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New Ansamycin Derivatives Generated by Simultaneous Mutasynthesis

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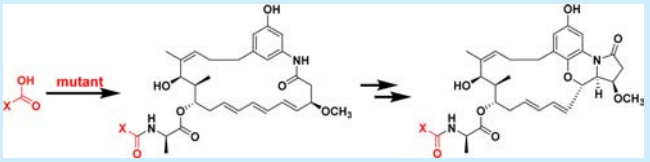
Supporting Information

ABSTRACT: The conversion from triene- to diene-typed ansamycins is clarified step by step in *Streptomyces seoulensis* IFB-A01. Such an intertype convertibility is adopted to establish for the first time the simultaneous mutasynthesis of both types of C17-benzene ansamycins (C17BAs). Three of the newly generated unnatural compounds showed potent cytotoxicity.



Owing to the unpredictable chemodiversity, natural products (viz., secondary metabolites from the biological viewpoint) have occupied a unique region of chemical space, which is not yet substantially overlapped by commercialized chemicals/pharmaceuticals and other small molecule libraries.[1] Source analysis of approved small molecule drugs indicates that polyketides are approximately five times more likely to possess drug activity than other natural product categories, and macrocyclic polyketides seem to have even higher activity.[2] Ansamycins are a unique class of bacterial macrocyclic polyketides with a broad range of bioactivities.[2d,3] As a subclass of this polyketide family, the C17-benzene ansamycin (C17BA) such as trienomycin A (**1**) is architecturally distinct in its macrolactam formed through an amide linkage to an aryl ring (Figure 1a).[4] The fascinating chemistry and promising bioactivity of C17BAs have driven collectively the advancement of the synthetic methodology under the necessity of supplying and modifying such a family of complex compounds.[2d,5] Most structurally complicated ansamycins continue to be prepared through fermentation with or without subsequent semisynthesis.[2d] Moreover, chemical derivatization approaches are limited by the complex functionality of the ansamycin scaffold. As a result of their synthetic complexity, our understanding of the structure−activity relationships of the ansamycins remains incomplete.[6] Another bottleneck remains in the drug discovery owing to the poor structural diversity of compound libraries.[7] A particular case in this regard is the C17BA polyketides that consist surprisingly of only one diene- and 43 triene-typed ansamycins (Figure 1a).

To diversify ansamycins, the mutasynthetic approach has been established by, but so far limited to, the supplementation of 3-amino-5-hydroxybenzoic acid (AHBA)-derived "unnatural substrates" (technically called mutasynthon) into the biosynthetic machinery of mutated bacterial producers of C15-benzene ansamycins (C15BAs, strikingly different from C17BAs) such as geldanamycin.[8] However, few attempts have been made to generate new C17BAs through "mutasynthetic operations" of the C-11 substituted motif, which is another determinant for bioactivities of this class of compounds.[9] A previous failure was reported for the restoration of ansatrienin production by a bacterial strain deprived of the gene (*chcA*) encoding 1-cyclohexenylcarbonyl coenzyme A (CHC-CoA) reductase.[10a] As illustrated by the trienomycin A (**1**) structure (Figure 1b), a cyclohexanecarboxylic acid (CHC)-derived side chain is anchored via an L-alanine residue to the C-11 position of a macrolactam. A previous study on the biosynthesis of ansatrienin A in *Streptomyces collinus* has revealed the CHC-CoA is the direct precursor of the cyclohexanate moiety of the ansatrienin A, and five genes including *ans J*−*M* and *chcA* were identified to be involved in the formation of the CHC-CoA.[10] Thus, the CHC-CoA related biosynthetic genes could be used as an important genetic target for mutasynthesis of trienomycin A (**1**). Additionally, the coproduction of trienomycin A (**1**) and benzoxazomycin (**2**) by *Streptomyces* sp. MJ672-m3[11] encouraged us to hypothesize that the conversion from triene- to diene-typed ansamycins might occur in some microbes. Obviously, this intertype convertibility would permit the simultaneous mutasynthesis of more (at least two) C17BA derivatives upon supplementation of a single substrate (mutasynthon). To test this hypothesis, here we demonstrated for the first time that conversion from triene to diene was due to the tandem oxidation and subsequent [4 + 2] intramolecular Diels−Alder cyclization (Figure 1b). Through supplementing different CHC-like unnatural precursors, the mutant strain of *S. seoulensis*, which lacked the ability to biosynthesize CHC-CoA, was able to simultaneously produce both triene- and diene-type anasamycins with the modifiable C-11 dipeptidyl motif (Figure 1). Some of the newly generated unnatural compounds showed potent cytotoxicity against HepG2 and MCF-7 tumor cell lines.

