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NOTE

New ansamycin analogues from the mutant strain of *Streptomyces seoulensis*

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Ansamycin antibiotics, derived from 3-amino-5-hydroxybenzoic acid (AHBA), are a group of microbial metabolites that exhibit an extensive range of biological activities such as antitumor, antiviral and antibacterial.[1–4] The C17-benzene ansamycins (C17BAs), distinguished by their 21-membered macrolactam formed through an amide linkage to the AHBA moiety, have a unique feature with a carboxylic acid moiety attached via an L-alanine residue to the polyketide backbone.[5–9] It is worth noting that the bioactive diversity highly depends on the structure of the acyl chain attached at C-11 and the aromatic ring of C17BAs.[10,11] During our screening for new bioactive C17BA analogs, four known C17BAs, trienomycin A (**1**), benzoxazomycin (**2**), mycotrienin II (**3**) and mycotrienin I (**4**), were isolated from the fermentation broth of *Streptomyces seoulensis* IFB-A01 (derived from *Penaeus orientalis Kishi-nouye* gut).[12] The conversion from **1** to **2** through the intermediates of **3** and **4** has been clarified step by step, together with the discovery of the gene cluster for biosynthesis of **1** in our previous study,[12] which sets up the stage for the further mutasynthesis.

The mutant strain *S. seoulensis* IFB-A01-C, whose genes related to the biosynthesis of the carboxylic side chain (cyclohexanecarboxylic acid, CHC) have been deleted, lost the ability to produce compounds **1–4** completely.[12] However, in the mutant strain three new analogs (**1a–1c**) were detected as the major HPLC peaks. These compounds had not been isolated previously from the wild-type strain, presumably because of the relatively low yield or overlap by other major peaks. To see whether the wild-type strain can also produce these two products, an LC–MS experiment was conducted to analyze the crude extracts from wild-type and mutant strains. The result unambiguously showed that the wild-type strain indeed can produce **1a–1c** with the production yields about 10–20 times lower than those in mutant strain (Supplementary Figure S1). Encouraged by these results, scale-up fermentation of the mutated strain was performed. Isolation, structural elucidation and bioactivity assay of these C17BAs analogs are described in this paper.

The culture method and fermentation of the mutant strain of *S. seoulensis* IFB-A01 were performed according to the earlier report.[12] After 7-day scale-up fermentation, the fermentation (20 l) was extracted three times with ethyl acetate, and evaporation of the solvent under reduced pressure provided a residue for subsequent investigation. Subsequent purification of the column chromatography (CC) fractions that contained the target compounds was accomplished by Sephadex LH-20 and semipreparative HPLC. In particular, purification of the fourth CC fraction derived from the mutant strain culture by gel filtration over Sephadex LH-20 in MeOH and by semipreparative HPLC (50% acetonitrile/H_2O, 2 ml min^{-1}) to afford pure compounds.

In the beginning, compounds **1a–1c** were obtained from the extracted fraction. From the NMR analysis, it was found that the structures of the isolated three compounds differed from **1** only in the side chain at C-11. Compound **1a** and **1b** were identified as trienomycin B and E, respectively, by comparing their NMR data (Supplementary Figures S5 and S6) with the reported ones.[7,8] Compound **1c**, an amorphous powder, had a molecular formula of $C_{33}H_{46}N_2O_7$ revealed by its $[M+Na]^+$ ion peak at 605.3190 (calcd. for $[C_{33}H_{46}N_2O_7Na]^+$ 605.3197) in its high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS) spectrum. The ^1H NMR spectrum of **1c** revealed that conjugated triene signals at δ_H 5.58–5.64 (2H, m, H-4 and H-9), δ_H 6.0 ~ 6.2 (4H, m, H-5, H-6, H-7 and H-8), 1,3,5-trisubstituted benzene ring (giving three broadened singlets at δ_H 6.50 (H-19), 7.30 (H-21) and 6.41 (H-23), a methoxy singlet at δ_H 3.26 (H-26), and three methyl resonances at δ_H 1.75 (br s, H-25), 1.35 (d, J = 7.2 Hz, H-29) and 0.86 (d, J = 6.8 Hz, H-24), suggesting that it shared the same ansa ring moiety and alanine moiety as those of **1** (Figure 1 and Supplementary Table S2), which was supported by the ^1H–^1H COSY correlations from H-2/H-3/H-4/H-5/H-6/H-7/H-8/H-9/H-10/H-11/H-12/H-13, H-15/H-16/H-17, NH-28/H-29 and HMBC correlation of H-2/C-1, NH-1/C-19, H-19/C-17, H-11/C-27, H-29/C-27 and H-28/C-30. However, the HMBC correlations of C-30 (δ_C 178.1) with two methyl groups H-32 (δ_H 1.02) and H-33 (δ_H 1.06) suggested that the identified isobutyl group was anchored at the 27-alanine through an amide linkage. Thus, the compound **1c** was a new triene ansamycin derivative produced by the mutant strain (Supplementary Figures S7 – S12).

