NOTE

Creation of novel reveromycin derivatives by alcohol-added fermentation

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Reveromycin A, a polyketide compound with a distinctive spiroacetal core structure (Figure 1), was isolated from *Streptomyces reveromyceticus* SN-593 as an inhibitor of epidermal growth factor-induced mitogenic activity.¹ It has various functions,^{2–6} and its robust anti-osteoclastic activity^{7–10} is its most prominent feature as a drug lead. To generate reveromycin derivatives that have greater activity, we recently identified a biosynthetic gene cluster of reveromycin A by gene disruption and complementation analysis.¹¹ We identified reveromycin T as the biosynthetic intermediate of reveromycin A in feeding experiments.¹¹ To determine the biosynthetic origin of polyketide carbon sources of reveromycin A, we also fed [¹³C]acetate and [¹³C]propionate dissolved in alcohol. During the purification of ¹³C-incorporated species, we observed an unexpected compound using a photodiode array detector that was attached to an LC/MS instrument and speculated it to be a novel reveromycin derivative. In this study, to identify the structure of this compound, we performed large-scale fermentation of *S. reveromyceticus* SN-593 in the presence of methanol. In addition, ethanol-added fermentation was performed, resulting in the production of another novel reveromycin derivative. Based on these experiments, two new reveromycin derivatives were isolated and determined to be reveromycin T 1-methyl ester (**1**) and 1-ethyl ester (**2**) by spectroscopic and chemical method. We report the creation of these novel derivatives and their structures, which have activities against cancer cell lines.

S. reveromyceticus SN-593 was precultured in a cylindrical flask that contained 70 ml of SK2 medium (1.4% starch, 0.35% glucose, 0.35% yeast extract, 0.21% bacto-peptone, 0.21% meat extract and 0.014% KH₂PO₄, 0.042% MgSO₄·7 H₂O) for 72 h at 28 °C on a rotary shaker at 150 r.p.m. One milliliter of the seed culture was transferred to cylindrical flasks that contained 70 ml of reveromycin production medium (2% potato dextrose, 1% malt extract, 1% dried yeast, 5% TABLE LAND tomato juice, 0.1% K₂HPO₄, 0.1% NaCl, 0.03%

MgSO₄·7 H₂O, 0.01% NaNO₃, 0.005% ZnSO₄·7 H₂O and 0.005% CuSO₄·5 H₂O) and cultured under the same conditions. After 72 h, 0.7 ml methanol or ethanol was added to a flask, which was cultured further for 48 h. A control culture that lacked alcohol was prepared under the same conditions. The culture broth was extracted with ethyl acetate and analyzed by photodiode array detector-LC/MS. The unidentified peaks were observed on the UV chromatograms at 238 nm for the methanol- and ethanol-added culture broths, in which reveromycin A production decreased to ~60%, as shown in Figure 2. These peaks were determined to be reveromycin derivatives, with typical UV absorption spectra for reveromycins with a $\lambda_{\max}$ value of ~240 nm by photodiode array detector analysis.¹ The molecular weights for peaks 1 and 2 were 558 (m/z : 557 [M-H][–]) and 572 (m/z : 571 [M-H][–]) by electrospray ionization tandem MS analysis in the negative mode, respectively, suggesting that they are methyl or ethyl derivatives of reveromycin T¹¹ that have not been isolated. To isolate them, 5.0 and 1.4 l fermentation cultures were begun for the addition of methanol and ethanol, respectively. The methanol-added fermentation broth yielded 5.47 g of ethyl acetate extract, which was separated by C₁₈-HPLC to obtain **1** as a colorless oil (81.1 mg). Colorless oil; [α]_D²⁰ –133 (c 0.15, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 240 (4.58), 265 (4.24, sh) nm; ¹H NMR and ¹³C NMR data, see Table 1; HR electrospray ionization m/z : 557.3440 [M-H][–] (calcd C₃₃H₅₁O₇, 557.3478). The ethanol-added fermentation broth generated 1.2 g of ethyl acetate extract, which was subjected to SiO₂ flash column chromatography, C₁₈-HPLC and preparative TLC to obtain **2** as a colorless oil (12 mg). Colorless oil; [α]_D²⁰ –155 (c 0.4, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 238 (4.85), 264 (4.57, sh) nm; ¹H NMR and ¹³C NMR data, see Table 1; HR fast atom bombardment MS m/z : 571.3638 [M-H][–] (calcd C₃₄H₅₃O₇, 571.3635).

