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Activating a Cryptic Ansamycin Biosynthetic Gene Cluster To Produce Three New Naphthalenic Octaketide Ansamycins with *n*-Pentyl and *n*-Butyl Side Chains

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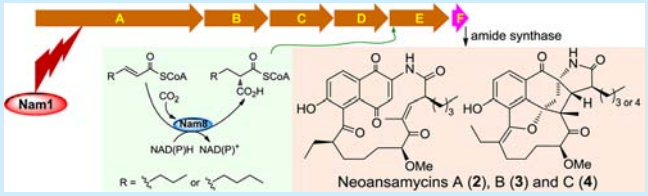
Supporting Information

ABSTRACT: Genome mining is a rational approach to discovering new natural products. The genome sequence analysis of *Streptomyces* sp. LZ35 revealed the presence of a putative ansamycin gene cluster (*nam*). Constitutive overexpression of the pathway-specific transcriptional regulatory gene *nam1* successfully activated the *nam* gene cluster, and three novel naphthalenic octaketide ansamycins were discovered with unprecedented *n*-pentylmalonyl-CoA or *n*-butylmalonyl-CoA extender units. This study represents the first example of discovering novel ansamycin scaffolds via activation of a cryptic gene cluster.



Ansamycins are an important family of natural products that cover a range of biological activities, including the RNA polymerase inhibitor rifamycin,[1] the Hsp90 inhibitor geldanamycin,[2] and the antiproliferative maytansinoids.[3] These macrolactams arise from a polyketide pathway that employs multidomain modular type I PKSs using 3-amino-5-hydroxybenzoic acid (AHBA) as the starter unit.[4] On the basis of the structures of AHBA-derived aromatic moieties, ansamycins are divided into benzenic and naphthalenic groups. Each group can be further classified according to the numbers and types of extender units recruited. So far, about 200 ansamycins have been reported. By analogy to the biosynthesis of type I polyketides such as macrolactones, we speculate that there could be more ansamycin scaffolds; e.g., hexaketide, dodecaketide, and tridecaketide backbones, are awaiting discovery and exploitation.[5] Moreover, we recently isolated two macrodilactams, juanlimycins A and B, from *Streptomyces* sp. LC6 for the first time among the reported ansamycins.[6]

The intriguing structures and promising bioactivity of ansamycins encouraged us to search for more new ansamycin skeletons. Herein, we report the activation of a cryptic ansamycin gene cluster in *Streptomyces* sp. LZ35 by constitutive overexpression of a LuxR family transcriptional regulatory gene. This led to the discovery of three novel naphthalenic ansamycins, named neoansamycins A–C. Notably, two different unusual extender units, *n*-pentylmalonyl-CoA and *n*-butylmalonyl-CoA, were incorporated in the last step of polyketide chain elongation. In addition, the *nam8* gene encoding a crotonyl-CoA carboxylase/reductase was found to be involved in the production of *n*-pentylmalonyl-CoA and *n*-butylmalonyl-CoA. This study illustrates the potential in exploring the huge reservoir of cryptic ansamycin biosynthetic gene clusters to obtain novel ansamycins, which will increase the probability of identifying new bioactive molecules and possible drug leads.

Streptomyces sp. LZ35 was identified as an AHBA-positive strain with the potential to produce ansamycins.[7,8] Genome sequence analysis of the strain LZ35 revealed presence of three ansamycin gene clusters, one of them is a new cryptic ansamycin gene cluster (*nam*) (Figure 1, Table S3 and Scheme S1 (Supporting Information, GenBank accession no. KJ590158). The putative PKSs within this cluster, designated NamA–E, contain eight modules, implying that octaketide ansamycins could be their synthetic products. Bioinformatics analysis of the eight acyltransferase (AT) domains in NamA–E revealed that the possible extender units for the assembly of