The C-11 acyl moiety of **1a – 1c** might be derived from 3-methylbutanoic, 4-methylpentanoic and isobutyric CoAs, respectively, which are produced from the fatty-acid biosynthetic pathway.[13,14] Therefore, deletion of CHC biosynthesis genes might give these acids

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more chance to be involved in the biosynthetic pathway of trienomycin A.

We have demonstrated that the conversion from the triene to diene anasamycin involved one step of enzymatically catalyzed monooxygenation at C-19 followed by two spontaneous reactions, oxidation and intramolecular Diels–Alder reaction (Figure 2).[12] Thus, the diene-typed ansamycins compounds **2a** and **2c** were obtained in the cultures of the mutant strain. Compound **2a**, a white amorphous powder, was analyzed to have a molecular formula of $C_{34}H_{46}N_2O_8$ by its $[M+Na]^+$ ion peak at 633.3153 (calcd for $[C_{34}H_{46}N_2O_8Na]^+$ 633.3146) by its HR-ESI-MS spectrum. The ^1H NMR spectrum of **2a** (Table S3) displayed a conjugated diene system (resonating at δ_H 5.54 (1H, dd, $J=15.2$, 9.2 Hz, H-6), 6.70 (1H, dd, $J=15.6$, 10.4 Hz, H-7), 6.13 (1H, dd, $J=15.6$, 9.2 Hz, H-8) and 5.84 (1H, dt, $J=15.6$, 7.6 Hz, H-9)), 1,2,3,5-tetrasubstituted benzene ring originated doublets at δ_H 7.98 (H-21) and 6.41 (H-23), a methoxy singlet at δ_H 3.40 (H-26) and three methyl groups (signifying at δ_H 0.93 (d, $J=8.0$ Hz, H-24), 1.77 (s, H-25) and 1.12 (d, $J=7.2$ Hz, H-29)), suggesting that it shared the same macrocyclic lactam and alanine moiety with those of **2**.[11] Moreover, the ^1H NMR data of **2a** showed the connection of the alanine residue to the 3-methylbutyl residue that gave similar proton signals at δ_H 2.12 (2H, m, H-31), 2.03 (1H, m, H-32), and 0.91 and 0.92 (3H each, d, $J=6.4$ Hz, H-33 and H-34) to those of **1a**. Thus, compound **2a** was a new member to the diene ansamycin family. For compound **2c**, its molecular formula was determined as $C_{33}H_{44}N_2O_8$ using HR-ESI-MS ($[M+Na]^+$ at *m/z* 619.30152, calcd for $[C_{33}H_{44}N_2O_8Na]^+$ 619.2990), a CH_2 group less than compound **2a**. The ^1H NMR spectrum of **2c** suggested that it possessed the same macrocyclic lactam scaffold as those of **2a** (Supplementary Table S3 and Supplementary Figure S13). In particular, the ^1H NMR spectral data of **2c** revealed that the substitution moiety of the alanine residue was an isobutyl group as evidenced from the ^1H NMR signals at δ_H 2.41 (1H, m, H-31), 1.05 (6H, d, $J=6.8$ Hz, H-32 and H-33). In

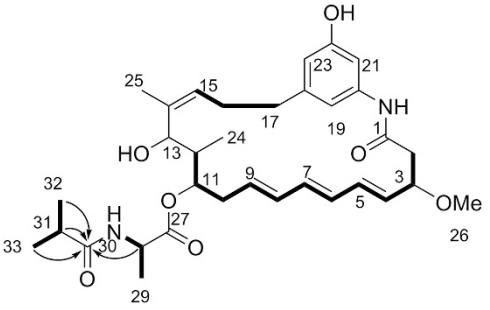

Figure 1 ^1H–^1H COSY (–) and Key HMBC (→) correlation of **1c**.

