, which was fractionated by silica gel flash column chromatography with 25% hexanes/ethyl acetate and 10% MeOH/CHCl₃. The fractions containing **3** were collected and further purified by PTLC (2-butanone). The product bands were collected and eluted with 20% MeOH/CHCl₃. The elution fractions were analyzed by LC-MS as described in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Dell A, Williams DH, Morris HR, Smith GA, Feeney J, Roberts GC. *J Am. Chem. Soc.* 1975; 97:2497–2502. [PubMed: 1133418]
2. Glund K, Schlumbohm W, Bapat M, Keller U. *Biochemistry.* 1990; 29:3522–3527. [PubMed: 2354149]
3. Marahiel MA, Stachelhaus T, Mootz HD. *Chem. Rev.* 1997; 97:2651–2674. [PubMed: 11851476]
4. Schwarzer D, Finking R, Marahiel MA. *Nat. Prod. Rep.* 2003; 20:275–287. [PubMed: 12828367]
5. Walsh CT. *Science.* 2004; 303:1805–1810. [PubMed: 15031493]
6. Romero F, Espliego F, Perez Baz J, Garcia de Quesada T, Gravalos D, de la Calle F, Fernandez-Puentes JL. *J Antibiot. (Tokyo).* 1997; 50:734–737. [PubMed: 9360617]
7. Okada H, Suzuki H, Yoshinari T, Arakawa H, Okura A, Suda H, Yamada A, Uemura D. *J Antibiot. (Tokyo).* 1994; 47:129–135. [PubMed: 8150706]
8. Takahashi K, Koshino H, Esumi Y, Tsuda E, Kurosawa K. *J Antibiot. (Tokyo).* 2001; 54:622–627. [PubMed: 11592498]
9. Searle MS, Hall JG, Denny WA, Wakelin LP. *Biochem. J.* 1989; 259:433–441. [PubMed: 2719658]
10. Quigley GJ, Ughetto G, van der Marel GA, van Boom JH, Wang AH, Rich A. *Science.* 1986; 232:1255–1258. [PubMed: 3704650]
11. Viswamitra MA, Kennard O, Cruse WB, Egert E, Sheldrick GM, Jones PG, Waring MJ, Wakelin LP, Olsen RK. *Nature.* 1981; 289:817–819. [PubMed: 7464949]
12. Lee JS, Waring MJ. *Biochem. J.* 1978; 173:115–128. [PubMed: 687363]
13. Lingham RB, Hsu AH, O'Brien JA, Sigmund JM, Sanchez M, Gagliardi MM, Heimbuch BK, Genilloud O, Martin I, Diez MT, Hirsch CF, Zink DL, Liesch JM, Koch GE, Gartner SE, Garrity GM, Tsou NN, Salituro GM. *J Antibiot. (Tokyo).* 1996; 49:253–259. [PubMed: 8626240]
14. Watanabe K, Hotta K, Praseuth AP, Koketsu K, Migita A, Boddy CN, Wang CC, Oguri H, Oikawa H. *Nat. Chem. Biol.* 2006; 2:423–428. [PubMed: 16799553]
15. Challis GL, Naismith JH. *Curr. Opin. Struct. Biol.* 2004; 14:748–756. [PubMed: 15582399]
16. Ciardelli TL, Chakravarty PK, Olsen RK. *J Am. Chem. Soc.* 1978; 100:7684–7690.
17. Low CM, Olsen RK, Waring MJ. *FEBS Lett.* 1984; 176:414–420. [PubMed: 6386526]
18. Fox KR, Olsen RK, Waring MJ. *Biochim. Biophys. Acta.* 1982; 696:315–322. [PubMed: 7066328]
19. Lee JS, Waring MJ. *Biochem. J.* 1978; 173:129–144. [PubMed: 687364]
20. Praseuth AP, Wang CC, Watanabe K, Hotta K, Oguri H, Oikawa H. *Biotechnol. Prog.* 2008; 24:1226–1231. [PubMed: 19194935]
21. Patel HM, Walsh CT. *Biochemistry.* 2001; 40:9023–9031. [PubMed: 11467965]
22. Vevodova J, Graham RM, Raux E, Schubert HL, Roper DI, Brindley AA, Scott AI, Roessner CA, Stamford NP, Stroupe ME, Getzoff ED, Warren MJ, Wilson KS. *J Mol. Biol.* 2004; 344:419–433. [PubMed: 15522295]
23. Konz D, Marahiel MA. *Chem. Biol.* 1999; 6:R39–R48. [PubMed: 10021423]
24. See the Supporting Information for details.

25. Malkinson JP, Anim MK, Zloh M, Searcey M, Hampshire AJ, Fox KR. J Org. Chem. 2005; 70:7654–7661. [PubMed: 16149796]
26. Schmoock G, Pfennig F, Jewiarz J, Schlumbohm W, Laubinger W, Schauwecker F, Keller U. J Biol. Chem. 2005; 280:4339–4349. [PubMed: 15569690]
27. Koketsu K, Oguri H, Watanabe K, Oikawa H. Chem. Biol. 2008; 15:818–828. [PubMed: 18721753]
28. Alfredson TV, Maki AH, Waring MJ. Biopolymers. 1991; 31:1689–1708. [PubMed: 1793810]
29. Praseuth AP, Praseuth MB, Oguri H, Oikawa H, Watanabe K, Wang CC. Biotechnol Prog. 2008; 24:134–139. [PubMed: 18173279]
30. Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd ed.. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
31. Pfeifer BA, Wang CC, Walsh CT, Khosla C. Appl. Environ. Microbiol. 2003; 69:6698–6702. [PubMed: 14602630]

Consensus	VLDEGXGXG	
PchF	LLELGAGTA	
Ecm7 M1	VLELGAGTG	(G1000/G1002/G1004)
Ecm7 M2	VLEIGVGSG	(G2439/G2441/G2443)

Figure 1.

Sequence alignment of the conserved motif I of methyltransferases with the homologous region in the pyochelin NRPS (PchF) and Ecm7 methyltransferase domains 1 (M1) and 2 (M2).

Figure 2.

LC–MS analyses of TANDEM **3** extracted from the culture of the engineered *E. coli*. A) The total ion count spectrum from the LC–MS analysis of **3** extracted from the culture of the engineered *E. coli*. B) The MS spectrum of the HPLC peak at 18.48 min of **3** extracted from the culture of the engineered *E. coli*, and its comparison to C) the LC–MS analysis of the authentic reference for **3**.^[25]

1 **2:** R=CH₃**3:** R=H **Scheme 1.**

Structures of natural (echinomycin **1**, triostin A **2**) and synthetic (TANDEM **3**) quinomycin antibiotics.

Scheme 2.

Proposed biosynthetic scheme for TANDEM 3. Abbreviations are: AMP, adenosine 5'-monophosphate; A, adenylation; C, condensation; E, epimerization; M*, inactive methylation; T, thiolation; and TE, thioesterase. Ecm1 adenylates and transfers L-tryptophan-derived quinixoline-2-carboxylic acid (QXC) to the fatty acid biosynthesis acyl carrier protein FabC.^[26] Ecm6 accepts QXC-S-FabC as the starter for the peptide core formation. Ecm7 contains a TE domain capable of peptide chain homodimerization and cyclorelease.^[27] Ecm17 accepts the des-*N*-tetramethyl product as its substrate for disulfide bond formation to give 3.