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NOTE

New pyridone alkaloids JBIR-130, JBIR-131 and JBIR-132 from *Isaria* sp. NBRC 104353

Takahiro Hosoya[1], Motoki Takagi[1] and Kazuo Shin-ya[2]

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A wide variety of screening programs for biologically active compounds have been carried out in our laboratory, employing several assays of fungal metabolites. In the course of our screening program for inhibitors of a cancer target, dynactin-associated protein, a new steroidal compound, JBIR-14, was obtained by activity-guided isolation from the culture extract of an entomopathogenic fungus *Isaria* sp. NBRC 104353.[1,2] During the process of isolation of JBIR-14, new cytotoxic compounds, JBIR-130 (**1**), JBIR-131 (**2**) and JBIR-132 (**3**), were discovered. The new pyridone alkaloids **1** –**3** were purified, and their structures were determined. Herein, the isolation, structure elucidation and biological activities of **1** –**3** are reported.

EXPERIMENTAL PROCEDURE

General

Optical rotations were measured by using a SEPA-300 polarimeter (Horiba, Kyoto, Japan). UV and IR spectra were measured by using a DU730 UV/Vis spectrophotometer (Beckman Coulter, Brea, CA, USA) and an FT-720 spectrophotometer (Horiba), respectively. NMR spectra were collected in CD_3OD or acetone-d_6 by using an NMR System 600 NBCL spectrometer (Varian, Palo Alto, CA, USA), with the residual solvent peaks referenced to δ_C 49.1 and δ_H 3.31 p.p.m. for CD_3OD and δ_C 29.9 and δ_H 2.05 p.p.m. for acetone-d_6. The optimizations of HMBC and HSQC spectra for appropriate couplings were 8 and 140 Hz, respectively. High-resolution ESI-MS (HRESIMS) data were recorded by using a LCT-Premier XE MS (Waters, Milford, MA, USA). Normal-phase medium pressure liquid chromatography was performed on a Purif-Pack Si-60 column (Shoko Scientific, Yokohama, Japan). Analytical reversed-phase HPLC was performed on a CAPCELLPAC column (4.6 mm i.d. × 150 mm; SHISEIDO, Tokyo, Japan) coupled with a 2996 photodiode array detector (Waters) and a 3100 mass detector (Waters). Analytical reversed-phase ultra performance liquid chromatography (Waters) was performed using a BEH ODS column (2.1 mm i.d. × 50 mm; Waters) coupled with an ACQUITY ultra performance liquid chromatography photodiode array eλ detector (Waters) and an LCT-Premier XE MS (Waters). Preparative reversed-phase HPLC was performed on a CAPCELLPACK column (20 mm i.d. × 150 mm) coupled with an L-2455 photodiode array detector (Hitachi High Technologies, Tokyo, Japan).

Fermentation

Isaria sp. NBRC 104353 (National Institute of Technology and Evaluation, Tokyo, Japan) was cultivated in 50-ml test tubes containing 15 ml of potato dextrose broth (24 g l^{-1} potato dextrose; BD Biosciences, San Jose, CA, USA). The test tubes were shaken on a reciprocal shaker (320 r.p.m.) at 27 °C for 3 days. Aliquots (1 ml) of the culture were transferred to 100 ml Erlenmeyer flasks with a medium consisting of 3 g oatmeal (Quaker, Chicago, IL, USA) and 10 ml V8 Mix Juice (Campbell Soup Company, Camden, NJ, USA) and statically incubated at 27 °C for 14 days.

Isolation

The culture (20 flasks) was extracted with 80% aqueous solution of acetone. After filtration and evaporation of the solvent under reduced pressure, the aqueous concentrate was extracted with ethyl acetate (100 ml × 3). The organic layer was dried over Na_2SO_4 and then evaporated to dryness. The dried residue (1.2 g) was purified by silica gel medium pressure liquid chromatography (Purif-Pack Si-60) using a hexane/ethyl acetate gradient solvent system (100:0 to 85:15) and $CHCl_3$/methanol (MeOH) mixture (100:0, 98:2, 95:5, 90:10, 80:20, 30:70 and 0:100). A fraction (200 mg) eluted with $CHCl_3$/MeOH (95:5) was then subjected to preparative reversed-phase HPLC with 80% aqueous MeOH containing 0.1% formic acid (flow rate, 8 ml min^{-1}) to give **1** (retention time = 19.5 min, 2.3 mg), **2** (retention time = 23.0 min, 2.0 mg) and **3** (retention time = 12.5 min, 0.8 mg).

