

NOTE

Structures and biological activities of novel 4'-acetylated analogs of chrysomycins A and B

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Two new 4'-acetylated analogs of chrysoicin were discovered during the screening for antitumor agents from the metabolites of actinomycetes. Their structures and physicochemical properties were determined by standard spectrometric analyses. Their cytotoxicities and antimicrobial activities were evaluated against a panel of cancer cell lines and microbes. While acetylation reinforced the cytotoxicity of chrysoicin B, it weakened the activity of chrysoicin A. Chrysoicin A and its acetylated analog showed high cytotoxicity toward most of the cancer cells with IC_{50} s less than 10 ng ml^{-1} . The 4'-acetyl-chrysoicin A was predominantly observed in nuclei at concentrations where the autofluorescence was observable. Chrysoicins were effective toward Gram-positive bacteria. The 4'-acetylated-chrysoicin A and B had MICs of $0.5\text{--}2 \mu\text{g ml}^{-1}$ and 2 to greater than $64 \mu\text{g ml}^{-1}$, respectively, toward Gram-positive bacteria including MRSA and VRE.

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Chrysoicins are benzonaphthopyranone glycosides found in *Streptomyces* spp.¹ Analogous compounds possessing the same chromophore, such as gilvocarcins, ravidomycins and polycarcins, are also known.^{2–5} This group of compounds has strong antitumor properties and mild toxicities toward mouse. The antitumor activities of chrysoicins were evaluated in mice up to $256\text{--}512 \text{ mg kg}^{-1}$ (i.p.) and elongation of survival times (~200%) were reported.⁶ The LD_{50} values of gilvocarcins were $>300 \text{ mg kg}^{-1}$ (i.v.) and $>1000 \text{ mg kg}^{-1}$ (p.o. and i.p.) and they showed similar effects on the elongation of the survival time of tumor-burdened mice.^{7,8} Their inhibition of topoisomerase II and cross-linking of histone H3 and heat-shock protein GRP78 to DNA may be involved in the antitumor activity.^{9,10} The related compound elsamitrucin was evaluated in several cancers in phase II studies.^{11,12} Elsamitrucin showed moderate activity including 13% partial and 7% minor responses, and 30% stable disease in patients with refractory or relapsed non-Hodgkin's lymphoma.¹²

Chrysoicin is a classic antibiotic first reported in 1955.¹³ Although its antiphage and antibacterial activities and some physicochemical properties were described, the test samples contained a mixture of analogs¹ with unknown structures. There were reports on the isolation and structure determination of chrysoicins A and B during the 1970s and 1980s, including studies of virenomycins as the synonym.^{1,14–17} Recently, chrysoicin C was reported as the reduced form of chrysoicin A and the existence of two other analogs, chrysoicins D and E, were indicated.¹⁸ Here, we describe the structures, physicochemical properties, cytotoxicity and antimicrobial activities of new 4'-acetylated analogs of chrysoicins A (1) and B (2) (Figure 1).

During our regular screening for antitumor compounds from the metabolites of actinomycetes using multiple human cancer cell lines and healthy mouse primary culture cells obtained from several organs, the culture broth extract of *Streptomyces* sp. strain MG271-CF2 showed about 100-fold stronger toxicity toward some cancer cells compared with normal cells. While the 16S ribosomal RNA gene sequence of the strain showed high homology with that of *Streptomyces durmitorensis* (strain MS405^T, 1399 bp/1406 bp, 99.5%), *S. aureus* (strain B7319^T, 1377 bp/1384 bp, 99.5%) and *S. kanamyceticus* (strain NBRC13414^T, 1373 bp/1384 bp, 99.2%), the gene sequence showed rather lower homology with those of the other chrysoicin-producing species *S. virens*¹⁴ (strain NRRL B-24331, 1331 bp/1390 bp, 95.8%) and *S. sporoverrucosus*¹⁸ (strain NRRL B-16379, 1363 bp/1406 bp, 96.9%).

