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Pyrrolidinone diterpenoid from *Isodon excisus* and inhibition of nitric oxide production in lipopolysaccharide-induced macrophage RAW264.7 cells

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ABSTRACT

A new pyrrolidinone diterpenoid, excisusin F (**1**), was isolated from the aerial parts of *Isodon excisus* (Lamiaceae), together with four known compounds, and their structures were determined mainly by NMR (1D and 2D) and mass spectrometry. Excisusin F (**1**) and inflexarabdonin E (**3**) showed potent inhibitory effects of LPS-induced nitric oxide production in RAW264.7 cells with the IC_{50} value of 10.4 and 3.8 μM, respectively.

Isodon species spreads widely in nature and are genus of the family Lamiaceae consisting of more than 150 species. Major secondary metabolites of genus of *Isodon* are *ent*-kauranoids including C-20 non-oxygenated *ent*-kaurane, C-20 oxygenated *ent*-kaurane, 6,7-*seco*-*ent*-kaurane, 8,9-*seco*-*ent*-kaurane, *ent*-kaurane dimers, *ent*-abietane and abietane skeletons.[1–3] These substances showed various bioactivities, such as cytotoxic, antibacterial, antitumor, and anti-inflammatory activities.[4–10] *Isodon excisus* is widely distributed in Korea, which has been mainly used as a traditional folk medicine for the treatment of detoxification and gastrointestinal disorders.[11,12]

Our previous paper reported the isolation and characterization of twelve diterpenoids from CH_2Cl_2-soluble fraction of the methanolic extract of *I. excisus*.[13] In a continuation of the phytochemical research on this plant, we now report the isolation and structural elucidation of a new pyrrolidinone diterpenoid, excisusin F (**1**) from the CH_2Cl_2 fraction, along with four known compounds, inflexarabdonin D (**2**),[14] inflexarabdonin E (**3**),[14,15] corchoionol A (**4**),[16,17] and corchorifatty acid B (**5**) (Fig. 1).[18,19] We examined the inhibitory effects of these compounds on nitric oxide (NO) generation using lipopolysaccharide (LPS) induced murine macrophage RAW264.7 cells.

The air-dried aerial parts of *I. excisus* (1.6 kg) were pulverized and extracted with MeOH (15 L × 3) at room temperature. After removing solvent, this extract was suspended in suitably diluted with water, and partitioned and removed their solvent successively to give *n*-hexane and CH_2Cl_2-soluble extract. The CH_2Cl_2-soluble extract (13.7 g) was subjected to column chromatography on silica gel (9 × 25 cm) eluting with CH_2Cl_2/MeOH (1:0) in increasing proportion of MeOH, to yield seven fractions (IEA/IEG). Fraction IEE (1.4 g) was further applied to column chromatography over silica gel (3 × 20 cm) eluting with *n*-hexane/acetone (5:1, 3:1, 2:1) to yield ten fractions (IEE-1/IEE-10). Fraction IEE-8 was subjected to flash column chromatography on RP-18 (2 × 30 cm) eluting MeCN/H_2O (30:70) affording three fractions (IEE-81/IEE-83). Fraction IEE-3 was further purified by means of semi-preparative HPLC eluting with MeCN/H_2O (35:65, v/v) at the flow rate of 6.5 mL/min to yield compound **1** (2.9 mg). Fraction IEE-10 was purified by semi-preparative HPLC [MeCN/H_2O (35:65, v/v), 6.5 mL/min] to give compounds **2** (2.9 mg) and **3** (2.5 mg). Fraction IEG (2.6 g) was further fractionated over silica gel column and eluted with *n*-hexane/acetone (3:1, 3:2, 1:1, 0:1) to obtain six fractions (IEG-1/IEG-6). Fraction IEG-3 (0.5 g) was purified by a RP-18 column (2 × 30 cm) using MeCN/H_2O (30:70, v/v) as the eluent to give nine fractions (IEG-31/IEG-39). Fraction IEG-33 was rechromatographed over a silica gel column eluting with CH_2Cl_2/MeOH (20:1) to compounds **4** (2.5 mg) and **5** (35 mg).

Compound **1** was obtained as white amorphous powder and has a molecular formula of $C_{26}H_{37}NO_6$ determined by HRESIMS which showed a quasi-molecular formula ion peak at *m*/*z* 504.2581 $[M+COOH]^-$ (calcd for $C_{26}H_{37}NO_6COOH$, 504.2597). This formula indicated 9 degrees of unsaturation. The UV spectrum indicated

