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Isopimarane diterpene glycosides, isolated from endophytic fungus *Paraconiothyrium* sp. MY-42

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ABSTRACT

Six isopimarane diterpenes, compounds **1**–**6**, were isolated from the endophytic fungus *Paraconiothyrium* sp. MY-42. Compound **1** possesses a 19-glucopyranosyloxy group. Its structure was first elucidated by spectroscopic data analysis and finally confirmed by X-ray crystallography, whereas structures **2**–**6** were mainly elucidated based on the analysis of spectroscopic evidence. Compounds **2** and **3** showed moderate cytotoxicities against the human promyelocytic leukemia cell line HL60 (IC_{50} 6.7 μM value for **2** and 9.8 μM for **3**).

1. Introduction

Endophytes live inside healthy tissues and produce bioactive metabolites which enhance the growth and competitiveness of host plants and protect them from herbivores and plant pathogens. They are emerging as an attractive source for discovering new bioactive compounds. Indeed, cytotoxic, antimicrobial, antiviral, and anticancer metabolites have been isolated from the cultures of endophytic fungi (Gunatilaka, 2006). Our ongoing efforts to identify new compounds from endophytes, in the forest trees of the northeastern region of Japan, resulted in the isolation of a novel cytotoxic polyketide from *Allantophomopsis* (Shiono et al., 2010). All forest trees in temperate regions have endophytic fungi. There is an enormous amount of microorganisms with a vast range of genetic diversity, which all play important roles in the ecosystem of this forest area. (Saikkonen, 2007). In our ongoing chemical screening for novel compounds from the metabolites of endophytic fungi isolated from the beech tree stem, we isolated six new compounds **1**–**6** from an unpolished rice culture of *Paraconiothyrium* sp. MY-42. Here, we report the isolation and structure elucidation of these compounds as well as their cytotoxic effects against HL60 cells.

2. Results and discussion

Compound **1** was obtained as colorless prisms, and its molecular formula was determined by HRFABMS as being $C_{26}H_{42}O_7$ on the basis of the quasimolecular ion peak observed at *m/z* 465 $[M\text{-}H]^-$. The IR spectrum indicated presence of hydroxyl (3375 cm^{-1}) and glycosidic linked (1050 cm^{-1}) moieties, whereas the ^1H and ^{13}C NMR spectra were similar to virescenoside Q (19-(β-D-mannopyranosyloxy)isopimara-7,15-dien-3β-ol) (Afiyatullov et al., 2002). In the ^{13}C NMR spectrum of **1**, the six signals at δ_C 62.8, 71.6, 73.5, 74.9, 75.5, and 101.8 were assigned to a glucopyranose moiety instead of mannopyranose, which was observed in virescenoside Q. HMBC experiments were carried out in order to elucidate the structure of **1** (Fig. 1). The relative stereochemistry of the aglycone moiety was deduced from NOEs from Me-20 to Me-17, and from Me-20 and H_2-19. The equatorial position of the hydroxyl group at C-3 was deduced by an observation of the coupling pattern and constant (d, $J_{2',3'} = 9.9$ Hz) in the ^1H NMR spectrum of **1**. To confirm this assumption, an X-ray crystal analysis was performed using suitable crystals. Fig. 2 shows a perspective ORTEP drawing of **1**.

To clarify the absolute stereochemistry of **1**, this compound was subjected to hydrolysis with 1 N CF_3COOH to liberate D-glucose and **1a** (Polonsky et al., 1972); the liberated D-glucose was identified by a direct comparison of its optical rotation value with that of an authentic sample. Thus, **1** was characterized as 19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol.

Compound **2** was isolated as a white amorphous powder. Its molecular formula was determined to be $C_{28}H_{45}NO_7$ on the basis of HRFABMS. The IR spectrum of **2** showed an amide carbonyl absorption peak at 1653 cm^{-1}. A comparison of the ^1H and ^{13}C NMR spectroscopic data of **2** with those of **1** showed that the signals due to the aglycone moiety agreed well (Table 1). It is