The small-scale separation of the culture extract by octadecylsilica (ODS)-HPLC, HR-MS, and UV analyses indicated that chrysoicins A, B, and comparable amounts of two new analogous compounds 1 and 2 existed in the bioactive fractions. These two analogs are more hydrophobic, have similar UV spectra, and molecular weights of 42 Da larger (550 and 538 Da) than those of chrysoicins A and B (Supplementary Table 1), suggesting they are new monoacetylated analogs of chrysoicins. The new analogs are not the presumed analogs chrysoicins D and E because they are more hydrophilic than chrysoicins A and B and their molecular weights are 526 and 524 Da as determined by liquid chromatography-MS profiling, respectively.¹⁸ To enable structural determination and biological testing, larger-scale production and purification were attempted. The ethyl acetate extract of the producing strain culture was dissolved in methanol and left until

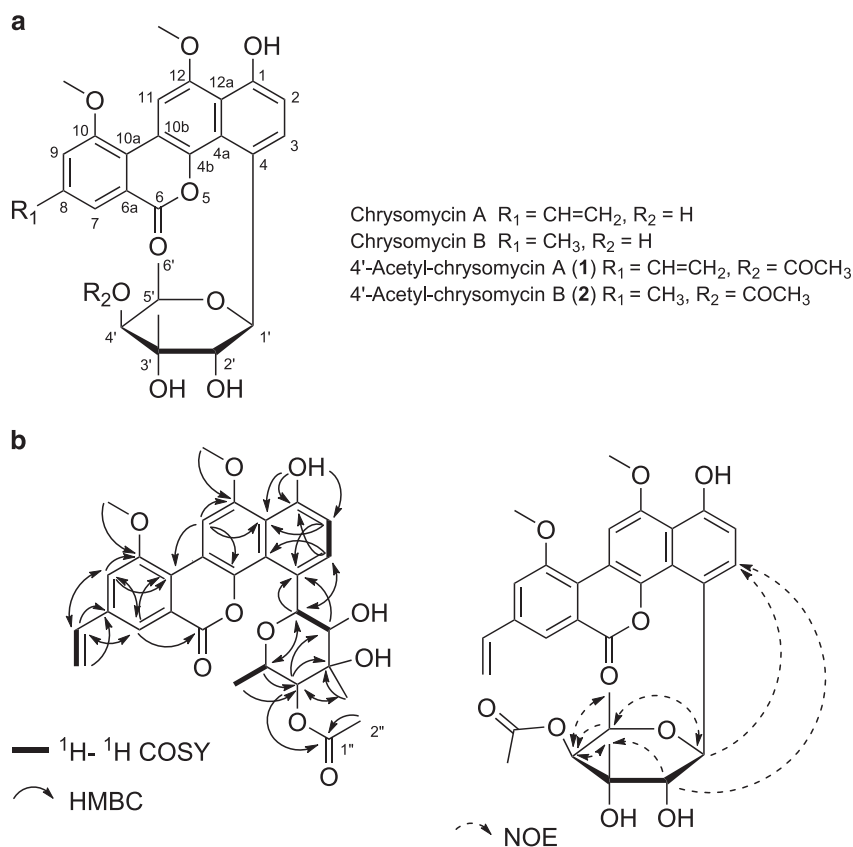


Figure 1 (a) Structures of chrysomycins and new acetylated analogs **1** and **2**. (b) ^1H - ^1H COSY, HMBC, and NOE correlations of **1**.