*JBIR-130 (**1**)*: yellow amorphous solid; $(\alpha)_D^{25}$ +11.9 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 387 (4.2), 255 (4.2) nm; IR (KBr) ν_{max} 3425, 1716, 1635 and 1434 cm^{-1}; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1; HRESIMS *m/z* 402.1921 (M − H)$^-$ (calcd for $C_{22}H_{28}NO_6$, 402.1917), see Supplementary Figure S1.

*JBIR-131 (**2**)*: yellow amorphous solid; $(\alpha)_D^{25}$ +36.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 337 (4.2), 277 (4.2) nm; IR (KBr) ν_{max} 3430, 1735, 1604 and 1438 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$) and ^{13}C NMR

[1]Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), 2-4-7 Aomi, Koto-ku, Tokyo, Japan and [2]Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo, Japan
Correspondence: Dr M Takagi, Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.
E-mail: motokitakagi03@gmail.com
or Dr K Shin-ya, Biomedicinal Information Research Centre (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.
E-mail: k-shinya@aist.go.jp
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Table 1 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data for JBIR-130 (1), JBIR-131 (2) and JBIR-132 (3)

	JBIR-130 (1)[a]		*JBIR-131 (2)*[b]		*JBIR-132 (3)*[c]	
Position	δ_C, *type*	δ_H *(J in Hz)*	δ_C, *type*	δ_H *(J in Hz)*	δ_C, *type*	δ_H *(J in Hz)*
2	174.6, C		172.1, C		172.0, C	
3	119.6, C		117.7, C		101.4, C	
4	160.1, C		158.3, C		157.7, C	
5	107.5, C		106.9, C		68.3, CH	4.19, br s
6	139.2, CH	8.07 s	139.0, CH	8.10 s	74.3, CH	4.97, br s
7	195.4, C		207.8, C		172.0, C	
8	128.7, CH	7.97 d (18.0)	43.1, CH_2	3.30 br	120.0, CH	7.03 br d (12.6)
9	146.9, CH	7.65 dd (18.0, 13.8)	27.0, CH_2	2.43 br	144.9, CH	7.41 dd (1.0, 12.6)
10	130.5, CH	6.48 dd (17.4, 13.8)	130.4, CH	5.65 br	130.0, CH	6.49 dd (13.8, 12.6)
11	145.0, CH	6.80 dd (17.4, 13.8)	131.6, CH	6.08 dd (14.0, 10.5)	144.6, CH	6.78 br
12	130.1, CH	6.28 dd (18.0, 13.8)	128.6, CH	5.99 dd (15.0, 10.5)	129.9, CH	6.27 dd (15.0, 10.8)
13	148.3, CH	5.97 dd (18.0, 9.6)	139.0, CH	5.45 dd (15.0, 8.0)	148.2, CH	5.96 dd (15.0, 8.4)
14	40.2, CH	2.21 m	38.5, CH	2.06 m	39.6, CH	2.21 m
15	30.6, CH_2	1.40 m	29.9, CH_2	1.36 m	30.0, CH_2	1.40 m
16	12.1, CH_3	0.93 t (8.7)	11.8, CH_3	0.89 t (7.5)	11.9, CH_3	0.82 t (7.8)
17	20.2, CH_3	1.08 d (8.7)	20.1, CH_3	1.01 d (7.5)	20.0, CH_3	1.01 d (7.8)
1′	71.7, C		83.8, C		131.0, C	
2′, 6′	34.7, CH_2	2.46 m, 1.65 br d (15.0)	35.8, CH_2	2.46 m, 1.65 br d (13.0)	129.8, CH	7.15 d (8.4)
3′, 5′	31.5, CH_2	1.81 m	31.6, CH_2	1.81 m	115.3, CH	6.70 d (8.4)
4′	70.6, CH	3.67 m	75.8, CH	3.67 m	157.7, C	

[a]CD_3OD
[b]CD_3OD - $CDCl_3$ (4:1)
[c]Acetone-d_6

(150 MHz, $CDCl_3/CD_3OD$), see Table 1; HRESIMS *m/z* 404.2051 (M – H)$^-$ (calcd for $C_{22}H_{30}NO_6$, 404.2073), see Supplementary Figure S8.