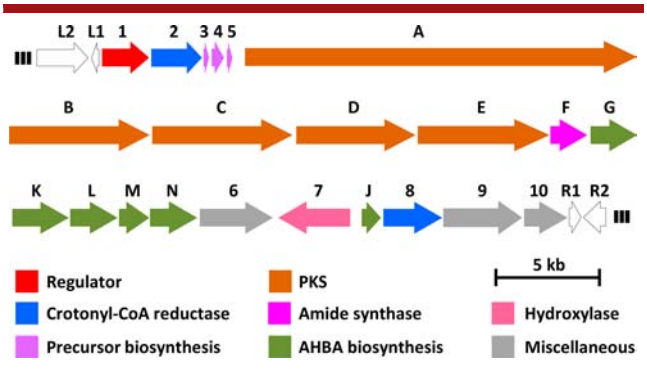


Figure 1. Organization of the *nam* gene cluster.

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polyketide chain could be different from those in the known octaketide ansamycins, i.e., geldanamycins[9] and ansamitocins,[10] and thus that the products of this gene cluster could have novel scaffolds (Table S4). However, none of the predicted ansamycins was obtained by large-scale fermentation under our experimental conditions. Indeed, reverse transcription PCR (RT-PCR) analysis indicated that all of the genes examined were expressed very poorly in SR101, a geldanamycin-nonproducing mutant of the strain LZ35,[8] indicating that this ansamycin gene cluster was silent or poorly expressed under the laboratory conditions (Figure S1d).

Several strategies have been developed to activate silent or cryptic biosynthetic gene clusters, including manipulation of culture conditions,[11,12] using a promoter exchange strategy,[13,14] genetic manipulation of regulatory genes,[15] synthetic biology strategies,[16] and heterologous expression of gene clusters in different host strains.[17,18] Previously, two different classes of ansamycins, geldanamycins and hygrocins, have been isolated from strain LZ35,[19,20] which means the supply of starter unit AHBA is not responsible for the silence of the *nam* gene cluster. To induce the expression of the *nam* gene cluster, we first conducted a promoter replacement. Since the structural genes, PKS (*namA-E*) and amide synthase (*namF*) genes, are located in a single operon, we introduced the strong constitutive promoter *ermE** upstream of the operon of *namA* in the strain SR101 to generate the strain SR101*namA-ermE** (Figure S1a,b). One extra peak (**1**) was identified and obtained in large-scale fermentation of SR101*namA-ermE** (Figure S1c). The molecular formula of **1** was determined to be $C_{17}H_{19}NO_6$ on the basis o f analysis of the high-resolution ESIMS *m/z* 334.1270 $[M + H]^+$ (calcd for $C_{17}H_{20}NO_6$, 334.1285). The structure of **1** was elucidated by extensive analysis of one- and two-dimensional (2D) NMR spectroscopy (^1H, ^{13}C, heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) (Figure 2, Table S6, Figures S2a and S6−10). Compound **1** shares a similar structure as SY4b, which is an intermediate accumulated in the *rifF* (rifamycin amide synthase gene) and *rif-orf19* (rifamycin 3-(3-hydroxyphenyl)propionate hydroxylase gene) deletion mutants of *Amycolatopsis mediterranei* S699.[21,22] These results showed that the promoter replacement only partially activated the *nam* gene cluster. Indeed, RT-PCR analysis indicated that only the transcriptional levels of *namA* and *namF* genes were increased by this promoter replacement (Figure S1d). Thus, other uninduced genes beyond PKS (*namA−E*) and amide synthase (*namF*) genes are required for the production of the expected final products.

Members of the LuxR family have been previously described as activators of polyketide biosynthesis.[15] Analysis of the *nam* gene cluster identified the *nam1* gene as encoding a putative positive regulator of the LuxR family transcriptional factors. Thus, we decided to examine whether constitutive overexpression of the *nam1* gene could lead to activation of the whole gene cluster. The *nam1* gene was cloned into the conjugative and integrative vector pJTU824, placing it under the control of the *ermE** promoter. The resulting construct was integrated into the chromosome of the strain SR201, which is the geldanamycin and hygrocin nonproducing mutant,[8] to create the strain SR201*nam1*OE, and the control strain SR201pJTU824 containing the empty pJTU824 vector was also prepared. Comparative transcriptional analysis with quantitative real-time PCR (qRT-PCR) showed that the transcriptional levels of six genes *nam2, namA, namF, namK,*