sufficient lime precipitate mainly consisting of chrysomycins was formed. The precipitate was used as the source of the purification of **1** and **2**. A previous report described that the separation of chrysomycins A and B was difficult and after subjecting the compounds to triacetylation, they were finally separated by HPLC.¹ The new analogs **1** and **2** were also difficult to separate. As they were monoacetylated compounds and selective deacetylation could not be used after the separation, the triacetylation strategy could not be applied. Several liquid chromatography columns were tested in an attempt to purify each analog. While most of the columns were not suitable for separating the analogs, CHP-20 provided the best separation of **1** and **2**. Because the analogs were not completely purified after a single separation, CHP-20 separations were performed repeatedly. ODS-HPLC was used to remove other impurities. Although there was still some material that was not purified (~20 mg each), 11.5 mg of **1** and 31.1 mg of **2** were isolated from the culture of 1620 g of barley medium (720 g grain). The physicochemical properties of **1** and **2** were analyzed as shown in Supplementary Table 1. The UV and IR spectra and optical rotation were consistent with those of chrysomycins and gilvocarcins^{2,15}, although the effect of 4'-acetylation on these physicochemical properties was unclear. The signs of optical rotations of **1** and **2** in acetic acid and methanol were opposite. A similar phenomenon was reported for chrysomycins, in which the signs were positive in acetic acid and negative in dimethyl sulfoxide (DMSO).¹⁵

The structures of **1** and **2** were determined by comparing their NMR and HR-MS data with that published for chrysomycins A and B¹

(Figure 1, Table 1, Supplementary Figure 1 and Supplementary Table 1). The planar structures of **1** and **2** except for the acetylated moiety determined by 2D NMR correlations matched with those of chrysomycins A and B, respectively. The acetyl group was connected to the hydroxyl group at C-4' ($\delta_{\text{C}} 77.1$) of the sugar moiety as determined by the HMBC correlation from H-4' to C-1'' ($\delta_{\text{C}} 170.3$). Some ravidomycins have a 4'-acetylation modification in their six-membered ring sugar.³ The structures of chrysomycin A and ravidomycins are similar in that they share the same chromophore and similar sugars. The difference is at C-3', where the methyl group in chrysomycin A is changed to a N-dimethyl group and a hydroxyl group is deoxygenated in ravidomycins. It is expected that similar acetyltransferases may function to produce the new chrysomycin analogs and ravidomycins. The relative stereochemistry of the sugar moiety was determined by NOE, the optical rotation signs in acetic acid are same, and the absolute stereochemistry of the sugars of **1** and **2** were determined to be consistent with those of chrysomycins.

The cytotoxicities of chrysomycins toward cancer cells were very potent compared with the clinically used anticancer agent doxorubicin (Table 2 and Supplementary Table 2). Consistent with published reports,⁵ the analogs carrying a vinyl group, such as chrysomycin A and **1**, showed especially strong cytotoxicity. Their IC_{50} values in sensitive cell lines were less than 0.1 ng ml^{-1} . Because of the acetylation, the cytotoxicity of **1** was weaker than that of chrysomycin A. In contrast, the cytotoxicity of **2** was more than that of chrysomycin B. This discrepancy may be caused by the changes induced by the acetylation in the cellular permeation and