JBIR-132 (3): yellow amorphous solid; $(\alpha)_D^{25}$ –145.7 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 395 (4.1), 216 (4.2) nm; IR (KBr) ν_{max} 3430, 1710, 1662 and 1459 cm^{-1}; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1; HRESIMS *m/z* 382.1643 (M – H)$^-$ (calcd for $C_{22}H_{24}NO_5$, 382.1654), see Supplementary Figure S14.

Cell-growth inhibitory assay

The human lung carcinoma cell line A549 was used in this study. The cells were cultured in DMEM medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) at 37 °C in a humidified incubator with 5% CO_2. The cytotoxic activity was estimated using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) colorimetric assay. A549 cells were incubated in 96-well plates at a density of 1×10^4 cells per well in 100 µl of medium overnight, after which they were treated with compounds at various concentrations for 48 h. Next, 10 µl of WST-8 reagent solution (Cell Counting Kit; Dojindo, Kumamoto, Japan) was added, and the plate was incubated in a humidified incubator with 5% CO_2 for 1 h at 37 °C. The absorbance of the formazan dye formed was measured at 450 nm. Actinomycin D was used as a positive control (IC_{50} = 8.5 nM).

Flow cytometry

A549 cells were exposed to **1** at 5 µM for 48 h. The treated cells were collected, washed with phosphate-buffered saline, fixed with cold 70% ethanol for 2 h on ice, and then stained with propidium iodide solution (50 µg ml^{-1} of propidium iodide and 10 µg ml^{-1} of RNase A in phosphate-buffered saline) at room temperature in the dark for 30 min. The DNA contents were determined using a flow cytometer (Millipore, Billerica, MA, USA).

Compounds **1**–**3** were isolated from the acetone extract of the fungus *Isaria* sp. NBRC 104353 by medium pressure liquid chromatography and HPLC. The ^{1}H and ^{13}C NMR data for **1**–**3** (Table 1) suggested that the basic structural skeletons were identical to those of pyridone alkaloids.[3–6] Detailed structural information was obtained from a series of 2D NMR experiments, including double-quantum filtered-correlation spectroscopy (DQF–COSY), and HSQC and HMBC spectroscopy (Figure 1a, Supplementary Figure S2–S6, S9–13, S15–19).

JBIR-130 (**1**) was obtained as a yellow amorphous solid, and its molecular formula was determined to be $C_{22}H_{29}NO_6$ by HRESIMS. The ^{1}H NMR, ^{13}C NMR and UV absorption spectra were significantly similar to those of militarinones A–D,[3,4] which have a 1,4-dihydroxy-(5-cyclohexyl)pyridone moiety and a side chain. The sequence from an olefinic methine proton H-8 (δ_H 7.97) to methyl protons H_3-16 (δ_H 0.93) via olefinic methine protons H-9 (δ_H 7.65), H-10 (δ_H 6.48), H-11 (δ_H 6.80), H-12 (δ_H 6.28), H-13 (δ_H 5.97) and H-14 (δ_H 2.21), which was in turn ^{1}H spin-coupled to methyl protons H_3-17 (δ_H 1.08) and methylene protons H_2-15 (δ_H 1.40), was established by the DQF–COSY experiment. The *E*-configuration of the carbon–carbon double bonds at C-8, C-10 and C-12 was confirmed from the vicinal coupling constants of 18.0, 17.4 and 18.0 Hz, respectively, (Table 1). ^{1}H–^{13}C long-range couplings from H-8 and H-9 to ketone carbonyl C-7 (δ_C 195.4) established the structure of the side chain moiety, as shown in Figure 1a.