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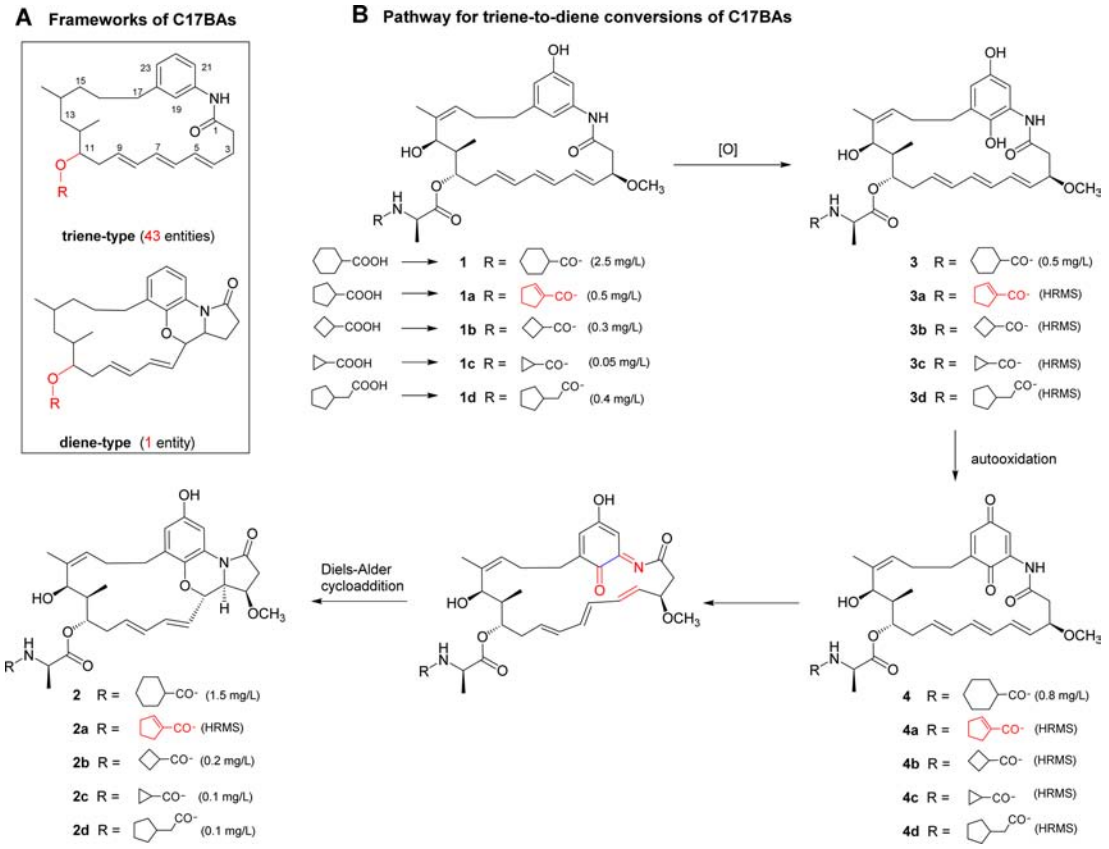

Figure 1. Frameworks and triene-to-diene conversions of C17-benzene ansamycins (C17BAs). Transformation of **1** to **2** via **3** and **4** found in the wild-type strain of *S. seoulensis* IFB-A01 was repeated in the culture of the CHC biosynthetic genes knockout strain by feeding cyclohexanecarboxylic acid (CHC). Conversions of **1a**−**1d** to **2a**−**2d** via **3a**−**3d** and **4a**−**4d** were ascertained in cultures of the mutant strain (IFB-A01-C) by supplementing with cyclopentanecarboxylic, cyclobutanecarboxylic, cyclopropanecarboxylic, and 2-cyclopentylacetic acids, respectively.

