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ORIGINAL ARTICLE

New pyranonaphthoquinones and a phenazine alkaloid isolated from *Streptomyces* sp. IFM 11307 with TRAIL resistance-overcoming activity

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Four new pyranonaphthoquinones (1–4) were isolated from the liquid culture of *Streptomyces* sp. IFM 11307. Additionally, one new phenazine derivative (5), along with the known phenazine-1,6-dicarboxylic acid (6) were identified. The chemical structure of compounds 1–6 was elucidated by 1D and 2D NMR spectroscopy together with CD spectral analysis. Compounds 1–4 significantly overcame tumor necrosis factor-related apoptosis-inducing ligand resistance in human gastric adenocarcinoma cell lines.
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Keywords: phenazine; pyranonaphthoquinones; *Streptomyces*; TRAIL

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent, as it can kill tumor cells selectively.[1] TRAIL-induced apoptosis initiated by the death receptor pathway involves the engagement of death receptors, formation of a death-inducing signaling complex, proteolytic activation of caspase-8 and, consequently, activation of caspase-3. However, considerable numbers of cancer cells are resistant to TRAIL. Overcoming TRAIL resistance and understanding the mechanisms underlying such resistance are, therefore, very important in anticancer drug discovery.[2]

In the course of our screening program for bioactive natural products from actinomycetes,[3–5] we collected soil and seawater samples from different areas of Japan. The crude extract of terrestrial *Streptomyces* sp. IFM 11307 drew our attention due to striking yellow bands on TLC, which gave a blue color reaction with 2 N NaOH and strong orange fluorescence under UV light (366 nm). Here, we report the isolation and structural elucidation of four new pyranonaphthaquinones (**1**–**4**) and a phenazine derivative (**5**) from the culture extract of *Streptomyces* sp. IFM 11307. Compounds **1**–**5** were evaluated for their activity in overcoming TRAIL resistance in human gastric adenocarcinoma (AGS) cell lines, as we are interested in screening studies targeting signaling molecules related to cancer diseases.[6]

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured with a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). IR spectra were measured by ATR (attenuated total reflection) on a JASCO FT-IR 230 spectrophotometer (JASCO). UV spectra were measured on a Shimadzu UV mini-1240 spectrometer (Shimadzu, Kyoto, Japan). The NMR spectra were recorded on JEOL JNM-A500 and JEOL JNM-ecp600 spectrometers (JEOL, Tokyo, Japan) with a deuterated solvent, the chemical shift of which was used as an internal standard (see supporting information). Mass spectra were recorded on an AccuTOF-T100LP (JEOL) mass spectrometer.

Identification of the *Streptomyces* sp. IFM 11307

The strain IFM 11307 was isolated on humic acid-vitamin agar,[7] from a soil sample collected from Yoro-keikoku, Ichihara-shi, Chiba prefecture, Japan in 2007. It was identified as *Streptomyces* sp. and deposited at the Medical Mycology Research Center, Chiba University, Japan with the code number IFM 11307. Identification of the strain was carried out by sequence analysis of 16S rRNA gene using the DDBJ-BLAST search.

Fermentation of the *Streptomyces* sp. IFM 11307

The *Streptomyces* sp. IFM 11307 was cultivated from the glycerol stock on the Waksman agar medium at 28 °C for 3 days. Pieces of agar (1 cm^2) cultures were used to inoculate 4×500 cm^3 Sakaguchi flasks each containing 100 ml of the Waksman medium under shaking (200 r.p.m.) at 28°C for 5 days. The subculture was used to inoculate 16×3 l round flask each containing 750 ml of the Waksman medium under the same conditions.

Extraction and isolation

The culture broth (12 l) was centrifuged at 3500 r.p.m. for 20 min then extracted three times with ethyl acetate. The organic layer was concentrated in *vacuo* to dryness to give 2.81 g of crude extract. The mycelial cake was extracted three times with acetone. After removal of acetone, the aqueous solution was extracted three times with EtOAc to yield 0.60 g residue. As the TLC of both extracts from the culture filtrate and mycelia showed the same composition, they were combined and concentrated under reduced pressure. The crude extract of *Streptomyces* sp. IFM 11307 (3.41 g) was subjected to silica

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gel 60 N flash column chromatography (φ 25×600 mm) using gradient of $CHCl_3$/MeOH to afford six fractions. Fraction II (224 mg) was subjected to Sephadex LH-20 (GE Healthcare BioScience, Uppsala, Sweden; φ 15×600 mm, $CHCl_3$/MeOH, 3:2) to give three subfractions Iia–IIc. Subfraction IIb (69 mg) was purified by preparative HPLC (Nomura Chemical Co., Ltd, Seto, Japan; Develosil ODS HG-5, 10×250 mm) to give compounds **1** (1.5 mg, R_t=26.5 min) and **3** (2.7 mg, R_t=28.3 min). The mobile phase was gradients of CH_3CN/H_2O at flow rate of 2.0 ml min^{-1}. Subfraction IIa (35 mg) was applied to preparative TLC (six plates, 20×20 cm, $CHCl_3$/5%MeOH) to yield compound **2** (2.4 mg). Compounds **4** (6.5 mg) and **5** (2.9 mg) were isolated from fraction III (130 mg) by Sephadex LH-20 chromatography (φ 15×600 mm, MeOH) followed by preparative TLC (five plates, 20×20 cm, CH_2Cl_2/15%MeOH). Compound **6** (8.3 mg) was precipitated when acetone was added to fraction IV and the mixture was kept overnight. It was finally purified by Sephadex LH-20 chromatography (φ 15×600 mm, MeOH).