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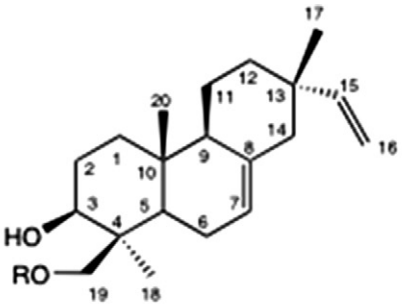
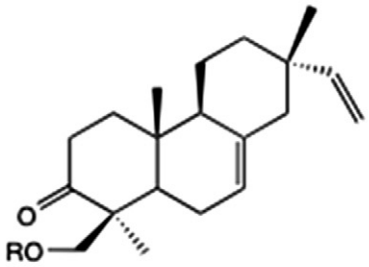


1 : R = α-D-glucopyranosyl

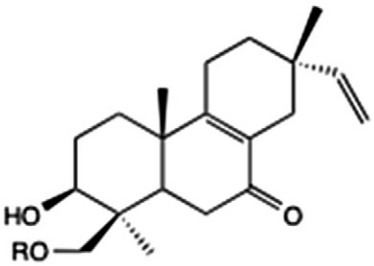
1a : R = H

2 : R = 2-acetamido-2-deoxy-α-D-glucopyranosyl



3 : R = α-D-glucopyranosyl

4 : R =2-acetamido-2-deoxy-α-D-glucopyranosyl



5 : R = α-D-glucopyranosyl

6 : R = 2-acetamido-2-deoxy-α-D-glucopyranosyl

proposed that **2** had the same aglycone as that of **1** and differed only in the sugar part. The signals at δ_H 2.20 (3H, s), δ_C 23.1, and δ_C 170.8 in the ^{1}H and ^{13}C NMR spectra showed the presence of an acetyl group in the sugar part of this molecule. In the HMBC spectrum, correlations were observed between δ_H 2.20 (3H, s), 8.74 (NH), and δ_C 170.8, indicating the presence of an acetamido sugar. Furthermore, in the acetyl derivative **2a**, ^{1}H–^{1}H coupling constants ($J_{2',3'} = 10.5$ Hz, and ($J_{3',4'} = 10.5$ Hz) and NOEs from H-2′ to H-4′, and from H-3′ to H-5′ were observed, indicating that the sugar moiety was glucosamine. The acetyl group was attached at the amino group of glucosamine by an HMBC correlation of H-2 to the acetyl carbonyl carbon of **2**. Stereochemistry at the anomeric position of the sugar moiety of **2** was assigned as α on the basis of ^{1}H–^{1}H coupling constants ($J_{2',3'} = 3.6$ Hz) in the ^{1}H NMR spectrum of **2**. An acid hydrolysis of **2** afforded **1a**, besides the D-glucosamine confirmed by specific rotation. Therefore, the structure of **2** was

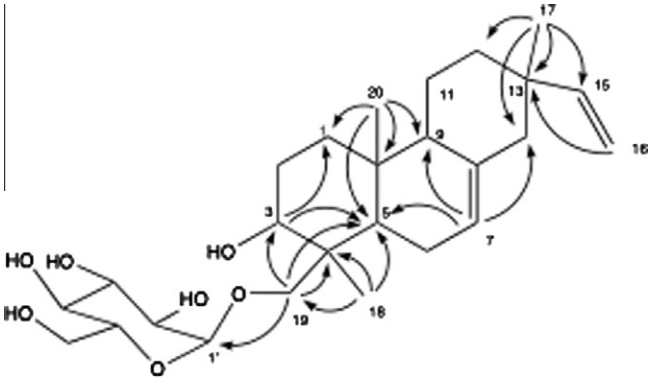


Fig. 1. Key HMBC correlations of **1**.

determined as 19-(2-acetamido-2-deoxy-α-D-glucopyranosyl-oxy)isopimara-7,15-dien-3β-ol.

Compound **3** showed spectroscopic characteristics similar to those of **1**. The molecular formula of **3**, $C_{26}H_{40}O_7$, was determined by HRFABMS. Its IR spectrum indicated absorptions for a carbonyl group (1707 cm^{-1}). The spectroscopic properties of **3** were similar to **1** apart from the presence of a ketone at C-3 (Table 2). This was confirmed by the connectivity found in the HMBC correlations between H_2-19 and C-3, and Me-18 and C-3. Thus, the structure **3** was fully characterized and identified as 19-(α-D-glucopyranosyl-oxy)isopimara-7,15-dien-3-one.

