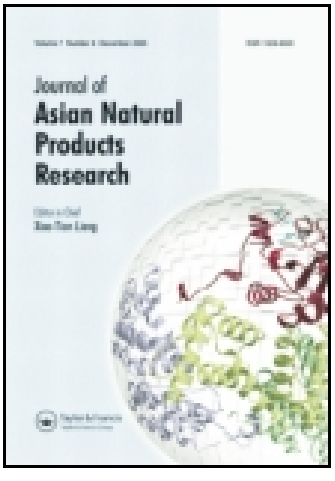

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Four new sesquiterpenoids from fruiting bodies of the fungus Inonotus rickii

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Four new sesquiterpenoids from fruiting bodies of the fungus *Inonotus rickii*

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Three new bisabolane sesquiterpenoids, inonotic acid A (**1**), 3-*O*-formyl inonotic acid A (**2**), inonotic acid B (**3**), and one new drimane sesquiterpenoid 3α,6β-dihydroxycinnamolide (**4**), were isolated from the fruiting bodies of mushroom *Inonotus rickii*. Their structures were elucidated by means of extensive spectroscopic methods. Compound **4** had moderate inhibitory activity on human colon cancer SW480 ($IC_{50} = 20.4$ μmol).

Keywords: *Inonotus rickii*; sesquiterpenoid; cytotoxicity

1. Introduction

The genus *Inonotus* distributed worldwide and comprised many medicinal species, such as *I. obliquus* (Pers.) Pilát, *I. levis* P. Karst., and *I. hispidus* (Bull.) P. Karst. [1]. Among them, *I. obliquus* served as a folk remedy to cure cancer, diabetes and heart diseases in northeast Asia for centuries [1,2]. Several categories of secondary metabolites isolated from this genus, such as terpenoids and polysaccharides, exhibited anti-inflammatory, anti-tumorigenic, antioxidative, and neuroprotective activities [2–10]. *I. rickii* is a wood-decaying fungus, which has not been reported for any chemical constituents yet. As our continuous search for new biological compounds from higher fungi, an investigation on chemical constituents of cultures of *I. rickii* resulted in the isolation of four new sesquiterpenoids (**1**–**4**) (Figure 1), including three bisabolane- and one drimane-type sesquiterpenoids. Their structures were elucidated by means of extensive spectroscopic data analysis. All compounds were screened for their cytotoxicities against five human cell lines.

2. Results and discussion

Compound **1** was isolated as a colorless oil. Its molecular formula was determined as $C_{15}H_{26}O_4$ by HREIMS at *m/z* 270.1829 $[M]^+$, with three degrees of unsaturation. The IR spectrum displayed absorption bands at 3434 cm^{-1} for hydroxy group and 1710 cm^{-1} for carboxyl group. The ^1H NMR data demonstrated the presence of three methyls (δ_H 1.15, 1.23, and 1.55), which were also indicated by the ^{13}C NMR spectrum. The DEPT spectrum, as well as HSQC spectrum, revealed another 12 carbon resonances, which were ascribed to 5 methylenes, 4 methines (including 1 olefinic carbon), and 3 quaternary carbons (including the carboxyl). The ^1H–^1H COSY spectrum displayed three fragments as shown in Figure 2. In the HMBC spectrum, the correlations from a methyl at

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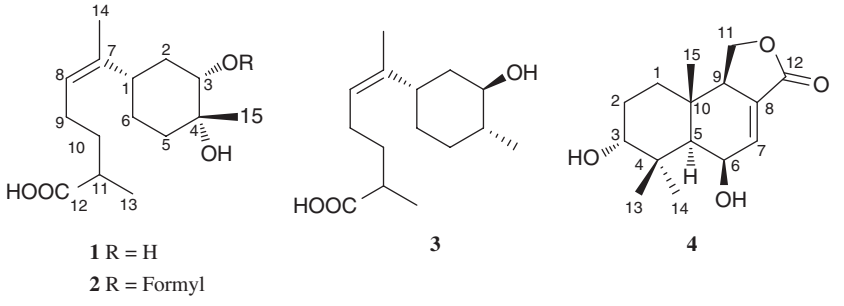

Figure 1. Structures of compounds **1**–**4**.