Fluorometric microculture cytotoxicity assay (FMCA)

AGS cells were seeded in a 96-well culture plate (6×10^3 cells per well) in 200 µl of RPMI medium containing 10% fetal bovine serum. Cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Then the test samples with or without TRAIL (Wako, Osaka, Japan, 100 ng ml^{-1}) at different doses were added to each well. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS), and 200 µl of PBS containing fluorescein diacetate (10 µg ml^{-1}) was added to each well. The plates were then incubated at 37 °C for 1 h, and fluorescence was measured in a 96-well scanning spectrofluorometer at 538 nm, following excitation at 485 nm.

(2S,9S,10S,3′S,4′S,6′S)-griseusin E (1): yellow solid; $[\alpha]_D^{22}$+54.9 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 424 (3.1), 252 (3.6) and 211 (4.1) nm; CD (MeOH) λ_{ext} (Δε) 452 (+0.2), 381 (+0.1), 334 (1.4), 273 (+4.4) and 214 (+6.9) nm; IR ν_{max} (ATR) ca 3394, 2921, 2852, 1732, 1646, 1456, 1035 and 800 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; (+)-HRESIMS *m/z* 499.1227 $[M+Na]^+$ (calcd. for $C_{23}H_{24}O_{11}Na$, 499.1216); (−)-HRESIMS *m/z* 475.1191 $[M-H]^-$ (calcd. for $C_{23}H_{23}O_{11}$, 475.1240).

(2S,10R,3′S,4′S,6′S)-4′-deacetyl-griseusin B methyl ester (2): yellow solid; $[\alpha]_D^{22}$+173.8 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 421 (3.5), 249 (4.0) and 211 (4.5) nm; CD (MeOH) λ_{ext} (Δε) 436 (+2.6), 355 (+2.4), 289 (7.2), 252 (+28.1) and 214 (+9.0) nm; IR ν_{max} (ATR) ca 3587, 2917, 2849,1637, 1456, 1275, 1086 and 799 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; (+)-HRESIMS *m/z* 441.1151 $[M+Na]^+$ (calcd. for $C_{21}H_{22}O_9Na$, 441.1162).

(2S,9S,10S,3′S,4′S,6′S)-4′-deacetyl-griseusin A (3): $[\alpha]_D^{22}$+148.2 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 421 (3.5), 249 (4.0) and 211 (4.5) nm; CD (MeOH) λ_{ext} (Δε) 459 (+6.1) 365 (+1.4), 320 (13.2), 260 (+48.6) and 213 (+51.3) nm; ^1H NMR (600 MHz, $CDCl_3$) δ 11.9 (1H, s, 4-OH), 7.69 (1H, m, H-6), 7.66 (1H, m, H-7), 7.30 (1H, d, *J*=7.4 Hz, H-5), 5.27 (1H, d, *J*=2.5 Hz, H-9), 4.79 (2H, m, H-3′, H-10), 4.19 (1H, m, H-6′), 4.16 (1H, m, H-4′), 3.03 (1H, dd, *J*=4.6, 14.0 Hz, Ha-11), 2.75 (1H, d, *J*=14.0 Hz, Hb-11), 2.07 (1H, m, Ha-5′), 1.93 (1H, ddd, *J*=2.7, 3.5, 14.1 Hz, Hb-5′), 1.26 (3H, d, *J*=6.5 Hz, H_3-7′); (+)-HRESIMS *m/z* 425.0811 $[M+Na]^+$ (calcd. for $C_{20}H_{18}O_9Na$, 425.0849).

(2S,10R,3′S,4′S,6′S)-4′-deacetyl-griseusin B (4): yellow solid; $[\alpha]_D^{22}$+162.4 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 427 (3.0), 248 (3.9) and 212 (4.2) nm; CD (MeOH) λ_{ext} (Δε) 453 (+0.36), 345 (+0.69), 289 (−1.7), 252 (+7.1) and 213 (+1.6) nm; ^1H NMR (600 MHz, $CDCl_3$) δ 12.11 (1H, s, 4-OH), 7.60 (1H, m, H-7), 7.58 (1H, m, H-6), 7.25 (1H, d, *J*=7.9 Hz, H-5), 4.68 (1H, d, *J*=3.9 Hz, H-3′), 4.46 (1H, m, H-10), 4.31(1H, m, H-6′), 4.15 (1H, m, H-4′), 2.88 (1H, dd, *J*=2.8, 17.2 Hz, Ha-9), 2.51 (1H, dd, *J*=8.1, 17.2 Hz, Hb-9),