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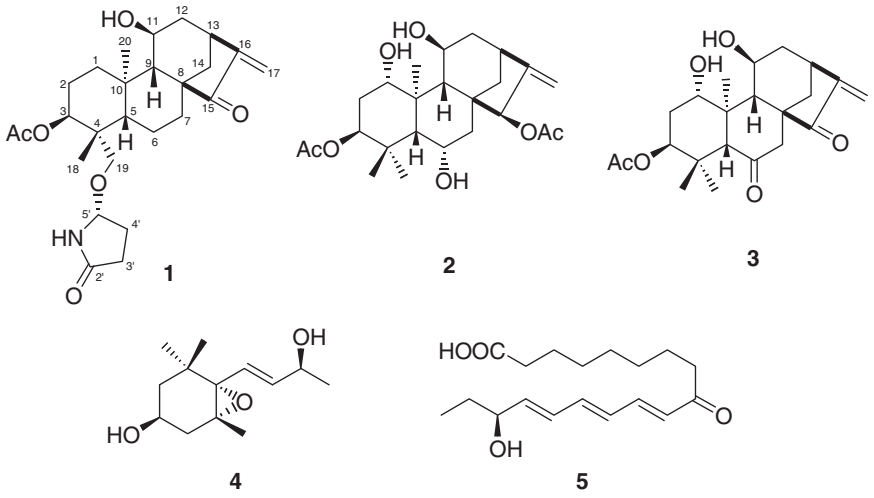

Figure 1. Structures of compounds **1**–**5**.

characteristic absorption bands for a five-membered ring ketone conjugated with an *exo*-methylene (241.2 nm).[13] The ^{1}H, ^{13}C and DEPT NMR spectra of **1** (see Table 1) showed the signals for three methyl groups including an acetyl group at δ_H 1.93 (3H, s), δ_C 170.2 and δ_C 20.9, seven methylene groups including an oxygenated methylene at δ_H 3.75 (d, J = 9.3 Hz) and 3.28 (d, J = 9.3 Hz), and δ_C 69.4, an *exo*-methylene signal at δ_H 6.00 (1H, s) and 5.23 (1H, s), and δ_C 151.6 and 111.2, five methine signals including two oxygenated methines at δ_H 5.31 (1H, br s) and 4.20 (d, J = 3.9 Hz), and δ_C 73.6 and 64.9, four quaternary carbons, one ketonic carbon at δ_C 208.7, and an ester carbonyl carbon at δ_C 170.2. These data suggested that **1** was an *ent*-kaurane skeleton diterpenoid with an acetoxyl group and a hydroxyl group. Also, the ^{1}H and ^{13}C NMR spectra of **1** displayed signals for the presence of two methylene groups at δ_H 2.01–2.16 (2H, m) and 2.55 (2H, m), and δ_C 28.5 and 28.8, an oxygenated methine group at δ_H 5.05 (d, J = 5.4 Hz) and δ_C 86.8, an amide group at δ_H 9.73 and δ_C 178.4. These data indicated that partial structure of **1** were quite similar to those of 5-hydroxypyrrolidin-2-one except the downfield of C-5′ (Table 1).[20–24] The location of the functional groups were assigned by the observed HMBC correlations from H-3 (δ_H 5.31) to C-1, C-5 and acetoxyl carbonyl carbon, from H-11 (δ_H 4.20) to C-8 and C-13, from H-19 (δ_H 3.75 and 3.28) to C-3, C-4, C-18, and C-5′. These correlations clearly indicated that an acetoxy group at C-3 and a hydroxyl group at C-11, respectively. Furthermore, 5-hydroxypyrrolidin-2-one derivative was connected by an ether linkage between C-19 and C-5′ (Fig. 2).

Table 1
NMR data for compounds **1**–**3** in pyridine-d_5 (500 and 125 MHz, δ in ppm)[a]

Position	1			2	3
	δ_H, mult (J Hz)	δ_C	HMBC	δ_C	δ_C
1α	1.59 m	33.2 t		77.6 d	74.8 d
1β	1.44 m				
2		22.9 t		34.5 t	33.5 t
3α	5.31 br s	73.6 d	1, 5, OAc	80.2 d	78.9 d
4		41.6 s		38.0 s	36.1 s
5β	1.65 over	50.5 d		49.2 d	59.4 d
6		18.7 t		66.8 d	211.4 s
7		34.8 t	8, 9, 15	47.6 t	51.2 t
8		50.7 s		44.3 s	55.0 s
9β	1.93 over	64.7 d	8, 11, 20	58.6 d	65.9 d
10		38.4 s		43.9 s	51.0 s
11α	4.20 d (3.9)	64.9 d	8, 10	66.9 d	65.1 d
12α	2.22 m	41.4 t	11, 13	43.6 t	40.9 t
12β	2.06 m				
13α	3.00 s	37.7 d		39.9 d	37.7 d
14α	2.30 d (11.8)	37.1 t	8, 13, 15, 16	38.5 t	37.6 t
14β	1.33 m				
15		208.7 s		83.4 d	205.5 s
16		151.6 s		154.1 s	150.9 s
17	6.00 s, 5.23 s	111.2 t	13, 15, 16	107.4 t	111.9 t
18	1.09 s	23.1 q	3, 4, 5, 19	28.3 q	26.5 q
19	3.75 d (9.3)	69.4 t	3, 4, 5, 5′	24.3 q	21.9 q
	3.28 d (9.3)				
20	0.92 s	17.9 q	1, 5, 9, 10	14.4 q	15.4 q
OAc		170.2 s		170.8 s	170.1 s
	1.93 s	20.9 q		21.0 q	20.8 q
				170.4	
				21.0	
1′-NH	9.73 s		4′, 5′		
2′		178.4 s			
3′	2.55 m	28.8 t	2′		
4′	2.16 m, 2.01 m	28.5 t	2′, 5′		
5′	5.05 d (5.4)	86.8 d			

[a] The assignments were based on DEPT, HMQC, and HMBC experiments.