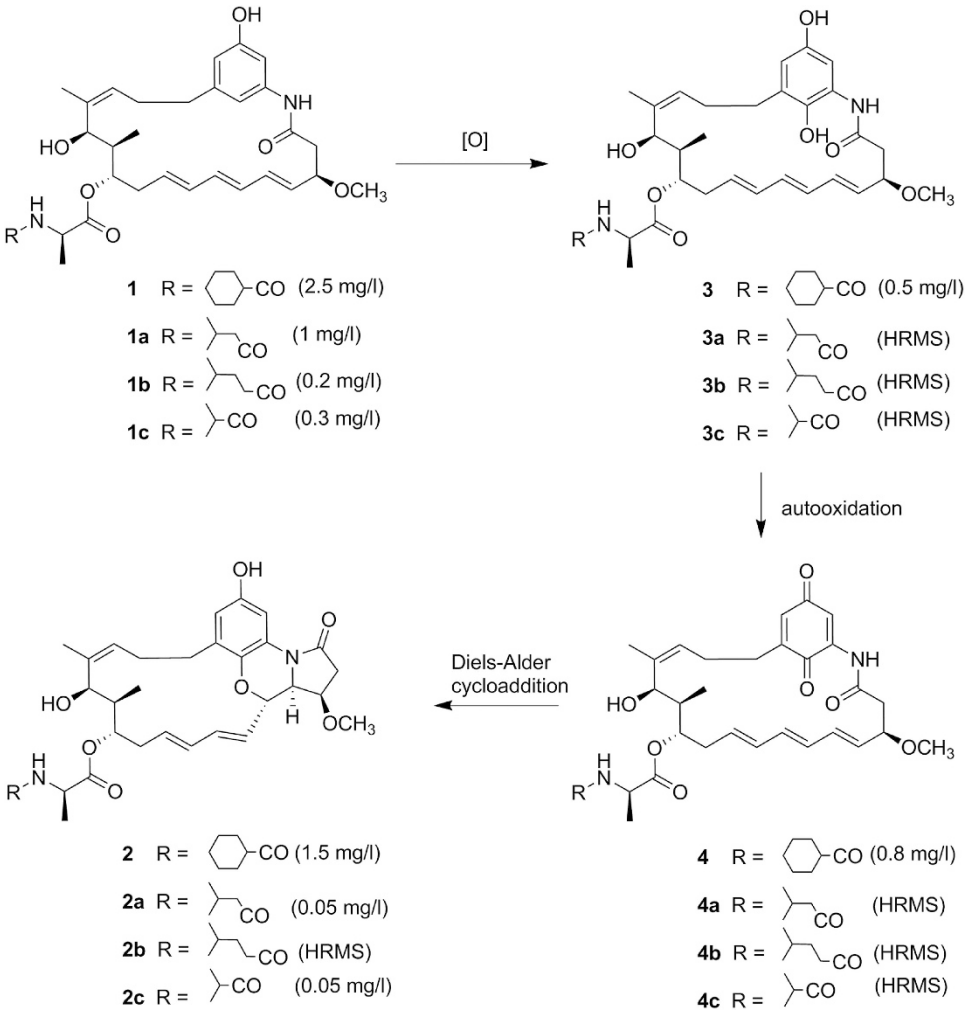

Figure 2 Compounds **1a**–**1c** to **2a**–**2c** were produced via **3a**–**3c** and **4a**–**4c** by the mutant strain of *Streptomyces seoulensis* IFB-A01 (IFB-A01-C).

addition, compounds **2b**, **3a** – **3c** and **4a** – **4c** were ascertained by the high-resolution LC–MS analysis in the cultures of the mutant strain (Supplementary Figure S2). However, isolated yields of these compounds were not enough for structural identification.