Compound **4** had the molecular formula $C_{28}H_{43}NO_7$ by HRFABMS. The signals observed in the ^{1}H and ^{13}C NMR spectra of **4** were similar to those of **3** (Table 2). Based on the chemical shifts and coupling constant analyses in the ^{1}H and ^{13}C NMR resonances, compound **4** was found to contain the same aglycone as **3**. The only difference between **3** and **4** originated from the sugar moiety attached at C-19 of the aglycone. When the ^{1}H and ^{13}C NMR spectroscopic data of **4** were compared with those of **2**, the ^{1}H and ^{13}C NMR data of the sugar moiety of **4** were in agreement with those of **2**. Unambiguous signal assignments in the ^{1}H and ^{13}C-NMR spectra of **4** were based on HMBC experiments. Thus, **4** was identified as 19-(2-acetamido-2-deoxy-α-D-glucopyranosyloxy) isopimara-7,15-dien-3-one.

Compound **5** had the molecular formula $C_{26}H_{40}O_8$ by HRFABMS. The IR spectrum showed absorption bands at 3339 (OH) and 1643 (C = O) cm^{-1}. The UV absorption 249 nm is characteristic of an α,β-unsaturated carbonyl group. With respect to **5**, its ^{1}H and ^{13}C NMR spectra showed the absence of the trisubstituted double bond resonancy, the presence of tetrasubstituted bond resonances, and a conjugated carbonyl group (δ_C 128.6, 164.0, and 200.0) (Table 3). The location of the α,β-unsaturated ketone in **5** was confirmed by analysis of its HMBC spectrum, which showed correlations from H-6 to C-8, and H-14 to C-7 and 9. The NMR spectroscopic data of the aglycone moiety were in good agreement with those of viresc-enoside P (19-(β-D-altropyranosyloxy)-3β-hydroxyisopimara-7,15-dien) (Afiyatullov et al., 2002). The ^{1}H and ^{13}C NMR spectra of **5** showed one anomeric signal [δ_H 5.26 (1H, br. s, H-1'), δ_C 101.1] and the carbon resonances of the sugar moiety were essentially the same as those of **1** and **3**. The proposed structure of **5** was also supported by HMBC experiments. Thus, **5** was identified as 19-(α-D-glucopyranosyloxy)-3β-hydroxyisopimara-8,15-dien-7-one.

Compound **6** has the molecular formula, $C_{28}H_{43}NO_8$, which was determined by HRFABMS. The spectroscopic data of **6** were very similar to those of **5**. A careful examination of ^{1}H and ^{13}C NMR spectra indicated that **6** was structurally identical to **5** with respect to the aglycone (Table 3). The ^{1}H and ^{13}C NMR spectroscopic data of the sugar moiety of **6** were essentially identical to those, which corresponded with **2** and **4**. Therefore, the structure of **6** was

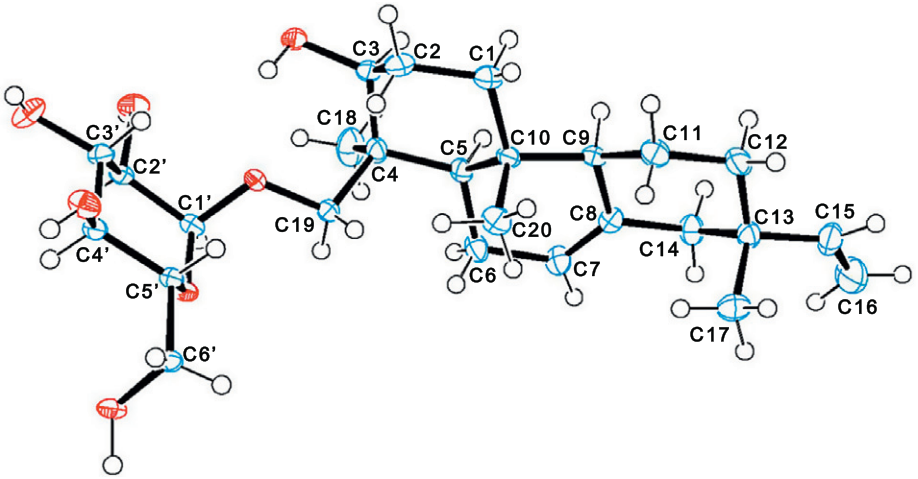


Fig. 2. ORTEP drawing of **1**.

Table 1
^{1}H and ^{13}C NMR spectroscopic data for **1** and **2** in C_5D_5N.