The relative configurations of the acetoxyl group at C-3 and hydroxyl group at C-11 were established by a NOESY experiment. The acetoxyl group at C-3 and hydroxyl groups at C-11 were shown to be in the β and β orientations, respectively, as deduced from the cross-peaks of δ_H 5.31 (H-3) with δ_H 3.75 (H-19a) and 3.28 (H-19b), and of δ_H 4.20 (H-11) with δ_H 1.59 (H-1α) and 0.92 (H-20α) (Fig. 2). The absolute configuration of **1** was determined by the measurement of the circular dichrome (CD) spectrum. The negative Cotton effect of 341 nm (−0.8) corresponding to an enone system was the same as that of *ent*-kaurane. Also, the negative Cotton effect near 209 nm (−9.2) was reported to have (*R*)-configuration by an octant rule as (*R*)-5-hydroxypyrrolidin-2-one.[23] Therefore, compound **1** was elucidated as 3β-acetoxy-11β-hydroxy-19-[5(*R*)-hydroxypyrrolidin-2-one]-*ent*-kaur-16-en-15-one, named excisusin F.[25] It is noteworthy that the identification of pyrrolidinone containing *ent*-kaurane diterpenoid from *I. exisus*, although the genus *Isodon* is an important source of *ent*-kaurane diterpenoids.

All isolates were evaluated for their inhibitory effects of NO production and excisusin F (**1**) and inflexarabdonin E (**3**) showed the significant inhibitory effects with IC_{50} values of 10.4 and 3.8 μM, respectively (Table 2). The cell viability measured by CCK-assay indicated that active compounds had no significant cytotoxic effects.[26]

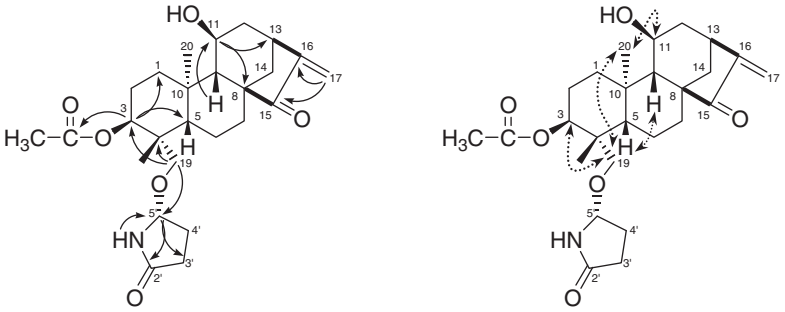

Figure 2. Key correlations of compound **1** in HMBC (H→C) and NOESY (◄- - -►) spectra.

Table 2
Inhibitory effects of the isolated compounds **1**–**5** on the LPS-induced NO production in RAW264.7 cells

Compound	IC_{50} (μM)
1	10.4 ± 0.3[a]
2	>40.0
3	3.8 ± 0.2
4	>40.0
5	>40.0
Aminoguanidine[b]	32.2 ± 0.1

[a] Data are mean ± SD from three separate experiments.
[b] Aminoguanidine was used as a positive control.

Acknowledgments

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25. Excisusin F (**1**): white amorphous powder; $[\alpha]_D^{25}$ −50.4 (*c* 0.18, MeOH); CD (*c* 1.09×10^{-3} M, MeOH) λ_{max} nm (Δε) 341 (−0.8), 242 (+0.8), 209 (−9.2); UV (MeOH) λ_{max} nm (log ε): 241 (4.12); ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR: see Table 1; ESI-MS (positive mode) *m*/*z*: 482 $[M+Na]^+$; HRESIMS (negative mode) *m*/*z* 504.2581 (calcd for $C_{26}H_{37}NO_6COOH$, 504.2597).
26. *Determination of NO production and cell viability assay*: The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. Briefly, RAW264.7 cells were seeded into 96-well tissue culture plates at a density of 2×10^5 cells/mL, and stimulated with 1 μg/mL of LPS in the presence or absence of compounds. After incubation at 37 °C for 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess containing equal volumes of 2% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.2% (w/v) of *N*-(1-naphthyl)ethylenediamine solution to determine nitrite production. Absorbance was measured in a microplate reader at 550 nm against a calibration curve with sodium nitrite standards. The remaining cells after Griess assay were used for viability with the CCK (Cell Counting Kit, Dojindo, Tokyo, Japan)-based colorimetric assay.[27,28]
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