Previous studies have reported that C17BAs have good cytotoxic activity *in vitro* and *in vivo*.[15–17] Thus, all isolated compounds were administered to cultured human hepatic carcinoma cells (HepG2) and human breast cancer cells (MCF-7) for cytotoxic assay. As expected, all triene-ansamycin analogs showed cytotoxicities. Clearly, **1b** and **1c** were found to be more cytotoxic against tumor cell lines than **1**, especially **1b** showed equal activity against HepG2 to that of the positive control, doxorubicin (Table S4). Moreover, diene-typed ansamycins were investigated by lipopolysaccharide (LPS)-induced interleukin-6 (IL-6) production in RAW264.7 to understand their anti-inflammatory activity. Our current result showed that the production of IL-6 in the macrophage cell line RAW264.7 can be suppressed by **2** and **2a** without detectable cytotoxic effects. The diene-based ansamycin **2a** showed much stronger anti-inflammatory activity than **2** (Table S5). Inflammatory responses have an important part in numerous diseases including rheumatoid arthritis, atherosclerosis and cancer.[18] This result suggests that **2a** may serve as a potent bioactive lead compound for the treatment of inflammatory diseases.

The structural diversity of natural products determines the applicability of the compound library as lead molecule sources for new drugs and agrochemicals.[19] However, it seems that it becomes harder and harder to discover new metabolites from *Streptomyces* strains after long-time investigation. In this work, we have explored an effective approach for mining new bioactive natural components by combining mutasynthesis and primary metabolism. The described method may be generally applicable in setting up multiply diversified compound libraries and valuable for drug discovery efforts.

EXPERIMENTAL PROCEDURE

General experimental procedures

LC hyphened with HR-ESI-MS (LC–MS) analysis on an Agilent 6210 TOF LC–MS spectrometer (Agilent technologies, Santa Clara, CA, USA). NMR data were recorded on Bruker DRX-600, DRX-500 or DRX-400 NMR spectrometers using solvent as internal standard. Silica gel (200 – 300 mesh) for CC and silica GF_{254} (10 – 20 μM) for TLC were performed on GF254 (Qingdao Marine Chemical Factory, Qingdao, Shandong Province, China). The semi-preparative HPLC was accomplished over a Hitachi L-7110 pump equipped with a Hypersil Octadecylsilyl (ODS) column (5 μM, 250 × 10 mm) from Thermo Fisher Scientific, Inc. (Waltham, MA USA).

Anti-inflammatory activation

The murine macrophage cell line RAW264.7 was purchased from the China Cell Bank at SIBS (Shanghai, China). RAW264.7 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin (100 U ml^{-1})/streptomycin (100 mg ml^{-1}). In brief, 1.0×10^5 cells ml^{-1} were placed in a 96-well plate, incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. Cells were treated with test samples at 0.01, 0.1, 1, 10 and 100 μM, followed by incubation at 37 °C for an additional 24 h. The cells were incubated for 3 h after adding cell counting kit-8 solution (10 μl) to each well. The absorbance was measured using a microplate reader at 450 nm. The average OD formed in control cells was taken as 100% viability, and the results of treatments were expressed as a percentage of the control. In all, 1.2×10^5 cells ml^{-1} were placed in a 24-well plate and incubated overnight, treated with compounds confirmed to be safe to RAW264.7 cells for 2 h, then exposed to LPS (L4391, Sigma-Aldrich, Co., St Louis, MO, USA) for 24 h. The release of IL-6 in the culture supernatants was measured by enzyme-linked immunosorbent assay using Mouse IL-6 ELISA Kit (Cusabio Life Science, Wuhan, Hubei Province, China).

Cytotoxicity

All of the compounds were evaluated for cytotoxicity against two cell lines, HepG2 and MCF-7 (both from Jiangsu Provincial Center for Disease Prevention and Control, Nanjing, Jiangsu Province, China), using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] colorimetric method.[20] For experiments cells were maintained in DMEM/RPMI-1640 plus 10% FBS, and the test compounds were dissolved in dimethyl sulfoxide. Doxorubicin HCl was used as a positive control, and the medium without compounds as a negative control in the bioassay.