Table 1 ^1H and ^{13}C NMR data for compounds 1 and 2

	1[b]		***2***[b]	
Position	δ_H	δ_C	δ_H	δ_C
2	—	99.1	—	99.4
2a	—	146.9	—	146.3
3	—	190.2	—	187.8
3a	—	116.7	—	115.2
4	—	162.9	—	161.8
4-OH			12.13 (1H, s)	—
5	7.21 (1H, d, 8.2)	125.7	7.24 (1H, d, 7.9)	125.1
6	7.60 (1H, t, 8.2)	137.7	7.59 (1H, t, 7.9)	136.3
7	7.54 (1H, d, 8.2)	119.7	7.56 (1H, d, 7.9)	119.1
7a	—	133.2	—	131.4
8	—	183.9	—	183.1
8a	—	140.9	—	139.8
9a	4.50 (1H, d, 2.2)	60.7	2.86 (1H, dd, 3.2, 16.2)	28.4
9b	—		2.39 (1H, dd, 11.8, 16.2)	
10	4.45 (1H, m)	68.9	4.42 (1H, m)	63.6
11a	2.84 (1H, d, 15.6)	36.5	2.77 (1H, dd, 3.1, 15.4)	39.7
11b	2.69 (1H, dd, 3.6, 15.6)		2.69 (1H, dd, 9.6, 15.4)	
12	—	172.9	—	171.1
13	3.64 (3H, s)	52.3	3.74 (3H, s)	52.3
3′	4.70 (1H, d, 4.1)	68.6	4.66 (1H, d, 4.2)	68.1
4′	5.18 (1H, ddd, 12.9, 4.7, 4.1)	71.2	4.07 (1H, ddd, 13.1, 4.2, 3.1)	68.8
4′-OCO<u>C</u>H$_3$	2.00 (3H, s)	21.2		
4′-O<u>C</u>OCH$_3$	—	173.1		
5′a	1.80 (1H, m)	37.5	2.06 (1H, m)	39.6
5′b	1.75 (1H, ddd, 13.2, 4.7, 2.1)		1.88 (1H, ddd, 2.4, 3.1, 14.2)	
6′	4.20 (1H, m)	63.6	4.24 (1H, m)	61.4
7′	1.08 (3H, d, 6.3)	20.7	1.25 (3H, d, 6.3)	20.7

Underline indicates signals of CH_3 or C=O.
[a]Spectra were measured in CD_3OD.
[b]Spectra were measured in $CDCl_3$.

2.82 (1H, dd, *J*=3.1, 15.0 Hz, Ha-11), 2.69 (1H, d, *J*=15.0 Hz, Hb-11), 2.10 (1H, m, Ha-5′), 1.90 (1H, m, Hb-5′), 1.25 (3H, d, *J*=6.0 Hz, H_3-7′); (+)-HRESIMS *m/z* 427.0992 $[M+Na]^+$ (calcd. for $C_{20}H_{20}O_9Na$, 427.1005).

Yorophenzine (5): yellow solid; $[\alpha]_D^{22}$+47.3 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 449 (3.3), 370 (3.7) and 251 (4.2) nm; CD (MeOH) λ_{ext} (Δε) 287 (−0.77), 258 (−0.14), 244 (+0.15), 225 (−0.33) and 207 (+0.91) nm; IR ν_{max} (ATR) ca 3722, 2921, 2852, 1698, 1456, 1036 and 669 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) δ 8.80 (1H, d, *J*=8.5 Hz, H-7), 8.49 (1H, d, *J*=8.5 Hz, H-9), 8.32 (1H, d, *J*=7.4 Hz, H-2), 8.08 (1H, t, *J*=8.5 Hz, H-8), 7.99 (1H, d, *J*=7.4 Hz, H-3), 4.73 (1H, dd, *J*=4.3, 8.2 Hz, H-2′), 4.05 (3H, s, 1′-OMe), 3.84 (1H, dd, *J*=8.2, 14.0 Hz, H-3′β), 3.49 (1H, dd, *J*=4.3, 14.0 Hz, H-3′α), 1.96 (3H, s, H_3-5′) p.p.m.; ^{13}C NMR (125 MHz, CD_3OD) δ 173.2 (C-4′), 168.4 (6-COOH), 167.9 (C-1′), 167.7 (1-COOH), 144.8 (C-4), 144.6 (C-9a), 142.7 (C-10a), 139.5 (C-5a), 137.9 (C-7), 138.5 (C-4a), 135.9 (C-9), 135.3 (C-2), 132.4 (C-8), 127.1 (C-3), 127.2 (C-1), 125.4 (C-6), 54.5 (C-2′), 53.1 (1′-OCH_3), 35.2 (C-3′), 22.7 (C-5′) p.p.m.; (+)-HRESIMS *m/z* 466.0692 $[M+Na]^+$ (calcd. for $C_{20}H_{17}N_3O_7SNa$, 466.0685); (−)-HRESIMS *m/z* 442.0695 $[M-H]^-$ (calcd. for $C_{20}H_{16}N_3O_7S$, 442.0708).

RESULTS AND DISCUSSION

The producing *Streptomyces* sp. IFM 11307 was isolated from soil sample collected from Chiba prefecture, Japan. The strain was determined to belong to the genus *Streptomyces* on the basis of 16S rRNA analyses. It showed 100% identity to *Streptomyces fimicarius* and 99.8% to *Streptomyces griseus*. Well-grown agar cultures of *Streptomyces* sp. IFM 11307 served to inoculate 4×500 cm^3 Sakaguchi flasks, each containing 100 ml of the Waksman medium.[8] The flasks were incubated at 28 °C while shaking at 200 r.p.m. for 5 days. The seed culture (10 ml) was used to inoculate 16×3-l flasks, each containing 750 ml of the same medium, which were incubated using similar conditions. After centrifugation and extraction of the culture broth (12 l), working up of the crude extract resulted in the isolation of six compounds (**1–6**; Figure 1).