Table 1 ^1H and ^{13}C NMR data of **1** and **2** in $\text{DMSO}-d_6$

| 1 | | | | | 2 | | | | |
|----------------------|------------------------------------|---|------------------------|-------------------------------------|---------------------|------------------------------------|--------------------------------|------------------------|----------------------------------|
| Position | δ_{C} (multiplicity) | δ_{H} (J, in Hz) | NOE | HMBC | Position | δ_{C} (multiplicity) | δ_{H} (J, in Hz) | NOE | HMBC |
| 1 | 153.5 (s) | | | | 1 | 153.5 (s) | | | |
| 2 | 112.1 (d) | 6.98 (1H, d, 8.3) | | 1, 4, 12a | 2 | 111.8 (d) | 6.96 (1H, d, 8.3) | | 1, 4, 12a |
| 3 | 129.1 (d) | 7.76 (1H, d, 8.3) | | 1, 4a, 1' | 3 | 129.1 (d) | 7.75 (1H, d, 8.3) | | 1, 4a, 1' |
| 4 | 126.9 (s) | | | | 4 | 126.7 (s) | | | |
| 4a | 125.3 (s) | | | | 4a | 125.3 (s) | | | |
| 4b | 142.3 (s) | | | | 4b | 141.9 (4b) | | | |
| 6 | 159.8 (s) | | | | 6 | 159.9 (s) | | | |
| 6a | 122.0 (s) | | | | 6a | 121.6 (s) | | | |
| 7 | 119.1 (d) | 8.00 (1H, brs) | | 6, 9, 10a, 8-CH=CH ₂ | 7 | 121.3 (d) | 7.78 (1H, brs) | | 6, 10a, 9, 8-CH ₃ |
| 8 | 138.9 (s) | | | | 8 | 140.6 (s) | | | |
| 9 | 114.8 (d) | 7.73 (1H, brs) | | 7, 10, 10a, 8-CH=CH ₂ | 9 | 119.2 (d) | 7.50 (1H, brs) | | 7, 10, 10a, 8-CH ₃ |
| 10 | 157.5 (s) | | | | 10 | 157.0 (s) | | | |
| 10a | 122.9 (s) | | | | 10a | 120.9 (s) | | | |
| 10b | 113.4 (s) | | | | 10b | 113.6 (s) | | | |
| 11 | 101.7 (d) | 8.49 (1H, brs) | | 4b, 10a, 12a | 11 | 101.8 (d) | 8.49 (1H, s) | | 4b, 10a, 12, 12a |
| 12 | 151.9 (s) | | | | 12 | 151.9 (s) | | | |
| 12a | 115.4 (s) | | | | 12a | 115.2 (s) | | | |
| 10-OCH ₃ | 56.3 (q) | 4.16 (3H, s) | | 10 | 10-OCH ₃ | 56.6 (q) | 4.10 (3H, s) | | 10 |
| 12-OCH ₃ | 56.8 (q) | 4.11 (3H, s) | | 12 | 12-OCH ₃ | 56.4 (q) | 4.11 (3H, s) | | 12 |
| 8-CH=CH ₂ | 135.2 (d) | 6.93 (1H, dd, 10.9, 17.6) | | 7, 8, 9 | 8-CH ₃ | 21.1 (q) | 2.50 (3H, brs) | | 7, 8, 9 |
| 8-CH=CH ₂ | 117.3 (t) | 5.50 (1H, d, 11.0), 6.14 (1H, d, 17.6) | | 8, 8- CH=CH ₂ | | | | | |
| 1' | 74.5 (d) | 6.03 (1H, d, 9.7) | 3, 5' | 3, 4, 2', 5' | 1' | 74.5 (d) | 6.03 (1H, d, 9.7) | 2', 5' | 3, 4, 2', 5' |
| 2' | 72.1 (d) | 3.69 (1H, d, 9.7) | 3, 3'-CH ₃ | 4, 1' | 2' | 72.1 (d) | 3.69 (1H, dd, 8.5, 9.7) | | 4, 1' |
| 3' | 71.6 (s) | | | | 3' | 71.6 (s) | | | |
| 4' | 77.1 (d) | 4.75 (1H, d, 1.1) | 6', 3'-CH ₃ | 2', 3', 1", 3'- CH ₃ | 4' | 77.1 (d) | 4.74 (1H, brd, 1.0) | 6', 3'-CH ₃ | 3', 3'-CH ₃ , 1" |
| 5' | 69.2 (q) | 4.71 (1H, dq, 1.1, 6.5) | 1', 4' | 1', 4', 6' | 5' | 69.2 (d) | 4.70 (1H, dq, 1.0, 6.6) | 1', 6' | 1', 4', 6' |
| 6' | 16.6 (q) | 0.87 (3H, d, 6.5) | 4', 5' | 4', 5' | 6' | 16.6 (q) | 0.86 (3H, d, 6.6) | 4' | 4', 5' |
| 3'-CH ₃ | 22.8 (q) | 1.16 (3H, s) | 4' | 3', 4' | 3'-CH ₃ | 22.9 (q) | 1.16 (3H, s) | 4' | 3', 4' |
| 1" | 170.3 (s) | | | | 1" | 170.3 (s) | | | |
| 2" | 20.7 (q) | 2.11 (3H, s) | | 1" | 2" | 20.7 (d) | 2.11 (3H, s) | | 1" |
| 1-OH | | 9.84 (1H, s) | | 1, 2, 12a | 1-OH | | 9.85 (1H, s) | | 1, 2, 12a |
| | | | | | 2'-OH | | 4.43 (1H, d, 8.5) | | 1', 2' |
| | | | | | 3'-OH | | 4.80 (1H, s) | | 2', 4' |