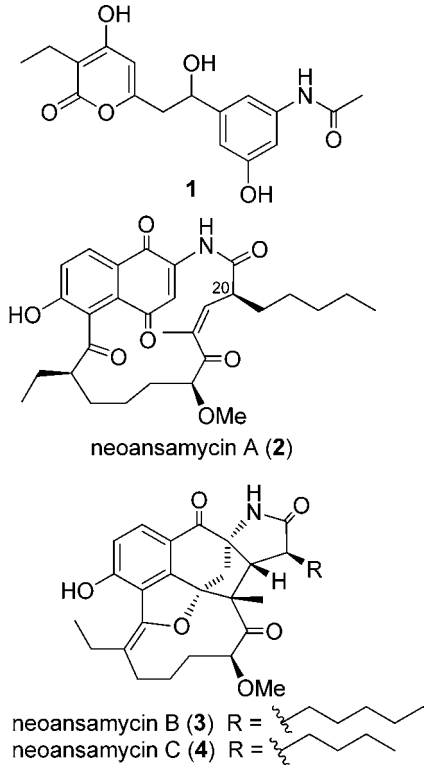

Figure 2. Structures of **1**−**4**, novel ansamycin intermediate, and neoansamycins from *Streptomyces* sp. LZ35.

nam7, and *nam8* of the *nam* gene cluster were upregulated in the strain SR201*nam1*OE as compared to the control strain SR201pJTU824 (Figure 3), suggesting that Nam1 might act as a pathway-specific activator of the *nam* gene cluster.

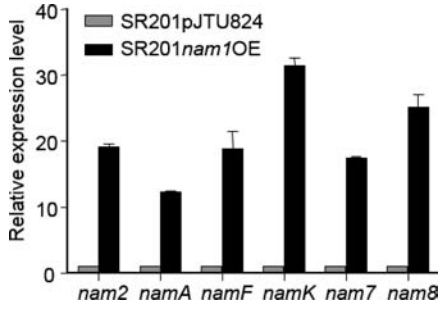

Figure 3. Activation of the *nam* gene cluster by overexpression of the LuxR family transcription regulator gene *nam1*. Transcription analysis of *nam2*, *namA*, *namF*, *namK*, *nam7*, and *nam8* was carried out by qRT-PCR. The transcription of Sigma factor *hrdB* gene was assessed as an internal control. The error bars represent standard deviations of the means of triplicate samples (see details in the Supporting Information 2.3). SR201pJTU824: the strain SR201 carrying the empty vector pJTU824; SR201*nam1*OE: overexpressing the transcriptional regulatory gene *nam1* under the control of the constitutive *ermE** promoter in the strain SR201.

HPLC analysis revealed that the SR201*nam1*OE strain produced one major product (**2**) compared with SR201pJTU824 (Figure S3b). In fact, large-scale cultivation of SR201*nam1*OE led to the isolation of compounds **2**−**4** (Figure 2). All of them were fully characterized by the analysis of HR ESI-MS and NMR data (Tables S7−S9 and Figures S11−29). The molecular formula ($C_{30}H_{37}NO_7$) of neo-

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ansamycin A (**2**) was deduced from the HR ESIMS data at *m/z* 524.2628 $[M + H]^+$. The planar structure of **2** was established by thorough analysis of ^1H, ^{13}C, and 2D NMR spectroscopic data (see details in the Supporting Information). Key double-quantum-filtered correlation (COSY), HMBC, and nuclear overhauser effect (NOE) correlations further elucidated the structure (Figure S2b). Neoansamycin B (**3**) with a quasimolecular ion at *m/z* of 508.2673 $[M + H]^+$ was determined to have the molecular formula $C_{30}H_{37}NO_6$, and identified as a "degradation" product of **2** as previously proposed for hygrocins.[23] The single-crystal X-ray diffraction experiment fully confirmed the proposed structure and the relative configuration (Figure 4, deposition no. CCDC 1027803). Neoansamycin C (**4**) was determined to have the molecular formula $C_{29}H_{35}NO_6$ according to the quasimolecular ion at *m/z* of 494.2514 $[M + H]^+$ and identified as an analogue of **3** with an *n*-butyl side chain rather than an *n*-pentyl one at C-20. The stereochemistry of **3** was proposed by comparison to that of **2** and by comparison with that of hygrocins.[19,23] The formation of **3** was proposed to be derived from **2** (Scheme 1).