Structure elucidation

Compound **1** was isolated as yellow solid that gave an orange fluorescence under UV light at 366 nm and a blue color reaction with 2 N NaOH. The ESI mass spectrum of compound **1** displayed in the positive ion mode a signal at *m/z* 499 $[M+Na]^+$. In the negative ion mode *m/z* 475 $[M-H]^-$ was visible, suggesting a molecular weight of 476 Da. The molecular formula was estimated as $C_{23}H_{24}O_{11}$ from the (+)-HRESI-MS *m/z* 499.1227 $[M+Na]^+$ (calcd. 499.1216, Δ +1.1 mmu). The UV spectrum of compound **1** showed absorption at λ_{max} 424, 252 and 211 nm, indicating the presence of a *peri*-hydroxy naphthoquinone moiety. The ^1H NMR spectrum of compound **1** was recorded in CD_3OD (Table 1) and showed three aromatic protons forming an ABC spin system (H-5, H-6 and H-7; δ_H 7.21 (d, 8.2), 7.60 (t, 8.2) and 7.54 (d, 8.2)), several oxymethine protons (between δ_H 5.18 and 4.20), one methoxy group [δ_H 3.64 (s)], four methylene protons as well as two methyl groups. The ^{13}C NMR spectrum of compound **1** depicted resonance for two quinone carbonyl groups (δ_C 190.2, 183.9), two ester carbonyl groups (δ_C 173.1 and 172.9), one oxygenated sp^2 carbon (δ_C 162.9), three sp^2 aromatic methine carbons (δ_C 137.7, 125.7 and 119.7) and four sp^2 quaternary carbons (δ_C 146.9, 140.9, 133.2 and 116.7). In the aliphatic region one quaternary carbon (δ_C 99.1), five methine carbons connected to oxygen atoms (δ_C 71.2, 68.9, 68.6, 63.6 and 60.7), one methoxy group (δ_C 52.3), two methylene signals (δ_C 37.5 and 36.5) and two methyl groups (δ_C 21.2 and 20.7) were observed. Analysis of the 2D NMR spectra (^1H-^1H COSY, HMQC and HMBC) of compound **1** gave two units A and B (Figure 2). The juglone moiety (unit A) in compound **1** was confirmed by the following HMBC correlations: H-7 (δ_H 7.54) to C-8 (δ_C 183.9), C-8a (δ_C 140.9), C-5 (δ_C 125.7) and C-3a (δ_C 116.7); H-6 (δ_H 7.60) to C-4 (δ_C 162.9) and C-7a (δ_C 133.2); and H-5 (δ_H 7.21) to C-3a (δ_C 116.7) and C-7 (δ_C 119.7). The partial structure of unit B was indicated by the HMBC couplings from H_3-7′ (δ_H 1.08) to C-6′ (δ_C 63.6) and C-5′ (δ_C 37.5); H_2-5′ (δ_H 1.80 and 1.75) to C-3′ (δ_C 68.6); H-4′ (δ_H 5.18) to C-2 (δ_C 99.1); 4′-$OCOCH_3$ (δ_H 2.00) to the acetate carbonyl group (δ_C 173.1); H-3′ (δ_H 4.70) to C-4′ (δ_C 71.2) and C-2 (δ_C 99.1); H_3-13 (δ_H 3.64) to C-12 (δ_C 172.9); and H-10 (δ_H 4.45) to C-12 (δ_C 172.9). The ^1H-^1H COSY NMR correlations between H-9–H-10–H_2-11 and H-3′–H-4′–H_2-5′–H-6′–H_3-7′ supported structure of unit B. The connectivity between units A and B was confirmed by the HMBC correlations of the oxymethine proton H-9 (δ_H 4.50) to the quinone carbonyl C-8 (δ_C 183.9) and the two sp^2 quaternary carbons C-2a (δ_C 146.9) and C-8a (δ_C 140.9). From these observations, the structure of compound **1** was elucidated as shown in

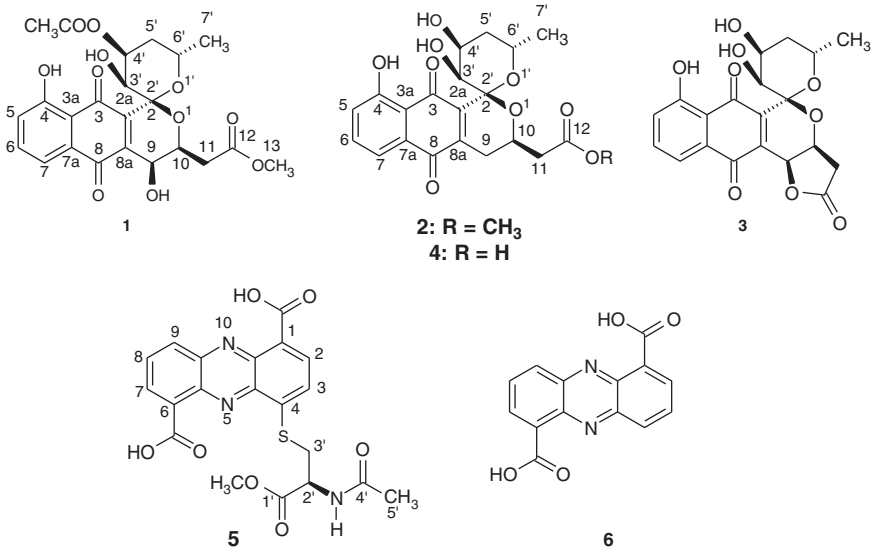

Figure 1 Structures of isolated compounds **1–6**.