The low-field resonance at 4-OH (δ_H 17.25) in DMSO-d_6 and the ^{13}C NMR chemical shift at quaternary carbon C-3 (δ_C 119.6), hydroxy carbon C-4 (δ_C 160.1) and C-7 (δ_C 195.4) constituted the enol form of a β-diketone system with strong electron delocalization, and together with HMBC correlations from 4-OH to aromatic carbons C-3, C-4 and C-5,[3] implied that the side chain is linked to the pyridone moiety at C-3. The equivalent signal pairs of the methylene protons at C-2′/C-6′ and C-3′/C-5′ suggested the presence of a 1,4-substituted cyclohexane ring. The HMBC coupling of the olefinic proton H-6 (δ_H 8.07) to C-1′ (δ_C 71.7) and of methylene

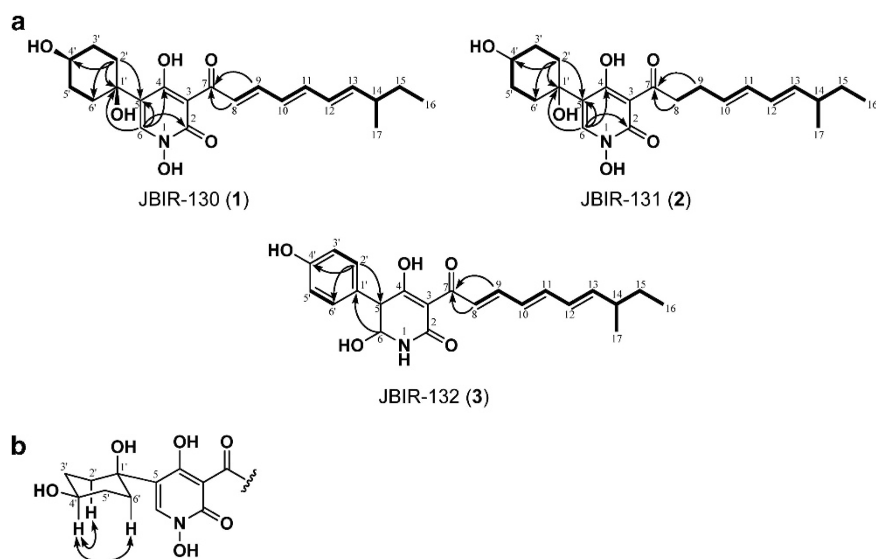

Figure 1 (**a**) Key correlations observed in 2D NMR spectra of **1**–**3**. The bold lines show ^{1}H–^{1}H DQF–COSY correlations, and the arrows show HMBC couplings. (**b**) Key NOESY correlations of **1**.

protons H-2′/H-6′ (δ_H 1.65/2.46) to aromatic carbon C-5 suggested that the 1,4-substituted cyclohexane ring is linked to the C-5 position of the pyridone moiety. Analysis of ^{13}C NMR chemical shifts of the pyridone moiety and HRESIMS data revealed the presence of an *N*-hydroxy-pyridone moiety, which was also reported for fungal metabolites such as militarinone A,[3] torrubielonoe[5] and farinosone B.[6] The relative configuration of the cyclohexyl moiety could be assigned on the basis of NOESY data (Figure 1b, Supplementary Figure S7). The NOESY correlations between methine proton H-4′ (δ_H 3.67) and methylene protons Hax-2′/Hax-6′ (δ_H 2.46) indicated that the ring was in chair conformation, with the 4′-OH adopting an equatorial orientation. The bulky pyridone moiety at C-1′ preferred an equatorial orientation. In addition, these NMR spectral data were consistent with the data for the derivatives, militarinone A[3] and torrubiellone A,[5] which have the pyridone moiety and the 4′-OH group in the equatorial orientation. The absolute configuration at methine carbon C-14 was unassigned. Accordingly, **1** was determined as 5-(1,4-dihydroxycyclohexyl)-1,4-dihydroxy-3-((2*E*,4*E*,6*E*)-8-methyldeca-2,4,6-trienoyl)pyridin-2(1*H*)-one.

JBIR-131 (**2**) was isolated as a light-yellow amorphous solid, and its molecular formula was determined as $C_{22}H_{31}NO_6$ on the basis of the HRESIMS data. Comparison of the ^{1}H NMR and ^{13}C NMR spectral data of **1** and **2** indicated that **2** had the same substructure of a 1,4-dihydroxy-(5-cyclohexyl)pyridone moiety and a reduced side chain. A sequence from methylene protons H_2-8 (δ_H 3.30) through H_2-9 (δ_H 2.43) to H-10 (δ_H 5.65) was observed by DQF–COSY, revealing that the triene moiety in **1** was replaced by a diene substructure in **2**. Thus, the structure of **2** was identified as 5-(1,4-dihydroxycyclohexyl)-1,4-dihydroxy-3-((4*E*,6*E*)-8-methyldeca-4,6-dienoyl)pyridin-2(1*H*)-one.