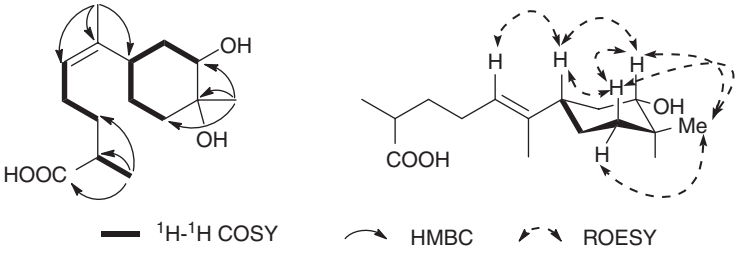

Figure 2. Key 2D NMR correlations of compound **1**.

δ_H 1.15 (3H, d, $J = 6.8$ Hz, H-13) to a methine (δ_C 39.0, C-11), a methylene (δ_C 33.6, C-10), and a carboxyl (δ_C 182.1, C-12) indicated the presence of an isobutyl moiety, of which a methyl was oxygenated to carboxyl. Meanwhile, the olefinic proton at δ_H 5.13 (1H, t, $J = 6.5$ Hz, H-8) correlated to C-1, C-14, and C-9 suggested connectivities from C-9 to C-1 (Figure 2). Besides, in the HMBC spectrum, correlations were observed from the rest methyl (δ_H 1.23, 3H, s, H-15) to C-3 (δ_C 74.0, methine) and C-4 (δ_C 71.8, quaternary carbon) and C-5 (methylene), as well as from H-1 (δ_H 2.15–2.20, 1H, m) to C-6 and C-5, indicating the existence of a six-membered ring. The above-mentioned data suggested that compound **1** possessed a similar structure to that of (1*S*,3*R*,4*R*,7*S*)-3,4-dihydroxy-α-bisabolol [11], except for the differences of the position of double bond and oxygenated carbons of C-7 and C-12 in compound **1**. The relative configuration of compound **1** was elucidated by rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY). From the ROESY spectrum, H-1, H-3, and H-5β correlated to each other, indicating that all of them were axial oriented, while the methyl (C-15) correlated to H-3, H-5β, and H-5α suggested that the methyl was β oriented (Figure 2). Therefore, compound **1** was established to be inonotic acid A, as shown in Figure 1.

Compound **2** was purified as a colorless oil, possessing a molecular formula as $C_{16}H_{26}O_5$ according to HREIMS at *m/z* 298.1777 $[M]^+$. The IR absorption bands at 3432 and 1711 cm^{-1} indicated the existence of hydroxy and carboxyl groups, respectively. Comparison of NMR data of **2** with those of **1** suggested that compound **2** should be an *O*-formyl derivative of **1**, which was supported by the HMBC correlations from H-16 at δ_H 8.09 (1H, s) to C-3 at δ_C 75.6 (d), as well as from H-3 at δ_H 4.88–4.90 (1H, m) to C-16 at δ_C 160.7 (d). Detailed analyses of 2D NMR data finally established the structure of **2** to be

3-*O*-formyl inonotic acid A, as shown in Figure 1.

Compound **3** was obtained as a colorless oil. Its molecular formula was established as $C_{15}H_{26}O_3$ by HREIMS at *m/z* 254.1885 $[M]^+$. The 1D NMR spectra were similar to those of compound **1** which suggested that they were the same type of sesquiterpenoids. Thorough analyses of 2D NMR spectra of compound **3** suggested that C-4 (δ_C 36.2) was not oxygenated in comparison with compound **1**. According to ROESY spectrum, H-1 correlated to H-6β, H-5β, and H-8, but not correlated to H-3α, while Me-15 did not correlate to H-5β, but to H-3α and H-5α. These signals suggested that both H-1 and 3-OH were β oriented, while Me-15 was α oriented (Figure 3). Therefore, compound **3** was characterized as inonotic acid B, as shown in Figure 1.