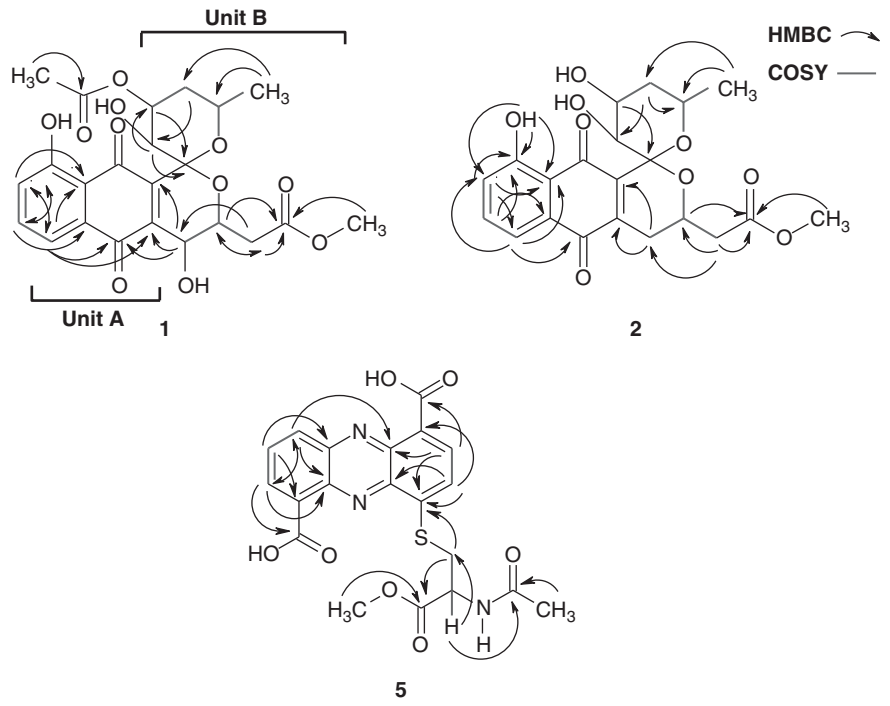

Figure 2 HMBC (⟶) and 1H-1H-COSY (——) NMR correlations of compounds **1**, **2** and **5**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

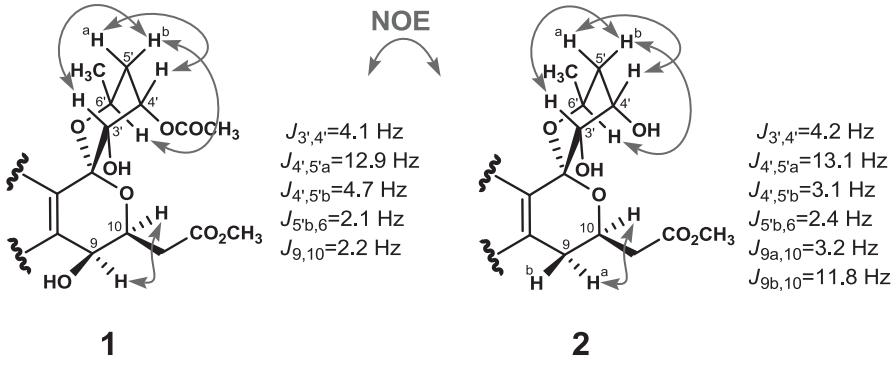

Figure 3 Selected NOE correlations and coupling constants of compounds **1** and **2**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Figure 1 and named as griseusin E. Its relative stereochemistry was determined by the NOE and coupling constants (Figure 3). The NOE observed between the vicinal protons H-3′ and H-4′ supported the conformation of C-3′ and C-4′ as depicted in Figure 3. This conformation was supported by the coupling constant of J=4.1 Hz between H-3′ and H-4′. Additionally, NOE correlations between H-4′ and H-5′a, and H-6′ and H-5′b suggested the configuration of the upper pyrane ring in griseusin E (**1**) shown in Figure 3. The *cis* relation of H-9 and H-10 was confirmed by the NOE between H-9 and H-10 ($J_{9,10}$=2.2 Hz). The absolute configuration of griseusin E (**1**) was determined using CD spectroscopy. It was reported that CD spectral analyses has been used as a tool to assign the absolute configuration of griseusins A and B by comparison with the related (+)-9-deoxygriseusin B and actionorhodin.[9,10] The CD spectrum of compound **1** (see supporting information) showed positive cotton effects at λ_{ext} 452, 381, 273 and 214 nm and one negative sign at λ_{ext} 334. In contrast, the structurally related (−)-griseusins A had negative cotton effects at λ_{ext} 460, 390 and 250 nm and positive sign at λ_{ext} 289 nm. Furthermore, the optical rotation of compound **1** ($[\alpha]_D^{22}$+54.9) has opposite sign to the reported value of griseusin A ($[\alpha]_D^{23}$–147.8).[11] These interesting findings suggested that compound **1** is the (+)-enantiomer of the structurally related (−)-griseusin A with configuration of 2*S*,9*S*,10*S*,3′*S*,4′*S*,6′*S*. The absolute configuration of compound **1** was also confirmed by comparing the CD data with different steroisomers of griseusins[12] and the synthetically known (+)-griseusin A.[13]

Compound **2** was obtained as yellow solid. The molecular weight of compound **2** was determined to be 418 Da from the positive ion mode of (+)-ESI mass spectroscopy. The molecular formula was determined to be $C_{21}H_{22}O_9$ by the (+)-HRESI-MS *m/z* 441.1151 $[M+Na]^+$ (calcd.