The molecular formula of compound **1** was determined to be C₃₃H₅₀O₇ by HR electrospray ionization MS. The ¹H NMR spectrum

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Figure 1 Structures of reveromycin derivatives.

showed five methyl signals and a methoxy signal at 3.69 p.p.m. (Supplementary Figure S1). It also showed eight olefinic proton signals and three signals that were attached to oxygenated carbons. Analysis of the ^{13}C NMR and HSQC data confirmed that **1** possessed 33 carbon atoms, including 2 carbonyl carbons and a methoxy carbon, among which 3 sp^3 methines were assigned to carbon atoms that bore an oxygen atom (Supplementary Figures S2 and S5). In addition, 1 sp^3 acetal carbon signal at 97.2 p.p.m. was observed, indicating that **1** had a 6,6-spiroacetal structure.¹² The ^{13}C NMR spectra also showed 10 olefin signals, suggesting the presence of five double bonds. These observations, in conjunction with the UV absorption spectrum and molecular formula, implied that **1** is a reveromycin derivative with an additional methoxy group and the absence of a succinyl group compared with reveromycin A. Its planar structure was elucidated by analysis of the 2D NMR data, as shown in Figure 1. The DQF-COSY and HSQC-TOCSY spectra revealed three spin systems from C-2 to C-7 with a methyl branch at C-4, C-9 to C-14 with a methyl branch at C-12, and C-16 to C-21 with a butyl branch at C-18 (Supplementary Figures S3 and S4). The connections between the spin systems were established by long-range correlations in the HMBC spectrum (Supplementary Figure S6), and the overall structure was established. The methoxy group was assigned to the C-1 position, based on the long-range correlations from the methoxy signal to C-1. The 6,6-spiroacetal core was confirmed, based on the HMBC correlations from H-14, H-17, and H-19 to the acetal carbon

Figure 2 UV chromatograms of alcohol-added fermentation broth.

and H-14 to C-16. The geometries of the double bonds at Δ^2 , Δ^6 and Δ^{20} were designated to have *E* configurations, based on the coupling constants in the ^1H NMR spectrum. The Δ^9 and Δ^{22} bonds were *E* configurations, based on the ^{13}C NMR chemical shift values of C-8-Me and C-22-Me. The absolute configuration at C-5 was determined to be *R* by the modified Mosher's method of the dimethyl ester that was converted from **1** by treatment with trimethylsilyl diazomethane.^{13,14} The configuration at C-15 was determined to be *S** by the NOESY correlations between H-11/H-13, H-16axial/H-18 and H-14equatorial/H-16axial in phase-sensitive NOESY spectrum (Supplementary Figures S7 and S8). The remaining stereochemistry including absolute configuration at C-15 assigning to be *S* was deduced to be the same as that of reveromycin A, comparing its NMR chemical shifts and optical rotations^{12,14} with those of spirofungins A and B.^{15–18} Based on these findings, the structure of **1** was determined as shown in Figure 1.

Compound **2** had a similar ^1H NMR spectrum as **1**, except for a newly observed ethoxy signal that comprised a triplet at 1.26 p.p.m., coupled with a quartet signal at 4.16 p.p.m. and the disappearance of a methoxy signal of **1** (Supplementary Figure S9). The molecular formula was established as $\text{C}_{34}\text{H}_{52}\text{O}_7$ by HR fast atom bombardment MS, which was identical to an ethyl ester form of **1**. The planar structure was elucidated in the same manner as that of **1**, based on an analysis of 2D NMR data (Supplementary Figures S10–S14). The attachment of the ethoxy group at C-1 was determined, based on the HMBC correlation from the quartet signal to C-1. The stereochemistry was deduced to be the same as **1**, based on a comparison of NMR data, and the structure was determined as shown in Figure 1.

The cytotoxic activities of compounds **1** and **2** and reveromycin A were evaluated against HL-60 (human promyelocytic leukemia cell line), K562 (human erythromyeloblastoid leukemia cell line) and tsFT210 (mouse temperature-sensitive mutant with a defect in *cdc2*) cells. Compared with reveromycin A, compounds **1** and **2** showed improved activity, exhibiting a 2–10-fold reduction against the HL-60 and K562 cell lines, with IC_{50} values of 0.9, 2.5 for **1**; 0.2, 0.7 for **2** and 2.7, 4.9 $\mu\text{g ml}^{-1}$ for reveromycin A. All three compounds showed moderate activity against tsFT210 cells, with IC_{50} values of 8.2, 11.4 and 8.5 $\mu\text{g ml}^{-1}$ for **1**, **2** and reveromycin A, respectively. In contrast, they did not show any antibacterial effects against *Escherichia coli* up to 50 $\mu\text{g ml}^{-1}$.