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1 Wehrli, W. & Staehelin, M. Actions of the rifamycins. *Bacteriol. Rev.* **35**, 290–309 (1971).
2 Balerna, M., Keller-Schierlein, W., Martius, C, Wolf, H. & Zähner, H. Naphthomycin, an antimetabolite of vitamin K1. *Arch. Mikrobiol.* **65**, 303–317 (1969).
3 Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E. & Neckers, L. M. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl Acad. Sci. USA* **91**, 8324–8328 (1994).
4 Sugita, M. *et al.* Studies on mycotrienin antibiotics, a novel class of ansamycins. I. Taxonomy, fermentation, isolation and properties of mycotrienins I and II. *J. Antibiot.* **35**, 1460–1466 (1982).
5 Umezawa, I. *et al.* Studies on a novel cytocidal antibiotic, trienomycin A. Taxonomy, fermentation, isolation, and physico-chemical and biological characteristics. *J. Antibiot.* **38**, 699–705 (1985).
6 Kim, C. G. *et al.* Biosynthesis of 3-Amino-5-hydroxybenzoic acid, the precursor of mC7N units in ansamycin antibiotics. *J. Am. Chem. Soc.* **118**, 7486–7491 (1996).
7 Funayama, S., Okada, K., Iwasaki, K., Komiyama, K. & Umezawa, I. Structures of trienomycins A, B and C, novel cytocidal ansamycin antibiotics. *J. Antibiot.* **38**, 1677–1683 (1985).
8 Nomoto, H. *et al.* Structural studies on minor components of trienomycin group antibiotics trienomycins D and E. *J. Antibiot.* **42**, 479–481 (1989).
9 Hosokawa, N. *et al.* New triene-ansamycins, thiazinotrienomycins F and G and a diene-ansamycin, benzoxazomycin. *J. Antibiot.* **53**, 886–894 (2000).
10 Sugita, M. *et al.* Studies on mycotrienin antibiotics, a novel class of ansamycins. III. The isolation, characterization and structures of mycotrienols I and II. *J. Antibiot.* **35**, 1474–1479 (1982).
11 Kawamura, T., Tashiro, E., Yamamoto, K., Shindo, K. & Imoto, M. SAR study of a novel triene-ansamycin group compound, quinotrierixin, and related compounds, as inhibitors of ER stress-induced XBP1 activation. *J. Antibiot.* **61**, 303–311 (2008).
12 Song, Y. N. *et al.* New ansamycin derivatives generated by simultaneous mutasynthesis. *Org. Lett.* **17**, 556–559 (2015).
13 Denoya, C. D. *et al.* A second branched-chain alpha-keto acid dehydrogenase gene cluster (*bkdFGH*) from *Streptomyces avermitilis*: its relationship to avermectin biosynthesis and the construction of a bkdF mutant suitable for the production of novel antiparasitic avermectins. *J. Bacteriol.* **177**, 3504–3511 (1995).
14 Li, Y., Luxenburger, E. & Müller, R. An alternative isovaleryl CoA biosynthetic pathway involving a previously unknown 3-methylglutaconyl CoA decarboxylase. *Angew. Chem. Int. Ed.* **52**, 1304–1308 (2013).
15 Komiyama, K. *et al.* Antitumor activity of trienomycin A on murine tumors. *J. Antibiot.* **40**, 1768–1772 (1987).
16 Nishio, M. *et al.* TMC-135A and B, new triene-ansamycins, produced by *Streptomyces* sp. *J. Antibiot.* **53**, 724–727 (2000).
17 Hosokawa, N. *et al.* Thiazinotrienomycins, new ansamycin group antibiotics. *J. Antibiot.* **48**, 471–478 (1995).
18 Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation, and cancer. *Cell* **140**, 883–899 (2010).
19 Cragg, G. M. & Newman, D. J. Natural products: a continuing source of novel drug leads. *Biochim. Biophys. Acta* **1830**, 3670–3695 (2013).
20 Cuadrado, I. *et al.* Labdanolic acid methyl ester (LAME) exerts anti-inflammatory effects through inhibition of TAK-1 activation. *Toxicol. Appl. Pharmacol.* **258**, 109–117 (2012).

Supplementary Information accompanies the paper on The *Journal of Antibiotics* website (http://www.nature.com/ja)