No		1		2
1	38.3	1.10 (1H, m) 1.75 (1H, br. d, 13.1)	38.4	1.13 (1H, m) 1.78–1.84*
2	29.1	1.82–1.86* 2.18–2.33*	28.8	1.78–1.84* 1.95–2.10*
3	79.2	3.53 (1H, br. d, 9.9)	78.8	3.55 (1H, br. dd, 10.0, 4.0)
4	42.2		42.4	
5	51.2	1.25–1.36*	51.2	1.28–1.36*
6	24.3	1.92–2.04* 2.18–2.33*	24.7	1.95–2.10* 2.34 (1H, m)
7	122.4	5.33 (1H, br. s)	122.4	5.35 (1H, br. s)
8	135.2		135.2	
9	52.1	1.56 (1H, m)	52.2	1.60 (1H, m)
10	35.3		35.4	
11	20.6	1.25–1.36* 1.43–1.46*	20.5	1.28–1.36* 1.46–1.49*
12	36.3	1.25–1.36* 1.43–1.46*	36.3	1.28–1.36* 1.46–1.49*
13	37.1	1.82–1.86*	37.1	1.78–1.84*
14	46.2	1.92–2.04*	46.2	1.95–2.10*
15	150.5	5.86 (1H, dd, 17.5, 10.7)	150.5	5.90 (1H, dd, 17.5, 10.5)
16	109.6	4.95 (1H, d, 10.7) 5.00 (1H, d, 17.5)	109.6	4.97 (1H, d, 10.5) 5.03 (1H, d, 17.5)
17	21.7	0.87 (3H, s)	21.6	0.91 (3H, s)
18	25.7	1.27 (3H, s)	24.6	1.36 (3H, s)
19	71.6	3.76 (1H, d, 10.3) 4.54 (1H, d, 10.3)	71.0	3.80 (1H, d, 10.2) 4.53 (1H, d, 10.2)
20	15.3	0.98 (3H, s)	15.5	1.01 (3H, s)
1′	101.8	5.26 (1H, d, 3.5)	99.2	5.55 (1H, d, 3.6)
2′	73.5	4.13 (1H, dd, 9.5, 3.5)	55.9	4.71 (1H, m)
3′	75.5	4.49–4.56*	73.0	4.50–4.57*
4′	71.6	4.18 (1H, t, 9.0)	72.6	4.27 (1H, t, 9.4)
5′	74.9	4.34–4.40*	74.7	4.36–4.40*
6′	62.8	4.34–4.40* 4.49–4.56*	62.5	4.36–4.40* 4.50–4.57*
$CH_3\underline{C}(O)$			23.1	
$\underline{CH_3}C(O)$			170.8	2.20 (3H, s)
NH				8.74 (1H, d, 7.3)

* Overlapped signals.

Table 2
^{1}H and ^{13}C NMR spectroscopic data for **3** and **4** in C_5D_5N.

No		3		4
1	38.1	1.90–2.05*	38.8	1.85–1.99*
2	36.6	2.40 (1H, br. d, 14.5) 3.19 (1H, td, 14.5, 5.2)	36.1	2.28 (1H, br. d, 14.6) 2.99 (1H, td, 14.6, 5.1)
3	214.2		213.5	
4	52.0		52.5	
5	51.2	1.62–1.67*	51.1	1.54–1.62*
6	24.6	1.90–2.05* 2.16 (1H, t, 12.4)	24.5	1.54–1.62* 2.10 (1H, m)
7	122.0	5.32 (1H, br.s)	121.8	5.29 (1H, br. s)
8	135.3		135.8	
9	53.4	1.62–1.67*	53.7	1.54–1.62*
10	35.3		35.4	
11	20.6	1.24–1.31* 1.35–1.45*	20.4	1.21–1.30* 1.29–1.40*
12	36.2	1.24–1.31* 1.35–1.45*	35.9	1.21–1.30* 1.29–1.40*
13	37.1		37.0	
14	46.1	1.90–2.05*	46.1	1.85–1.99*
15	150.4	5.86 (1H, dd, 17.5, 10.7)	150.4	5.82 (1H, dd, 17.5, 10.7)
16	109.7	4.97 (1H, d, 10.7) 5.03 (1H, d, 17.5)	109.8	4.93 (1H, d, 10.7) 5.00 (1H, d, 17.5)
17	21.7	0.88 (3H, s)	21.3	0.85 (3H, s)
18	22.2	1.30 (3H, s)	21.6	1.28 (3H, s)
19	72.5	3.58 (1H, d, 9.8) 4.52 (1H, d, 9.8)	71.8	3.41 (1H, d, 10.2) 4.57 (1H, d, 10.3)
20	15.4	1.21 (3H, s)	15.6	1.04 (3H, s)
1′	101.3	5.24 (1H, d, 3.7)	98.9	5.43 (1H, br. s)
2′	73.7	4.10–4.16*	55.7	4.70 (1H, m)
3′	75.4	4.53 (1H, m)	72.9	4.08 (1H, m)
4′	72.2	4.10–4.16*	72.6	4.37 (1H, m)
5′	75.1	4.28 (1H, m)	75.1	4.18 (1H, m)
6′	63.0	4.37 (1H, m) 4.59 (1H, t, 11.2)	62.8	4.28 (1H, m) 4.43 (1H, m)
$CH_3\underline{C}(O)$			170.6	
$\underline{CH_3}C(O)$			23.1	2.17 (3H, s)
NH				8.70 (d, 6.6)