We created and identified two new reveromycin derivatives, reveromycin T 1-methyl ester and 1-ethyl ester, by adding a small amount of related alcohol to culture broth of *S. reveromyceticus* SN-593 during the fermentation. Similar approach to create new metabolites has not been reported before, and this simple method

Table 1 ^1H and ^{13}C NMR chemical shifts of compounds **1** and **2** in methanol- d_4 .

Position	1		2	
	δ_{C}	δ_{H} (multiplicity, J in Hz)	δ_{C}	δ_{H} (multiplicity, J in Hz)
1	168.7	—	168.4	—
2	121.8	5.84 (dd, 15.6, 0.7)	122.3	5.83 (dd, 15.6, 1.5)
3	152.9	6.99 (dd, 15.6, 7.8)	152.7	6.98 (dd, 15.6, 7.6)
4	44.1	2.51 (m)	44.2	2.51 (m)
5	76.8	4.05 (dd, 7.3, 6.4)	76.9	4.06 (dd, 7.6, 6.6)
6	127.7	5.50 (dd, 15.6, 7.3)	127.8	5.50 (dd, 15.6, 7.6)
7	137.9	6.23 (d, 15.6)	137.9	6.24 (d, 15.6)
8	135.3	—	135.4	—
9	129.7	5.58 (t, 7.1)	129.7	5.59 (t, 7.1)
10	33.0	2.35 (m, 2H)	33.1	2.36 (m, 2H)
11	76.0	3.43 (m)	76.1	3.45 (ddd, 10.1, 4.0, 4.0)
12	34.9	1.36 (m)	34.9	1.36 (m)
13	28.7	1.44 (m, 2H)	28.8	1.46 (m, 2H)
14	37.2	1.42 ax. (m) 1.66 eq. (m)	37.3	1.42 ax. (m) 1.66 eq. (ddd, 12.1, 3.0, 3.0)
15	97.2	—	97.2	—
16	37.5	1.53 ax. (m) 1.76 eq. (m)	37.6	1.53 ax. (m) 1.76 eq. (m)
17	22.6	1.54 ax. (m) 1.75 eq. (m)	22.6	1.53 ax. (m) 1.76 eq. (m)
18	39.8	1.74 (m)	39.9	1.74 (m)
19	79.2	4.24 (br dd, 9.6, 4.1)	79.3	4.23 (dd, 9.6, 4.0)
20	135.5	6.55 (dd, 15.6, 9.6)	134.8	6.51 (dd, 15.6, 9.6)
21	137.97	6.32 (d, 15.6)	138.2	6.31 (d, 15.6)
22	152.8	—	151.0	—
23	121.0	5.82 (s)	122.5	5.83 (s)
24	170.4	—	171.5	—
25	32.7	1.09 (m, 2H)	32.8	1.10 (m) 1.13 (m)
26	30.0	1.28 (m, 2H)	30.1	1.22 (m) 1.28 (m)
27	23.8	1.27 (m, 2H)	23.9	1.28 (m, 2H)
28	14.3	0.87 (t, 7.1, 3H)	14.4	0.87 (t, 7.1, 3H)
Me-4	15.1	1.06 (d, 6.9, 3H)	15.2	1.07 (d, 7.1, 3H)
Me-8	12.9	1.74 (s, 3H)	13.0	1.74 (s, 3H)
Me-12	18.0	0.76 (d, 6.4, 3H)	18.0	0.77 (d, 6.6, 3H)
Me-22	14.6	2.24 (d, 0.9, 3H)	14.7	2.22 (s, 3H)
1'	51.9	3.69 (s, 3H)	61.4	4.16 (q, 7.3, 2H)
2'			14.6	1.26 (t, 7.3, 3H)

1 was recored at 500 MHz for ^1H and 125 MHz for ^{13}C . **2** was recored at 600 MHz for ^1H and 150 MHz for ^{13}C .

might be applied to other metabolites to expand their diversity for structure–activity relationship study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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