Our endeavor was initiated by screening our microbial library for the strain capable of coproducing triene- and diene-typed ansamycins. From our microbial library, *S. seoulensis* strain IFB-A01 isolated from the gut of *Penaeus orientalis* (the Chinese white shrimp) produced both **1** and **2** when fermented using an optimized medium (Figure 1, see "*Supplementary Methods*" in the Supporting Information). To test the convertibility from **1** to **2**, *S. seoulensis* IFB-A01 was regrown with any fluctuation in the medium monitored by LC-MS analysis. Compound **1** became detectable in the culture on the fourth day followed by a two-day plateau reflecting a continued accumulation of the metabolite. After that, the gradual increase of **2** was accompanied by the decrease of **1** in the culture, confirming the conversion of **1** into **2** (Figure S1). To understand the transformation step by step, a scaled-up culture of *S. seoulensis* was subjected to an LC-MS guided fractionation of intermediates formed in the biosynthetic assembly line for **1**. As expected, mycotrienins II (**3**) and I (**4**)[12] were isolated from the culture, and both could be the intermediates generated upon the production of **2** from **1** (Figure 1b). To verify the assumption, compound **1** was treated with the cell-derived protein to produce **3** (Figure 2). In view of the redox equilibrium between hydroquinone and benzoquinone, the conversion of **3** into **4** was hypothesized to occur in the presence of oxygen or air. This anticipation was validated by the conversion of **3** into **4** in a yield of 94% in air-exposed phosphate buffer (pH = 7) at 28 °C for 10 h (Figure 2). Interestingly, the subsequent [4 + 2] Diels−Alder cyclization of

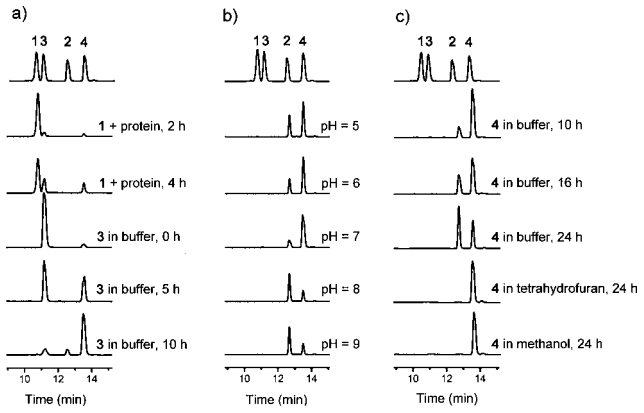

Figure 2. LC-MS evaluation for the production of **1**−**4** in cultures and reaction solution. (a) Convertibility tests of **1** by treating for 2 or 4 h with intercellular proteins of the WT strain, and of **3** into **2** via **4** in PBS buffer (pH = 7) within 0, 5, and 10 h. (b) Test for transformability of **4** into **2** in different pH solution buffers within 10 h. (c) Medium-dependence of transformability of **4** into **2** (tested separately in PBS buffer (pH = 7), tetrahydrofuran, and methanol).

4 was shown to proceed at room temperature in phosphate buffers between pH 5−9 (Figure 2).

Few attempts have been made to generate new C17BAs despite the incorporatability of 3-amino-5-hydroxybenzoic acid (AHBA)-derived mutasynthons into C15BAs such as geldanamycin.[8a−c] In the biosynthesis of trienomycin A (**1**) and benzoxazomycin (**2**), CHC-CoA is proposed as the source of

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the CHC-derived side chain in the C-11 position. Thus, the mutant strain lacking the ability to biosynthesize CHC-CoA could coproduce triene (such as **1**) and diene (such as **2**)-like compounds by adding a different CHC-like acid to its medium. To isolate the biosynthetic gene cluster for **1** and **2**, a fosmid library of *S. seoulensis* IFB-A01 was constructed and screened by a PCR method using two pairs of primer F/R-AHBA and F/R-chcA (see "*Cloning and analysis of the partial ansatrienin biosynthetic gene cluster*" in the Supporting Information). A partial biosynthetic gene cluster of trienomycin was cloned from one fosmid. The gene product showed high identity/similarity to that of ansatrienin A from *S. collinus* (Table S2).[10b,c]

To disrupt the biosynthetic pathway of CHC-CoA, four genes (*ansA1*−*A4*) involved in the CHC-CoA biosynthesis were inactivated in *S. seoulensis* IFB-A01 by double homologous recombination (Figure S2). The desired mutant (IFB-A01-C) was selected on the basis of an apramycin-sensitive and kanamycin-resistant phenotype and was further verified by a polymerase chain reaction followed by diagnostic digestion of restriction enzymes (Figure S3). The mutant strain is unable to produce **1**−**4**, but the production of **1**−**4** can be restorable by chemical complementation of CHC (Figure S2, see "*Supplementary Methods*" in the Supporting Information).