* Overlapped signals.

identified as 19-(2-acetamido-2-deoxy-α-D-glucopyranosyloxy)-3β-hydroxyisopimara-8,15-dien-7-one.

The new isopimarane diterpene glycosides, **1–6,** are structurally related to virescenosides A-C (Cagnoli-Bellavita et al., 1969; Cagnoli-Bellavita and Ceccherelli, 1970), M-X (Afiyatullov et al., 2000, 2002, 2004, 2006) from the fungus *Acremonium striatisporum* KMM 4401, which was isolated from a sea cucumber. Some virescenosides also exhibited cytotoxic activity (IC_{50} = 10–100 μM) against tumor cells of Ehrlich carcinoma and had cytotoxic effects ((IC_{50} = 2.7–150 μM) on developing see urchin as well. Furthermore, different biological properties have been reported for various pimarane derivatives, including TNF-α (Tumor necrosis factor-α) and IL-8 (Interleukin-8) secretion inhibition (Lam et al., 2003 and Cai et al., 2003), cyclooxygenase inhibition (Suh et al., 2001), nitric

Table 3
^{1}H and ^{13}C NMR spectroscopic data for **5** and **6** in C_5D_5N.

No	**5**		**6**	
1	33.8	1.24–1.30* 1.53 (1H, m)	33.8	1.25–1.30* 1.53 (1H, m)
2	28.4	1.94 (1H, m) 2.10 (1H, m)	28.2	1.98 (1H, m) 2.12–2.20*
3	77.6	3.50 (1H, dd, 11.4, 4.3)	77.5	3.48 (1H, dd, 11.6, 4.6)
4	42.5		42.6	
5	50.2	1.77–1.80*	49.8	1.76–1.80*
6	37.7	2.91(1H, br. d, 17.5) 3.70 (1H, t, 17.5)	37.5	2.95 (1H, dd, 17.7, 3.1) 3.20 (1H, dd, 17.7, 14.6)
7	200.0		199.4	
8	128.6		128.6	
9	164.0		164.3	
10	40.0		39.8	
11	23.1	1.24–1.30*	22.9	1.25–1.30*
12	34.9	1.24–1.30* 1.77–1.80*	34.6	1.25–1.30* 1.76–1.80*
13	34.6		34.6	
14	34.0	2.09 (1H, d, 17.3) 2.66 (1H, d, 17.3)	34.0	2.12–2.20* 2.62 (1H, d, 17.5)
15	145.8	5.77 (1H, dd, 17.2, 10.7)	145.8	5.78 (1H, dd, 17.5, 10.5)
16	111.6	4.96 (1H, d, 18.3) 5.03 (1H, d, 11.5)	111.7	4.95 (1H, d, 18.3) 5.00 (1H, d, 11.5)
17	28.3	0.99 (3H, s)	28.1	1.00 (3H, s)
18	24.0	1.35 (3H, s)	23.1	1.30 (3H, s)
19	71.6	4.05 (1H, d, 10.2) 4.53 (1H, d, 10.2)	70.7	4.01 (1H, d, 10.2) 4.51 (1H, d, 10.2)
20	16.9	1.43 (3H, s)	17.3	1.33 (3H, s)
1′	101.1	5.26 (1H, br. s)	98.6	5.61 (1H, d, 3.7)
2′	73.6	4.17 (1H, dd, 9.5, 3.5)	56.2	4.72 (1H, m)
3′	75.4	4.65 (1H, t, 8.7)	73.0	4.60 (1H, dd, 10.9, 8.0)
4′	72.0	4.26 (1H, t, 8.8)	72.7	4.33–4.45*
5′	74.6	4.37 (1H, m)	74.6	4.33–4.45*
6′	62.9	4.34–4.49*	62.5	4.62 (1H, d, 8.0) 4.30 (1H, d, 8.0)
$CH_3\underline{C}(O)$			23.2	2.20 (3H, s)
$\underline{CH_3}C(O)$			170.8	
NH				8.91 (d, 6.3)