Compound **4**, a colorless oil, had an $[M]^+$ peak at *m/z* 266.1518 ($C_{15}H_{22}O_4$) in the HREIMS. 1D NMR spectra in combination with HSQC spectrum demonstrated 15 carbons, which were classified into 3 methyls, 3 methylenes (1 oxygenated), 5 methines (2 oxygenated and 1 olefinic), and 4 quaternary carbons (1 lactone carbon and 1 olefinic carbon) (Table 2). All these data suggested that compound **1** was a drimane-type sesquiterpenoid with similarities to those of 2α,3β-dihydroxycinnamolide [12]. Preliminary analyses of 2D NMR data suggested that one OH should be placed at C-6 in **4** instead of at C-2 in 2α,3β-dihydroxycinnamolide, which was supported by the $^1H-^1H$ COSY correlations of H-5 at δ_H 1.73 (1H, d, $J = 4.9$ Hz) with H-6 at δ_H 4.61–4.63 (1H, m), and of H-6 with H-7 at δ_H 6.79 (1H, dd, $J = 3.5$, 3.6 Hz), as well as HMBC correlations from H-6 to C-5 at δ_C 47.8 (d) and C-7 at δ_C 135.8 (d). Detailed analyses of $^1H-^1H$ COSY and HMBC correlations suggested that the other parts were the same to those of 2α,3β-dihydroxycinnamolide. The relative configuration could be determined by a ROESY experiment. The correlations of H-15/H-2β, H-15/H-13, H-5/H-14, H-5/H-6, and H-5/H-9 suggested OH-6 to be β oriented, while Me-15 was β oriented and H-5 was α oriented. 3-OH was determined as α orientation due to coupling constants of H-3 (1H, dd, $J = 3.1$, 2.3 Hz). Therefore, compound **4** was identified as 3α,6β-dihydroxycinnamolide.

All new compounds were evaluated for their cytotoxicities against five human cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [13]. As a result, compound **4** showed moderate cytotoxic activity against human colon cancer SW480 with an IC_{50} of 20.4 μM.

3. Experimental

3.1 General experimental procedures

Optical rotations were obtained on a JASCO P-1020 digital polarimeter (Horiba, Kyoto, Japan). IR spectra were taken on a Bruker Tensor 27 FT-IR

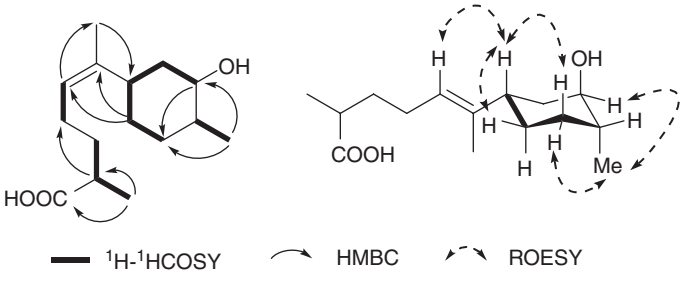

Figure 3. Key 2D NMR correlations of compound **3**.

spectrometer (Bruker, Karlsruher, Germany) with KBr pellets. UV spectra were obtained by using a Shimadzu UV-2401A spectrometer (Shimadzu, Kyoto, Japan). NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Bruker). HREIMS were measured on a Waters Autospec Premier P776 mass spectrometer (Waters, Milford, MA, USA). Silica gel 200–300 mesh (Qingdao Marine Chemical Inc., Qingdao, China) and Sephadex LH-20 (Amersham Biosciences, Upssala, Sweden) were used for column chromatography. Medium pressure liquid chromatography (MPLC) was performed on a Büchi Sepacore System equipped with pump manager C-615, pump modules C-605, and fraction collector C-660 (Büchi Labortechnik AG, Flawil, Switzerland), and columns packed with Chromatorex C-18 (40–75 μm, Fuji Silysia Chemical Ltd, Kasugai, Japan). Preparative HPLC was performed on an Agilent 1260 liquid chromatography system equipped with a Zorbax SB-C18 column (9.4 mm × 150 mm) (Agilent Technologies, Santa Clara, CA, USA).