To ascertain its mutasynthon scope, the mutant strain, IFB-A01-C, was cultured separately with a diversity of small molecule organic acids. As anticipated, compounds **1a**−**1d** and **2a**−**2d** were afforded by supplementing the mutant cultures with cyclopentanecarboxylic, cyclobutanecarboxylic, cyclopropanecarboxylic, and 2-cyclopentylacetic acids, respectively (see "*Structure Elucidation*" in the Supporting Information). However, no mutasynthetic product could be detected if supplementing bulkier (cycloheptanecarboxylic and 2-cyclohexylacetic acids) or heterocyclic mutasynthons (piperidine-4-carboxylic, tetrahydro-2*H*-pyran-4-carboxylic, tetrahydrofuran-3-carboxylic, and pyrrolidine-3-carboxylic acids). These data showed that the mutasynthons acceptable for the mutant strain seem limited to small molecule (≤7 carbons) and heteroatom-free fatty acids. The finding agrees with the substitution profile of identified natural ansamycins whose C-11 acyl chains are restricted to cyclohexanecarbonyl, cyclohex-1-enecarbonyl, 4-methylpentanoyl, 3-methylbutanoyl, 2-methylbutanoyl, and 2-methylbut-2-enoyl groups.[9] Surprisingly, the mutant strain IFB-A01-C produces compounds **1a** and **2a** with a cyclopent-1-ene-1-carbonyl motif, while culturing with supplementation of cyclopentanecarboxylic acid. This suggested that the mutant strain might have produced the α,β-desaturase capable of transforming cyclopentanecarboxylic acid into cyclopent-1-ene-1-carboxylic acid.

The production of low-abundance compounds **2a**, **3a**−**3d**, and **4a**−**4d** was ascertained by the high resolution LC-MS analysis in the cultures of the mutant strain IFB-A01-C (Table S9) although the scarcity of these compounds in cultures did not facilitate any fractionation of samples in the study. Despite the limited number of molecules of the ansamycin analogue library, the new triene-ansamycin members **1a**, **1b**, and **1d** were found to be more cytotoxic against HepG2 and MCF-7 tumor cell lines than trienomycin A (**1**) (Table 1), and the newly characterized diene-typed ansamycins **2b** and **2c** become more inhibitory against the lipopolysaccharide-induced intracellular IL-6 production of murine macrophage cell line RAW264.7 (Table S10).

Table 1. In Vitro Cytotoxicity IC_{50} (μM) of 1, and 1a−1d against HepG2 and MCF-7

compounds	HepG2	MCF-7
1	**>20**	**>20**
1a	12.18 ± 0.50	6.74 ± 1.21
1b	8.39 ± 0.14	7.33 ± 0.27
1c	>20	>20
1d	6.81 ± 0.21	>20
doxorubicin[a]	1.49 ± 0.08	2.18 ± 0.19

[a]Positive control; HepG2: human hepatic carcinoma cell; MCF-7: human breast cancer cell. ± Results are expressed as mean ± SEM (n = 3).

The chemodiversity of (unnatural) natural products determines the applicability of the compound library as lead molecule sources for new drugs and agrochemicals.[13] Actinomycetes have produced a magnitude of bioactive molecules, but the characterizable new metabolites from these microbes generally become fewer and fewer after long-time intense investigations. To deal with the frustration, a combined mutasynthesis/semisynthesis approach was introduced to generate a more diversified compound library using the gene blocked actinomyces mutant.[8a,d,e,14] This work has addressed the intertype conversion of ansamycins in *S. seoulensis* IFB-A01, a new microbial producer that can construct both types of ansamycin macrolactams. To our surprise, the triene-to-diene transformation of ansamycins includes two automatic reactions following the 19-oxygenation (Figure 1b). To overcome the long existing challenge in mutasynthetic operation of the C-11 motifs, our deletion of the CHC biosynthetic gene cluster gave the desired mutant strain that was able to produce asamycins upon exposure to exogenous CHC and other small molecule acids. This substantiates the simultaneous mutasynthesis of both types of ansamycins. In concert with the bioassay procedure, the bioactive molecules were identified from the ansamycin-based compounds. In aggregation, the simultaneous mutasynthesis approach established here for the first time is effective in generating multiply diversified compound libraries required for the drug discovery effort.

■ ASSOCIATED CONTENT

Supporting Information

Complete description of methods, additional tables, and figures, including full NMR data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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