* Overlapped signals.

oxide inhibition (Suh et al., 2004) and protein tyrosine phosphatase inhibition (Na et al., 2006). In this study, we examined the cytotoxic activities (Aburai et al., 2010) of the six isopimarane glucosides **1**–**6** against the human promyelocytic leukemia cell line HL60. The IC_{50} values of **1**–**6** and camptothecin were 11.2, 6.7, 9.8, 23.1, >100, >100 and 0.01 μM, respectively. Among these compounds, **1**, **2**, and **3** were found to exhibit moderate effects on HL60 cells, while the activities of other compounds were lower. To characterize cell apoptosis induced by **1**, **2**, and **3**, the DNA ladder formation was examined. Compounds **1**, **2**, and **3** caused the digestion of genomic DNA into ladders in a concentration-dependent manner in HL60 cells.

3. Conclusions

We have isolated a fungal endophyte, strain MY-42, from a beech branch and identified it as *Paraconiothyrium* sp. New compounds **1**–**6** were isolated from this fungal strain. In addition, although many isopimarane glycosides have been isolated from natural sources (Hanson, 2007, 2009), isopimaranes possessing an α-D-glucosamine unit are especially rare in nature. Afitatullov et al. reported the isolations of isopimarane glycosides and virescenosides, from marine-derived fungus *A. striatisporum* KMM 4401 where the cytotoxic active compounds have β-D-altropyranoside as a residue in most cases (Afiyatullov et al., 2000, 2002, 2004). Some structure–activity relationships within this isopimarane diterpene glycosides may be made. The following tendency was notable: isopimarane with a double bond Δ^7, such as **1**, **2**, **3**, and **4**, tended to suppress the growth of HL 60 cells; compounds with an enone moiety, such as **5** and **6**, were less effective. In addition, these results suggested that the cytotoxic activity was not influenced by the nature of the glycoside moiety at C-19. Interestingly, the structurally related isopimarane diterpenes, 16-(α-D-glucopyranosyloxy)isopimar-7-en-19-oic acid derivatives, which our group previously isolated from the ascomycete, *Xylaria polymorpha*, exhibited weak cytotoxicity toward the cancer cell line HL60 with IC_{50} values ranging from 132 to 327 μM (Shiono et al., 2009). It should be noted that the introduction of the sugar moiety at C-16 resulted in reduced cytotoxic activity.

4. Experimental

4.1. General procedures

Melting points were determined with Yanagimoto apparatus and are uncorrected. Optical rotations were measured with a Horiba model SEPA-300 polarimeter, whereas IR and UV spectra were recorded with JASCO J-20A and Shimadzu UV mini-1240 spectrophotometers, respectively. Mass spectra were obtained using a JEOL JMS-700 instrument, and ^{1}H and ^{13}C NMR spectra were acquired with a JEOL EX-400 spectrometer. Chemical shifts are given on the δ scale with TMS as an internal standard. Semipreparative HPLC was carried out with Shimadzu pump and UV LC-10A detector (set at 210 nm) on a Mitysil ODS column (150 × 6.0 mm i.d.) at a flow rate of 2.0 mL/min. Column chromatography (CC) was conducted on silica gel 60 (Kanto Chemical Co., Inc., Japan) and ODS (Fuji Silysia, Japan). TLC was carried out on Merck precoated silica gel plates (silica gel 60 F_{254}), and spots were detected by spraying with 10% vanillin in H_2SO_4 followed by heating, or by UV irradiation.