3.2 Fungus material

The fruiting bodies of *Inonotus rickii*, used as experimental material, were collected in Panzhihua, Sichuan province, China in September 2012, and were identified by Prof. Yu-Cheng Dai (Institute of Microbiology, Beijing Forestry University). A voucher specimen of *I. rickii* was deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (No. HFC20120711).

3.3 Extraction and isolation

The fresh fruiting bodies of *I. rickii* (7 kg) were extracted three times with ethanol (25 l) at room temperature. The extract was suspended in distilled water and partitioned successfully with ethyl acetate. The EtOAc layer was concentrated under reduced pressure to afford a crude extract (30 g), and the residue was subjected to silica gel column chromatography, eluted with a gradient of $CHCl_3$–MeOH (v/v 1:0 to 0:1) to obtain five fractions (A–E). Fraction D (8.4 g) was eluted by MPLC with MeOH–H_2O (30–100%) to obtain seven subfractions (D1–D7). Fraction D2 (78.2 mg) was subjected to silica gel column chromatography (petroleum ether–acetone; 2:1) to yield **1** (4.2 mg). Fraction D3 (325.4 mg) was separated by Sephadex LH-20 (acetone) column chromatography to afford **2** (6.3 mg) and **3** (2.2 mg). Fraction D6 (16.6 mg) was purified by silica gel column chromatography (petroleum ether–acetone; 6:1), followed by preparative HPLC (20–45%, CH_3CN–H_2O, 10 ml/min, 25 min, retention time: 13.25 min) to obtain **4** (1.1 mg).

3.3.1 *Inonotic acid A (**1**)*

A colorless oil, $[\alpha]_D^{20}$ − 13.6 (*c* 0.29, MeOH). UV (MeOH) λ_{max} nm (log ε): 202 (2.86). IR (KBr) ν_{max} cm^{-1}: 3433, 2936, 2879, 1711, 1460, 1378, 1188, 1034, 910. For ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) spectral data, see Table 1. HREIMS *m/z*: 270.1829 $[M]^+$ (calcd for $C_{15}H_{26}O_4$, 270.1831).

3.3.2 *3-O-Formyl inonotic acid A (**2**)*

A colorless oil, $[\alpha]_D^{21}$ − 16.7 (*c* 0.17, MeOH). UV (MeOH) λ_{max} nm (log ε): 202 (2.95). IR (KBr) ν_{max} cm^{-1}: 3432, 2936, 2879, 1711, 1460, 1377, 1193, 1033, 910. For ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) spectral data, see Table 1. HREIMS *m/z*: 298.1777 $[M]^+$ (calcd for $C_{16}H_{26}O_5$, 298.1780).

3.3.3 *Inonotic acid B (**3**)*

A colorless oil, $[\alpha]_D^{16}$ + 7.2 (*c* 0.24, MeOH). UV (MeOH) λ_{max} nm (log ε): 202 (3.07). IR (KBr) ν_{max} cm^{-1}: 3431,

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of **1**–**3** (δ in ppm, J in Hz).