441.1162, Δ –1.1 mmu). The ^{1}H NMR of compound **2** (Table 1) recorded in $CDCl_3$ showed signals assigned to a hydrogen-bonded phenolic hydroxyl group [δ_H 12.13 (s)] and protons of a 1,2,3-trisubstituted aromatic spin system (H-5, H-6 and H-7; δ_H 7.24 (d, 7.9), 7.59 (t, 7.9) and 7.56 (d, 7.9)) characteristic of the juglone moiety. In the aliphatic region, four oxymethine protons (H-3′, H-4′, H-6′ and H-10; δ_H 4.66 (d, 4.2), 4.07 (m), 4.24 (m) and 4.42 (m)), one methoxy group [δ_H 3.64 (s)], six methylene proton signals (between δ_H 2.86 and 1.88) as well as one methyl doublet [δ_H 1.35 (d, 6.3)] were observed. The ^{13}C NMR spectrum of compound **2** revealed the presence of three carbonyls and eight sp^2 aromatic carbons. In addition, compound **2** contained 10 sp^3 carbons including four oxygenated methines, one ketal, one methoxy group, three methylenes and one methyl signal. It was clear that the NMR data of compound **2** matched those of griseusin E (**1**). However, the doublet in the proton spectrum of compound **1** at H-9 [δ_H 4.50 (d, 2.2)] had disappeared and a new resonance arising from 2H was observed [δ_H 2.86 (dd, 3.2, 16.2) and 2.39 (dd, 11.8, 16.2)]. The corresponding change in the ^{13}C NMR spectrum was a shift from δ_C 60.7 to 28.4. Furthermore, the acetate group in griseusin E (**1**) was not observed in compound **2**. The connectivity of all protons and carbons was established by ^{1}H-^{1}H COSY, HMQC and HMBC data. The HMBC correlation of the –OCH_3 group (δ_H 3.74) to the carbonyl signal at C-12 (δ_C 171.1) confirmed the presence of the methyl ester group in compound **2**. After comparing these data with the literature,[11] the constitution of compound **2** was elucidated as the methyl ester of 4′-deacetyl-griseusin B (**4**). The relative configuration of compound **2** was established by analyses of the NOE spectra. The relative stereochemistry at C-3′, C-4′, C-6′ and C-10 was assigned on the basis of the NOE correlations between H-3′ and H-4′ ($J_{3',4'}$=4.1 Hz), H-4′ and H-5′b, H-6′ and H-5′a, and H-10 and H-9 (Figure 3). The specific rotation value ($[\alpha]_D^{22}$+173.8) and the CD spectrum of compound **2** (see supporting information) were of the opposite sign to that of 4′-deacetyl-griseusin B ($[\alpha]_D^{23}$–162).[11] These data conclusively reveal that compound **2** is the mirror image of the natural 4′-deacetyl-griseusin B having the 2*S*,10*R*,3′*S*,4′*S*,6′*S* configuration.

Compounds **3** and **4** were also isolated as yellow solids. The molecular formula of compound **3** was established as $C_{20}H_{18}O_9$ by the (+)-HRESIMS, which displayed a pseudomolecular ion at *m/z* 425.0811 $[M+Na]^+$ (Δ −3.7 mmu). Compound **4** had a molecular formula of $C_{20}H_{20}O_9$, revealed from the (+)-HRESIMS *m/z* 427.0992 $[M+Na]^+$ (calcd. 427.1005, Δ −1.3 mmu). The spectral data (including NMR and MS) of compounds **3** and **4** were identical with those of 4′-deacetyl-griseusin A and B, respectively, which indicated the same relative stereochemistry. A further interesting observation was that compounds **3** and **4** also possess opposite signs of optical rotation values and CD spectra to those of (−)-4′-deacetyl-griseusin A and B. Therefore, compounds **3** and **4** are the (+)-enantiomers of the natural 4′-deacetyl-griseusin A and B and have configurations of 2*S*,9*S*,10*S*,3′*S*,4′*S*,6′*S* and 2*S*,10*R*,3′*S*,4′*S*,6′*S*, respectively. According to the literature, compounds **1–4** belong to the family of pyranonaphthoquinone antibiotics that containing a 1,7-dioxaspiro [5.5] undecane ring system fused to a juglone moiety.[14] Several griseusins such as A–D and their derivatives have been isolated from alkaphilic *Nocardiopsis* sp. and *S. griseus*.[12,15] To the best of our knowledge, the isolated compounds **1–4** are new members, with (+) enantiomer, of this class of natural products.