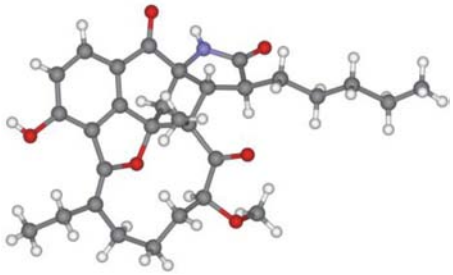
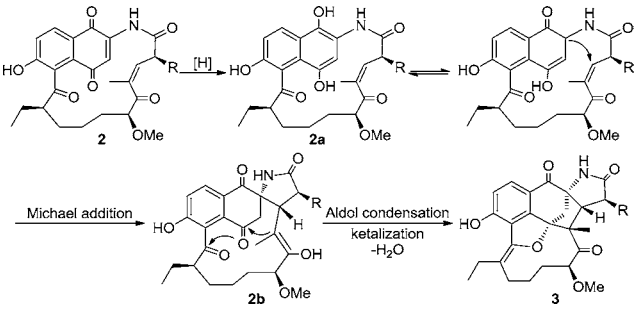


Figure 4. Single-crystal X-ray structure for neoansamycin B (**3**).

Scheme 1. Proposed Transformation of 2 to 3



Under reductive conditions, **2** is reduced to its phenol form **2a**, which undergoes an intramolecular Michael addition to produce the enolate intermediate **2b**, and then **2b** was transformed to **3** via an intramolecular aldol condensation, followed by the formation of a hemiacetal and subsequent dehydration to give an enol ether. Taken together, the proposed transformation of **2** to **3** represents a new type of intramolecular cyclization, producing a spiro γ-lactam moiety similar as that in hygrocins[19] and ansalactam A.[5]

The incorporation of either of two rare PKS extender units in the same position on the new structures represents another new feature of the neoansamycins.[24] The *n*-butyl/*n*-pentyl substituents at C-20 indicate that the AT domain of the PKS module 7 is able to recognize and activate both *n*-butylmalonyl-CoA (**7**) and *n*-pentylmalonyl-CoA (**10**) (Figure 5a). Crotonyl-