	1		**2**		**3**	
No.	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	39.1, d	2.15–2.20, m	39.8, d	2.07–2.13, m	39.7, d	2.17–2.21, m
2	33.9, t	1.87–1.92, m	31.3, t	1.88–1.92, m	38.7, t	1.78–1.82, m
		1.56, overlapped		1.67, overlapped		1.40, overlapped
3	74.0, d	3.61–3.62, m	75.6, d	4.88–4.90, m	71.4, d	3.85–3.87, m
4	71.8, s		70.1, s		36.2, d	1.47, overlapped
5	33.7, t	1.49, overlapped	34.7, t	1.60, overlapped	28.4, t	1.42, overlapped
		1.71, overlapped		1.70, overlapped		1.37, overlapped
6	26.2, t	1.52, overlapped	26.1, t	1.56, overlapped	31.5, t	1.62–1.64, m
		1.45, overlapped		1.50, overlapped		1.18, overlapped
7	139.4, s		139.3, s		140.4, s	
8	122.4, d	5.13, t (6.5)	122.8, d	5.12, t (6.6)	122.0, d	5.09, t (7.2)
9	25.6, t	2.00–2.05, m	25.6, t	2.00–2.04, m	25.6, t	1.99–2.03, m
10	33.6, t	1.46, overlapped	33.6, t	1.45, overlapped	33.7, t	1.44, overlapped
		1.71, overlapped		1.71, overlapped		1.69–1.75, m
11	39.0, d	2.40–2.46, m	38.9, d	2.41–2.47, m	39.0, d	2.41–2.47, m
12	182.1, s		182.1, s		182.7, s	
13	17.2, q	1.15, d (6.8)	17.2, q	1.16, d (6.6)	17.1, q	1.15, d (6.6)
14	14.7, q	1.55, s	14.6, q	1.55, s	14.5, q	1.54, s
15	26.6, q	1.23, s	27.3, q	1.18, s	18.6, q	0.93, d (6.6)
16			160.7, d	8.09, s		

2927, 2856, 1709,1633, 1455, 1378, 1212, 994. For ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) spectral data, see Table 1. HREIMS *m/z*: 254.1885 $[M]^+$ (calcd for $C_{15}H_{26}O_3$, 254.1882).

3.3.4 3α,6β-Dihydroxycinnamolide (*4*)

A colorless oil, $[\alpha]_D^{20}$ − 32.6 (*c* 0.07, MeOH). UV (MeOH) λ_{max} nm (log ε): 204 (2.93). IR (KBr) ν_{max} cm^{-1}: 3441, 2970, 2932, 2874, 1749, 1643, 1457, 1383, 1211, 1040, 918. For ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) spectral data, see Table 2. HREIMS *m/z*: 266.1510 $[M]^+$ (calcd for $C_{15}H_{22}O_4$, 266.1518).

3.4 Cytotoxicity assay

The cytotoxicity assay was performed according to the MTT method in 96-well microplates. Five human cancer cell lines: human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast cancer MCF-7, and human colon cancer SW480 cells were used in the cytotoxicity assay. All the cells

Table 2. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data of **4** (δ in ppm, J in Hz).

	4	
No.	δ_C	δ_H
1	33.7, t	1.75–1.81, m
		1.31–1.34, m
2	24.8, t	1.99–2.00, m
		1.55–1.60, m
3	77.6, d	3.45, dd (3.1, 2.3)
4	38.3, s	
5	47.8, d	1.73, d (4.9)
6	66.3, d	4.61–4.63, m
7	135.8, d	6.79, dd (3.5, 3.6)
8	128.3, s	
9	51.6, d	2.77–2.81, m
10	33.9, s	
11	67.7, t	4.44, dd (9.1, 9.4)
		4.10, dd (9.1, 9.4)
12	170.4, s	
13	25.2, q	1.33, s
14	27.7, q	1.16, s
15	15.9, q	1.06, s

were cultured in Roswell Park Memorial Institute-1640 or Dulbecco's modified Eagle medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) in 5% CO_2 at 37°C. Briefly, 100 μl of adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition with an initial density of 1×10^5 cells/ml. Each tumor cell line was exposed to the test compound at concentrations of 0.064, 0.32, 1.6, 8, and 40 μmol in triplicates for 48 h, with cisplatin (Sigma, St. Louis, MO, USA) as a positive control (IC_{50}: SW480, 12.0 μmol; SMMC-7721, 10.2 μmol; HL-60, 3.1 μmol; MCF-7, 17.5 μmol; A-549, 9.1 μmol). After compound treatment, cell viability was detected and cell growth curve was graphed. Surprisingly, compound **4** demonstrated moderate inhibitory activity on human colon cancer SW480 with IC_{50} 20.4 μmol.

Acknowledgments

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