Compound **5** was obtained as well from fraction III as an optically active ($[\alpha]_D^{22}$+47.3) yellow solid. It gave a positive color reaction with the Dragendorff's reagent and fluorescence under UV light at 254 nm. The molecular formula was determined as $C_{20}H_{17}N_3O_7S$ by the (–)-HRESIMS *m/z* 442.0695 $[M–H]^-$ (calcd. 442.0708, Δ–1.4 mmu). Compound **5** was rapidly identified as phenazine class antibiotic by considering the UV data (maxima were visible at 449, 370 and 251 nm) and the characteristic low-field chemical shifts of the aromatic protons.[3] The ^{1}H NMR spectrum of compound **5** in CD_3OD exhibited two *ortho*-coupled aromatic protons (δ_H 8.32 (1H, d, *J*=7.4 Hz) and 7.99 (1H, d, *J*=7.4 Hz)) and one 1,2,3-trisubstituted aromatic spin system (δ_H 8.80 (1H, d, *J*=8.5 Hz), 8.49 (1H, d, *J*=8.5 Hz) and 8.08 (1H, t, *J*=8.5 Hz)). The ^{1}H-^{1}H COSY NMR correlations between H-2–H-3 and H-7–H-8–H-9 supported this conclusion (Figure 2). The aliphatic region showed one methine signal (δ_H 4.73 (1H, dd, *J*=4.3, 8.2 Hz)), one methoxy group (δ_H 4.02 (3H, s)), one methylene (δ_H 3.84 (1H, dd, *J*=8.2, 14.0 Hz), 3.49 (1H, dd, *J*=4.3, 14.0 Hz)) and one methyl singlet (δ_H 1.97 (3H, s)). The ^{13}C NMR spectrum of compound **5** revealed four carbonyl groups (δ_C 173.2, 168.4, 167.9 and 167.7), five sp^2 aromatic methine carbons (δ_C 137.9, 135.9, 135.3, 132.4 and 127.1) and seven sp^2 quaternary carbons (δ_C 144.8, 144.6, 142.7, 139.5, 138.5, 127.2 and 125.4). In the aliphatic pattern, one methine carbon (δ_C 54.5), one methoxy group (δ_C 53.1), one methylene signal (δ_C 35.2) and methyl group (δ_C 22.7) were observed. Interpretation of the HMBC correlations of compound **5** suggested the presence of *para* substituted phenazine-1,6-dicarboxylic acid (H-2/C-10a, C-4, 1-COOH; H-3/ C-4a, C-1; H-7/C-9, 6-COOH, C-5a; H-8/C-9a, C-6 and H-9/C-7, C-5a, C-10a). Several biosynthetic studies have demonstrated that phenazine-1,6-dicarboxylic acid is a universal precursor for may phenazine secondary metabolites.[16,17] The HMBC spectrum also showed correlations of the methyl signal (δ_H 1.96) to C-4′ (δ_C 173.2) and both of methylene (δ_H 3.84, 3.49) and methoxy groups (δ_H 4.05) to C-1′ (δ_C 167.9). The ^{1}H and ^{13}C chemical shifts in compound **5** suggested the presence of an amino acid. The NMR signals were consistent with an *N*-acetyl-cysteine methyl ester.[18,19] Because the aromatic proton H-2 (δ_H 8.32) and H_2-3′ (δ_H 3.84, 3.49) coupled to C-4 (δ_C 144.8), the *N*-acetyl-cysteine methyl ester could be located at C-4 position. The absoulte streochemistry of compound **5** was proposed by comparing the CD spectra and the optical rtotation value with that of *N*-acetyl-L-cysteine and L-cysteine.[20] Two positive cotton effects at 207 and 244, and the three negative peaks at 225, 258 and 287 nm confirmed the L-configuration of the *N*-acetyl-cysteine methyl ester moiety in compound **5**. Thus, the structure of compound **5** was determined as shown in Figure 1, which we have named yorophenazine. From the literature, SB 212305 is the only phenazine antibiotic linked to *N*-acetyl-cysteine.[18] The known compound **6** was easily identified as phenazine-1,6-dicarboxylic acid based on the NMR data and by comparison with the reference values.[21]

Biological activity

We evaluated the bioactivity of compounds **1–5** for their ability to overcome TRAIL resistance in AGS cells. Recently, this cell line has been widely used as a model system for evaluating cancer cell apoptosis and is reported to be refractory to apoptosis induction by TRAIL.[22] To assess effects of the isolated secondary metabolites on cell viability in the presence and absence of TRAIL, AGS cells were treated with the indicated agents and subjected to the FMCA method.[23] Luteolin was used as a positive control, producing about 44% more inhibition along with TRAIL than the agent alone at 17.5 μM.[24] The assay results (Figure 4) showed that compound **1** at 0.5 and 1.5 μM exhibited 20% and 23% decreases, respectively, in cell viability in the presence of TRAIL (100 ng ml^{-1}) compared with in the absence of TRAIL. Compound **2** at 0.1 and 0.5 μM caused 28% and 27%

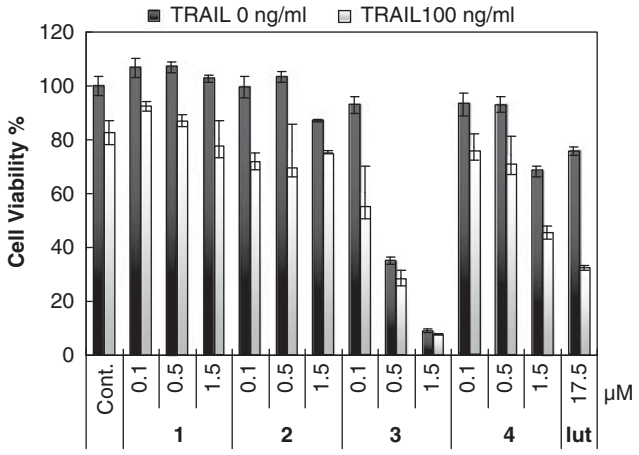

Figure 4 Effect of the isolated compounds **1**–**4** on the cell viability of AGS cells in the presence and absence of TRAIL. The s.e. bar represents the means (n=3 ± s.d.). Abbreviations: AGS, human gastric adenocarcinoma; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

decreases, respectively, in cell viability in the presence of TRAIL (100 ng ml^{-1}). Compound **3** at 0.1 μM proved to be the most active in this series, with 33% decrease in cell viability in the presence of TRAIL (100 ng ml^{-1}). Combined treatment of TRAIL and compound **4** at 0.5 μM resulted in 19% more inhibition than the agent alone. Compound **5**, however, did not produce any significant reduction in cell viability with TRAIL. These results suggest that pyranonaphthoquinones **1**–**4** had a synergistic effect in combination with TRAIL in AGS cell lines.