Chemical shifts of ^1H (600 MHz) and ^{13}C (150 MHz) NMR spectra were adjusted with solvent signal.

in the binding to DNA and binding proteins. Some DNA intercalators not only bind to nucleic DNA but also to mitochondrial DNA either preferably or non-selectively. The localization of **1** and **2** in the culture cells was observed by harnessing their autofluorescence (Figure 2). The signal of **1** showed a similar pattern to that of 4',6'-diamidino-2-phenylindole (DAPI) as it localized in the nuclei at high concentrations. The whole cells were obscurely bright and slightly stronger signals were observed around the nuclei at low concentrations. It seemed that it was more difficult for compound **2** to enter into the nuclei. Even at a high concentration, it showed similar patterns as that of the low concentration of **1**. Compounds **1**, **2**, and DAPI were strongly observed in the rounded cells, which were probably in M phase.

The collapse of the nuclear membrane might facilitate the access of the compounds to the nuclear DNA. The preference of mitochondrial DNA for the intercalator ethidium bromide (EtBr) led it to be localized in the nuclei at the high concentration but was localized in the mitochondria and nucleolus at the low concentration as previously reported.¹⁹ Such mitochondrial selective localization was not observed for **1**, **2**, and DAPI at the concentration at which their signals were observable.

While **1** and **2** showed no or low anti-fungal and anti-Gram-negative bacterial activity, they showed anti-Gram-positive bacterial activity (Supplementary Tables 3 and 4). Chrysomycin A and **1** showed strong anti-Gram-positive bacterial activity toward MRSA and VRE strains with MIC values of 0.5–2 $\mu\text{g ml}^{-1}$. This trend is

Table 2 Cytotoxicity of chrysomycin analogs

| | | <i>IC</i> ₅₀ (ng ml ⁻¹) | | | | |
|--------------|----------------|--|-------------|---------------|---------|-------------|
| | Cell line | Chrysomycin A | 1 | Chrysomycin B | 2 | Doxorubicin |
| Sensitive | ME-180 | 0.0059±0.0042 | 0.085±0.057 | 8.8±1.2 | 1.7±1.0 | 440±300 |
| | WER1-Rb-1 | 0.0082±0.0021 | 0.098±0.069 | 18±1.7 | 2.8±1.4 | <156.25 |
| | STC1 | 0.035±0.036 | 0.26±0.076 | 68±3.7 | 14±7.5 | <156.25 |
| Average | HS-Os-1 | 0.21±0.20 | 1.4±0.75 | 420±12 | 56±47 | >1250 |
| | COLO201 | 0.26±0.24 | 1.7±1.2 | 340±140 | 37±23 | >1250 |
| | GI-1 | 0.22±0.22 | 1.4±0.85 | 450±2.0 | 57±42 | >1250 |
| Resistant | VMRC-MELG | 5.6±0.5 | 14±12 | 780±22 | 300±210 | >1250 |
| | CACO-2 | 3.7±4.2 | 3.4±0.6 | 920±100 | 260±120 | >1250 |
| | TM-31 | 2.5±2.2 | 16±9.6 | 840±54 | 370±230 | >1250 |
| Normal Cells | Mouse kidney | 0.48±0.09 | 2.1±1.3 | 630±22 | 140±60 | >1250 |
| | Mouse pancreas | 2.6±2.3 | 7.7±6.3 | 770±19 | 180±70 | >1250 |
| | Mouse lung | 4.6±5.0 | 9.8±8.9 | 880±130 | 320±150 | >1250 |