The molecular formula of JBIR-132 (**3**) was identified to be $C_{22}H_{25}NO_5$ by HRESIMS. The ^{1}H and ^{13}C NMR spectroscopic data of **3** were similar to those of **1** except for the chemical shift of methine carbons C-5 (δ_C 68.3) and C-6 (δ_C 74.3) and the presence of a phenol moiety (C-1′ –C-6′). The DQF–COSY correlation between methine

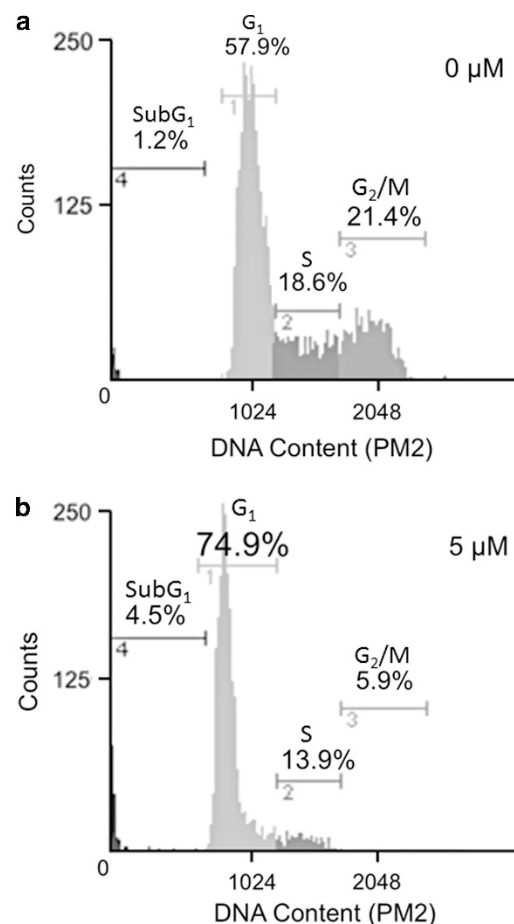

Figure 2 Mode of action of **1** on human lung cancer cell line A549.

proton H-5 (δ_H 4.19) and hydroxy proton H-6 (δ_H 4.97) and the HMBC correlation from H-6 (δ_H 4.97) to aromatic carbon C-1′ (δ_C 131.0) suggested that the 4-hydroxyphenyl substituent is attached to C-5 of the pyridone moiety. The relative configuration at H-5/H-6 was unclear because of the broad signals. Therefore, **3** was determined as 4,6-dihydroxy-5-(4-hydroxyphenyl)-3-((2*E*,4*E*,6*E*)-8-methyldeca-2,4,6-trienoyl)-5,6-dihydropyridin-2(1*H*)-one.

Pyridone alkaloids with a *cis*-(1,4-dihydroxy-cyclohexyl) or a 4-hydroxyphenyl moiety and a side chain have been found in secondary metabolites from fungi *Paecilomyces* sp.[3,4,6] and *Torrubiella* sp.,[5] as biologically active compounds. Militarinone A,[3] which was initially isolated from *Paecilomyces militaris*, showed neurotrophic effect in PC-12 cells. In addition, militarinone B–D[4] and farinosones A–C[6] have been reported, and militarinone D has been found to be cytotoxic against PC-12 cells.[4] Torrubiellones A[5] isolated from *Torrubiella* sp. exhibited antimalarial activity. Thus, we tested the cell growth-inhibitory activity of **1** –**3** against human lung carcinoma cells, A549. The IC_{50} values of **1** –**3** against A549 cells after 48 h were 87, 53 and $>125\,\mu M$, respectively. To explore the mode of action of **1**, A549 cells were treated with $5\,\mu M$ (which was relatively lower concentration than the IC_{50} value) of **1** for 48 h, and the cell cycle was examined by flow cytometry. As shown in Figure 2, **1** induced remarkable accumulation of A549 cells in the G_1 phase; this was concomitant with a decrease in the S and G_2/M phase population. The G_1 arrest was not accompanied by an increase in the sub-G_1 region of cells with fractional DNA contents, which is typical in the late stages of apoptosis. Thus, **1** arrested the cell cycle at the G_1 phase without evidence of cytotoxicity. A detailed study of the biological activity of **1** is underway.

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)