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1 Ishibashi, M. & Ohtsuki, T. Studies on search for bioactive natural products targeting TRAIL signaling leading to tumor cell apoptosis. *Med. Res. Rev.* **28,** 688–714 (2008).
2 Zhang, L. & Fang, B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther.* **12,** 228–237 (2005).
3 Abdelfattah, M. S., Toume, K. & Ishibashi, M. Isolation and structure elucidation of izuminosides A–C: a rare phenazine glycosides from *Streptomyces* sp. IFM 11260. *J. Antibiot.* **64,** 271–275 (2011).
4 Abdelfattah, M. S., Toume, K. & Ishibashi, M. Izumiphenazines AC: isolation and structure elucidation of phenazine derivatives from *Streptomyces* sp. IFM 11204. *J. Nat. Prod.* **73,** 1999–2002 (2010).
5 Ahmed, F., Ohtsuki, T., Aida, W. & Ishibashi, M. Tyrosine derivatives isolated from *Streptomyces* sp. IFM 10937 in a screening program for TRAIL-resistance overcoming activity. *J. Nat. Prod.* **71,** 1963–1966 (2008).
6 Ishibashi, M. & Arai, M. A. Search for bioactive natural products targeting cancer-related signaling pathways. *J. Synth. Org. Chem. Jpn.* **67,** 1094–1104 (2009).
7 Hayakawa, M. & Nonomura, H. Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *J. Ferment. Technol.* **65,** 501–509 (1987).
8 Waksman, S. A. *The Actinomycetes: Classification, Identification and Descriptions of Genera and Species* (Williams & Wilkins Co.: Baltimore, 1961) 2, 61–292.
9 Kometani, T., Takeuchi, Y. & Yoshii, E. Pyranonaphthoquinone antibiotics. 3. Synthesis of (+)-9-deoxygriseusin B and absolute configuration revision of griseusins A and B. *J. Org. Chem.* **47,** 4725–4730 (1982).
10 Tsuji, N., Kobayashi, M., Terui, Y. & Tori, K. The structures of griseusins A and B, new isochromanquinone antibiotics. *Tetrahedron* **32,** 2207–2210 (1976).
11 Igarashi, M. *et al.* 4′-Deacetyl-(-) griseusins A and B, new naphthoquinone antibiotics from an actinomycete. *J. Antibiot.* **48,** 1502–1505 (1995).
12 He, J. *et al.* Structure, derivatization, and antitumor activity of new griseusins from *Nocardiopsis* sp. *J. Med. Chem.* **50,** 5168–5175 (2007).
13 Kometani, T., Takeuchi, Y. & Yoshii, E. Pyranonaphthoquinone antibiotics. 4. Total synthesis of (+)-griseusin A, an enantiomer of the naturally occurring griseusin A. *J. Org. Chem* **48,** 2311–2314 (1983).
14 Sperry, J., Bachu, P. & Brimble, M. A. Pyranonaphthoquinones-isolation, biological activity and synthesis. *Nat. Prod. Rep.* **25,** 376–400 (2008).
15 Li, Y.- Q. *et al.* Griseusin D a new pyranonaphthoquinone derivative from a alkaphilic *Nocardiopsis* sp. *J. Antibiot.* **60,** 757–761 (2007).
16 Laursen, J. B. & Nielsen, J. Phenazine natural products:251658240 biosynthesis, synthetic analogues, and biological activity. *Chem. Rev.* **104,** 1663–1686 (2004).
17 Mentel, M. *et al.* Of two make one: The biosynthesis of phenazines. *ChemBioChem* **10,** 2295–2304 (2009).
18 Gilpin, M. L., Fulston, M., Payne, D., Cramp, R. & Hood, I. Isolation and structure determination of two novel phenazines from a *Streptomyces* with inhibitory activity against metallo-enzymes, including metallo-beta-lactamase. *J. Antibiot.* **48,** 1081–1085 (1995).
19 Ohnishi, Y. *et al.* Structures of grixazone A and B, a factor-dependent yellow pigments produced under phosphate depletion by *Streptomyces griseus*. *J. Antibiot.* **57,** 218–223 (2004).
20 Nan, J. & Yan, X.- P. A circular dichroism probe for L-cysteine based on the self-assembly of chiral complex nanoparticles. *Chem. Eur. J.* **16,** 423–442 (2010).
21 McDonald, M. *et al.* Biosynthesis of phenazine antibiotics in *Streptomyces antibioticus*: Stereochemistry of methyl transfer from carbon-2 of acetate *J. Am. Chem. Soc.* **121,** 5619–5624 (1999).
22 Srivastava, R. K. TRAIL/Apo-2 L: Mechanisms and clinical applications in cancer. *Neoplasia* **3,** 535–546 (2001).
23 Lindhagen, E., Nygren, P. & Larsson, R. The fluorometric microculture cytotoxicity assay. *Nat. Protoc.* **3,** 1364–1369 (2008).
24 Horinaka, M. *et al.* Luteolin induces apoptosis via death receptor 5 upregulation in human malignant tumor cells. *Oncogene* **24,** 7180–7189 (2005).

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