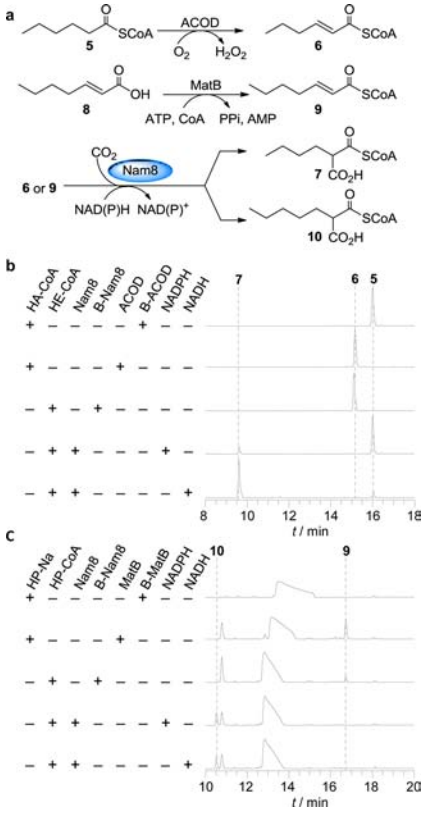


Figure 5. Nam8-catalyzed formation of *n*-butylmalonyl-CoA (**7**) and *n*-pentylmalonyl-CoA (**10**). (a) Preparation of 2-hexenoyl-CoA (**6**) and 2-heptenoyl-CoA (**9**) as the substrates of Nam8 and Nam8-catalyzed production of **7** and **10**. (b) HPLC analysis of Nam8 reaction with 2-hexenoyl-CoA. (c) HPLC analysis of Nam8 reaction with 2-heptenoyl-CoA, which was not able to detect **8**. The peak next to **10** was suggested to be 2-heptenoyl-AMP, an intermediate of MatB-catalyzed synthesis of **9**. The broad peak between **10** and **9** is a mixture of CoA and the degradations of MatB. MatB: malonyl-CoA synthetase, HA-CoA: hexanoyl-CoA (**5**), HE-CoA: 2-hexenoyl-CoA (**6**), B-Nam8: boiled Nam8, ACOD: acyl-CoA oxidase, B-ACOD: boiled acyl-CoA oxidase, HP-Na: sodium 2-heptenate (**8**), HP-CoA: 2-heptenoyl-CoA (**9**), B-MatB: boiled MatB.

CoA reductase/carboxylases (CCRs) are known to catalyze the biosynthesis of a variety of substituted malonyl-CoA employed as PKS extender units.[25,26] Four CCR genes were identified in the genome of LZ35, where two of them, *nam2* and *nam8*, are located in the *nam* gene cluster. Phylogenetic analysis of these four CCRs shows that Nam2 and the other two CCRs are probably involved in the biosynthesis of ethylmalonyl-CoA,[27] while Nam8 was grouped with RevT, which is proposed to produce butyl/pentylmalonyl-CoA for reveromycin biosynthesis (Figure S3a).[28] As predicted, deletion of *nam8* yielded a **2**-negative mutant, supporting the essential role of Nam8 in neoansamycin biosynthesis (Figure S3b).

To obtain direct evidence for the role of the *nam8* gene, we incubated purified Nam8 with candidate substrates, 2-hexenoyl-CoA (**6**) and 2-heptenoyl-CoA (**9**) that were synthesized in this study (Figure 5a), along with necessary cofactors NAD(P)H. The in vitro enzymatic activity assay demonstrated that Nam8-catalyzed the carboxylation of **6** and **9** in the presence of NAD(P)H (Figure 5b,c, Figures S4 and S5). Although it has been reported that NADH was unable to be used as the electron donor in CCR reactions,[26] our results showed that both NADH and NADPH were accepted by

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Nam8. Indeed, much more **7** was produced in the reaction mixture when Nam8 was incubated with **6** in the presence of NADH, compared with NADPH under the same conditions. However, the production of **10** when incubated Nam8 and **9** with NADPH was almost the same as in the presence of NADH; the reason for this is unclear at the moment.

Compounds **2** and **3** showed moderate antiproliferative activities against SW480, MDA-MB-231, HeLa, HepG2, and HL7702 cell lines. Compound **2** was shown to be most potent for the colorectal carcinoma cell line SW480 (IC_{50} = 6.7 μM) (Table S5). In addition, antimicrobial activities of neoansamycins were also investigated against *Staphylococcus aureus* ATCC 25923, *Mycobacterium smegmatis* mc^2 155, and *Candida albicans* 5314. The results showed that compounds **2** and **3** are selectively against *S. aureus* ATCC 25923 with an identical MIC value of 3.125 μg/mL (Table S5).

In conclusion, we have identified a cryptic ansamycin gene cluster by genome mining,[29,30] and successfully activated it by overexpression of the LuxR-type transcriptional regulatory gene *nam1*. Accordingly, we were able to isolate and fully characterize three novel naphthalenic ansamycins with different alkyl side chains. In addition, we demonstrated that the crotonyl-CoA carboxylase/reductase Nam8 is responsible for the formation of two rare extender units. Although various unusual extender units have been found in the same position of several polyketide derived structures, to our knowledge, this is the first example observed in ansamycins. The relaxed substrate specificity of neoansamycins could facilitate the bioengineering of novel ansamycins.[31]

■ ASSOCIATED CONTENT

Supporting Information

Complete description of methods and additional tables and figures, including structure elucidation and NMR data for compounds **1**−**4**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01686.

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Notes

The authors declare no competing financial interest.

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