The representative results of the cytotoxicities of chrysomycins for the sensitive, average, resistant human cancer cells and mouse primary culture cells. MTT assays were performed twice for one lot of chrysomycins A and B and two lots of **1** and **2**. The mean±s.d. of the compound is shown. Chrysomycins were tested at 0.1 pg ml⁻¹–1000 ng ml⁻¹. Doxorubicin was tested as a control at 156.25–1250 ng ml⁻¹.

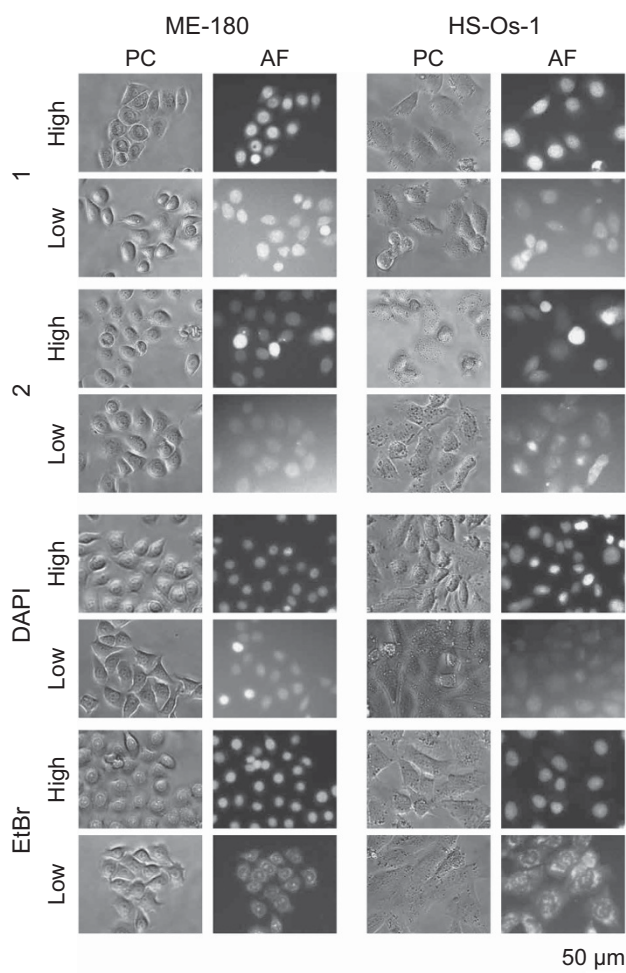


Figure 2 Localization of **1** and **2** in culture cells. Human cancer cell lines ME-180 and HS-Os-1 were treated with **1**, **2**, 4',6-diamidino-2-phenylindole (DAPI), and ethidium bromide (EtBr) at a high (10 µg ml⁻¹ for all compounds) and low (0.02 µg ml⁻¹ for EtBr and 0.1 µg ml⁻¹ for other compounds) concentration for 1.5 h and the compound autofluorescence was observed. Because the low concentration treatments emitted weak signals and exposed with stronger conditions, the backgrounds were brighter. PC and AF are phase contrast and autofluorescence, respectively.

consistent with previously reported antimicrobial activities of chrysomycins.^{13,16,20} The only difference was noted for *Mycobacterium smegmatis* where a crude mixture of chrysomycins was used and a MIC value of 0.6 µg ml⁻¹ was obtained, while the analogs reported herein had MIC values greater than 64 µg ml⁻¹.¹³ This difference might be caused by some impurities in their chrysomycin mixture.

Because their toxicity to mouse is often low and their cytotoxicity to tumor cells and anti-Gram-positive bacterial activity are high, chrysomycins, including the new analogs found in this study, could be good drug candidates for the treatment of these illnesses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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