4.2. The producing strain

An endophytic fungal strain *Paraconiothyrium* sp. MY-42 was isolated from the stem of a beech branch collected in October, 2008 at Mt. Gassan, Yamagata, Japan. The producing strain exhibited good growth at 25℃ on potato dextrose agar. Colonies on potato dextrose agar attained a diameter of 15–20 mm and were velvetinous and white, whereas the reverse side of the colonies on the agar was green to brown. The strain was identified based on analysis of the DNA sequences of the ITS1F and ITS1R regions of their ribosomal RNA gene. The ITS sequence (accession number: DDBJ; AB607046) matched with a fungal sequence of *Paraconiothyrium* sp. from GenBank. This strain was deposited in NBRC (NITE Biological Resource Center) at NITE (National institute of technology and Evaluation, Japan) (NBRC number: 107875).

4.3. Fermentation, extraction and isolation

The fungal strain MY-42 was cultivated on sterilized unpolished rice (total 1000 g, 20 g/petri dish × 50) at 25℃ for four weeks. The moldy unpolished rice was extracted with MeOH (2 L), and the MeOH extract was concentrated. The resulting MeOH concentrate was partitioned into *n*-hexane (0.5 L), EtOAc (1.5 L) and H_2O (0.5 L). Purification of the eluates was monitored by the characteristic intense blue coloration with 10% vanillin in H_2SO_4 on TLC plates. The EtOAc layer (5.3 g) was applied to a silica gel column with stepwise elution of *n*-hexane–EtOAc (100:0–0:100) and EtOAc–MeOH (50:50, 0:100), respectively, to afford fractions 1–1 to 1–13. Fractions 1–12 were subjected to ODS CC by eluting with H_2O and an increasing ratio of MeOH (100:0 to 0:100). The MeOH-H_2O (1:1, v/v) eluate (120 mg) was crystallized from MeOH to

afford **1** (35.2 mg) as colorless crystals. The filtrate was further separated by semipreparative ODS HPLC (MeOH–H_2O, 80:20) to give compounds **2** (15.4 mg), **3** (10.5 mg), **4** (5.2 mg). Fractions 1–13 were subjected to ODS CC by eluting with H_2O and an increasing ratio of MeOH (100:0–0:100) and three fractions obtained were purified by semipreparative ODS HPLC (MeOH–H_2O, 75:25) to give compounds **5** (3.4 mg) and **6** (4.2 mg).

4.4. 19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol (**1**)

Colorless crystals; mp 220–221 °C; $[\alpha]_D^{20}$ + 49.8 (*c* 0.31, MeOH); IR (KBr) ν_{max} 3375, 1630, 1050 cm^{-1}; for ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data, see Table 1; negative FABMS *m/z*: 465 $[M-H]^-$; negative HRFABMS *m/z:* 465.2860 $[M-H]^-$, (calcd for $C_{26}H_{41}O_7$, 465.2852).

4.5. 19-(2-acetamido-2-deoxy-α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol (**2**)

White amorphous powder; $[\alpha]_D^{20}$ + 88.6 (*c* 0.22, MeOH); IR (KBr) ν_{max} 3321, 1653, 1545, 1087, 1035 cm^{-1}; for ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data, see Table 1; negative FABMS *m/z*: 506 $[M-H]^-$; negative HRFABMS *m/z*: 506.3122 $[M-H]^-$, (calcd for $C_{28}H_{44}NO_7$, 506.3118).

4.6. 19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3-one (**3**)

White amorphous powder; $[\alpha]_D^{20}$ + 27.7 (*c* 0.45, MeOH); IR (KBr) ν_{max} 3398, 1707, 1637, 1030 cm^{-1}; for ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data, see Table 2; negative FABMS *m/z*: 463 $[M-H]^-$; negative HRFABMS *m/z*: 463.2699 $[M-H]^-$, (calcd for $C_{26}H_{39}O_7$, 463.2696).

4.7. 19-(2-acetamido-2-deoxy-α-D-glucopyranosyloxy)isopimara-7,15-dien-3-one (**4**)

White amorphous powder; $[\alpha]_D^{20}$ + 52.1 (*c* 0.20, MeOH); IR (KBr) ν_{max} 3366, 1707, 1640, 1552, 1122, 1037 cm^{-1}; for ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data, see Table 2; negative FABMS *m/z*: 504 $[M-H]^-$; negative HRFABMS *m/z*: 504.2969 $[M-H]^-$, (calcd for $C_{28}H_{42}NO_7$, 504.2961).

4.8. 19-(α-D-glucopyranosyloxy)-3β-hydroxyisopimara-8,15-dien-7-one. (**5**)

White amorphous powder; $[\alpha]_D^{20}$ + 148.0 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 249 (3.7); IR (KBr) ν_{max} 3339, 1643, 1373, 1032 cm^{-1}; for ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data, see Table 3; negative FABMS *m/z*: 479 $[M-H]^-$; negative HRFABMS *m/z*: 479.2643 $[M-H]^-$, (calcd for $C_{26}H_{39}O_8$, 479.2645).

4.9. 19-(2-acetamido-2-deoxy-α-D-glucopyranosyloxy)-3β-hydroxyisopimara-8,15-dien-7-one. (**6**)

White amorphous powder; $[\alpha]_D^{20}$ + 113.9 (*c* 0.45, MeOH); UV (MeOH) λ_{max} (log ε) 249 (3.7); IR (KBr) ν_{max} 3398, 1707, 1637, 1030 cm^{-1}; for ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data, see Table 3; FABMS *m/z*: 520 $[M-H]^-$; negative HRFABMS *m/z*: 520.2915 $[M-H]^-$, (calcd for $C_{28}H_{42}NO_8$, 520.2910).

4.10. X-ray Crystallography of **1**

Single crystals suitable for X-ray structure analysis were obtained by recrystallization from MeOH. X-ray diffraction data were collected at 173 K on a Rigaku Saturn CCD diffractometer with graphite monochromated Mo Kα radiation (λ = 0.71070 Å). The structure was solved by direct methods (SIR2004) (Burla et al., 2005) and expanded by using Fourier techniques (DIRDIF99) (Beurskens et al., 1999). A total of 16,248 reflections were measured and 2870 were unique (R_{int} = 0.044). Crystal data and refinement statistics are as follows: $C_{26}H_{42}O_7$, MW = 466.61, monoclinic, space group $P2_1$ (#4); a = 7.407(3), b = 9.809(4), c = 16.628(7) Å, β = 95.142(3)°, V = 1203.4(8) $Å^3$; Z = 2, D_{calc} = 1.288 g/cm^3, F_{000} = 508.00, μ(Mo Kα) = 0.917 cm^{-1}, final $R1$ = 0.0380 ($I > 2.00\sigma(I)$), R = 0.0397 (all data), $wR2$ = 0.1111 (all data), GOF = 1.009; Complete crystallographic data, as a CIF file, have been deposited with Cambridge Crystallographic Data Center (CCDC No. 764212). Copies can be obtained free of charge from: The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (e-mail: deposit@ccdc.cam.ac.uk).

4.11. Acid hydrolysis of **1** and **2**, and identification of sugars

A solution of **1** (7.0 mg) in 1 N CF_3COOH (10 mL) was heated at 100 °C for 3 h. After neutralizing with 1 N NaOH/H_2O, the solution was extracted with Et_2O (10 mL × 3) and the combined organic phase was evaporated to dryness under reduced pressure to yield aglycone **1a** (2.7 mg). The aqueous layer was evaporated to dryness under reduced pressure and subjected to silica gel CC to afford D-glucose (2.5 mg). The optical rotation value was as follows: $[\alpha]_D^{20}$ + 50.6 (*c* 0.9, H_2O, measured 24 h after dissolving in H_2O)(lit. + 53.3 (Zhang et al., 2001)).

A mixture of **2** (4.0 mg) and 3 N HCl (10 mL) was heated at 100 °C for 3 h. This reaction mixture was treated in the same way as described for **1** to afford aglycone **1a** and a sugar fraction. From the sugar fraction, D-2-amino-2-deoxy-D-glucose was isolated as described for **1**. The optical rotation value was as follows: $[\alpha]_D^{25}$ + 70.0 (*c* 0.2, H_2O, measured 24 h after dissolving in H_2O) (lit. + 72 (Armarego and Chai, 2009).

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Appendix A. Supplementary data

Supplementary data (The ^1H, ^{13}C NMR and 2D NMR spectra of compounds **1**–**6**, synthetic experiment and spectroscopic data, and methods in biological test) associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.04.016.

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