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RESEARCH ARTICLE

Annulohpoxylotol A and B, new sesquiterpenoids from the endophytic fungus *Annulohypoxylon truncatum*, are natural NF-κB inhibitors

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Abstract Two sesquiterpenoids, annulohpoxylotol A and B, were isolated along with five sterols from an ethyl acetate extract of cultures of the endophytic fungus *Annulohypoxylon truncatum* growing on leaves of *Zizania caduciflora*. The structures of the isolated compounds were established using one-dimensional (1D) and two-dimensional (2D)-NMR and mass spectrometry. The nuclear factor-kappa B (NF-κB) inhibitory activities of the isolated compounds stimulated with tumor necrosis factor-alpha (TNF-α) were measured using a luciferase reporter system. Annulohpoxylotol A (**1**) significantly inhibited NF-κB activation in a dose-dependent manner, with an IC_{50} of 7.11 μM, whereas annulohpoxylotol B (**2**) and ergone (**7**) moderately inhibited NF-κB transcriptional activity, with IC_{50} values of 19.24 and 17.51 μM, respectively.

Keywords *Annulohypoxylon truncatum* · Sesquiterpenoid · Endophytic fungus · NF-κB

Wei Li and Ya Nan Sun contributed equally to this work.

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Introduction

The term endophyte refers to a fungal or bacterial microorganism that colonizes the intercellular and intracellular healthy tissues or organs of plants but does not produce any apparent symptoms or have pathogenic effects on its host (Tan and Zou 2001; Gunatilaka 2006). Recently, endophytes have been recognized as a source of structurally novel and biologically active secondary metabolites. In particular, the family Xylariaceae, a large diverse group of ascomycetes, comprises over 1300 species with an almost cosmopolitan distribution (Stadler et al. 2013). Several secondary metabolites produced by representatives from some of these genera have been isolated and identified (Strobel et al. 2004; Zhang et al. 2006), however, the genus *Annulohypoxylon* has received less attention. Some studies have investigated the secondary metabolites of *A. boveri*, *A. ilanense*, *A. cohaerens*, and *A. squamulosum*, which include γ-lactones, benzoquinones, azaphilones, and furanoids (Cheng et al. 2011, 2012; Surup et al. 2013). In our ongoing research on metabolites from plant endophytes, we investigated the secondary metabolites in fermentation broth produced by the endophytic fungus *A. truncatum* (JS540), a strain isolated from the leaves of *Z. caduciflora*. A previou investigation showed that the metabolites of A. *truncatum* contain truncaquinones (Surup et al. 2016). However, terpenoid and sterol constituents and their nuclear factor-kappa B (NF-κB) inhibitory activity

have not been reported. NF-κB belongs to a family of proteins containing the Rel domain. The 5 NF-κB units that can form 15 transcription factors through homo- and hetero-dimerization. NF-κB plays an important role in the transcriptional regulation of numerous cytokines and adhesion molecules, and is the most extensively studied transcription factor in the immune system. The activation of NF-κB causes transcription at the κB site, which is involved in many diseases, including inflammatory disorders and cancer. Hence, inhibition of NF-κB signaling has become a therapeutic target for the treatment of inflammatory diseases and cancer (Rozieres et al. 2006; Kim et al. 2010; Kim and Kim 2011). In this study, seven secondary metabolites were isolated from an ethyl acetate extract of *A. truncatum* (Fig. 1), including two new sesquiterpenoids, together with five sterols. Here, we describe the isolation, structure elucidation, and NF-κB inhibitory activity of the isolated compounds, as well as the structure–activity relationships.

Materials and methods

General experimental procedures

Optical rotations were determined using a Jasco DIP-370 automatic polarimeter. The FT-IR spectra were measured using a Jasco Report-100 infrared spectrometer; The NMR spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz) and JEOL ECA 300 spectrometer (^{1}H, 300 MHz). The LCQ advantage trap mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.A.) was equipped with an electrospray ionization (ESI) source, and High-resolution electrospray ionization mass spectra (HR-ESI–MS) were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. Column chromatography was performed using a silica gel (Kieselgel 60, 70–230, and 230–400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60 F_{254} and RP-18 $F_{254}S$ plates (both 0.25 mm, Merck, Darmstadt, Germany).

Fungal material

The fungal strain (JS540) was isolated from leaves of *Z. caduciflora* collected from a swamp in Suncheon, South Korea. Leaf tissues were cut into small pieces (0.5 × 0.5 cm) and surfaces were sterilized with 2% sodium hypochlorite for 1 min, 70% ethanol for 1 min, and then washed with sterilized distilled water. Fungal strains were grown out from plant tissues after about 7 days incubation on malt extract agar (MEA, Difco) added to 50 ppm kanamycin, 50 ppm chloramphenicol, and 50 ppm Rose Bengal at 22 °C. The Fungal strain was identified by sequencing ITS regions with ITS1 and ITS4 primers. After a homology search against NCBI nt DB with a BlastN algorithm and phylogenetic analysis with ITS sequences from NCBI, JS540 was identified as *A. truncatum* by Dr. Soonok Kim from National Institute of Biological Resources (NIBR, Incheon, Korea). Fungus JS540 in 50% aqueous glycerol solution was stored in a −70 °C freezer at both NIBR and College of Pharmacy, Duksung Women's University (Seoul, South Korea). The fungal strain was

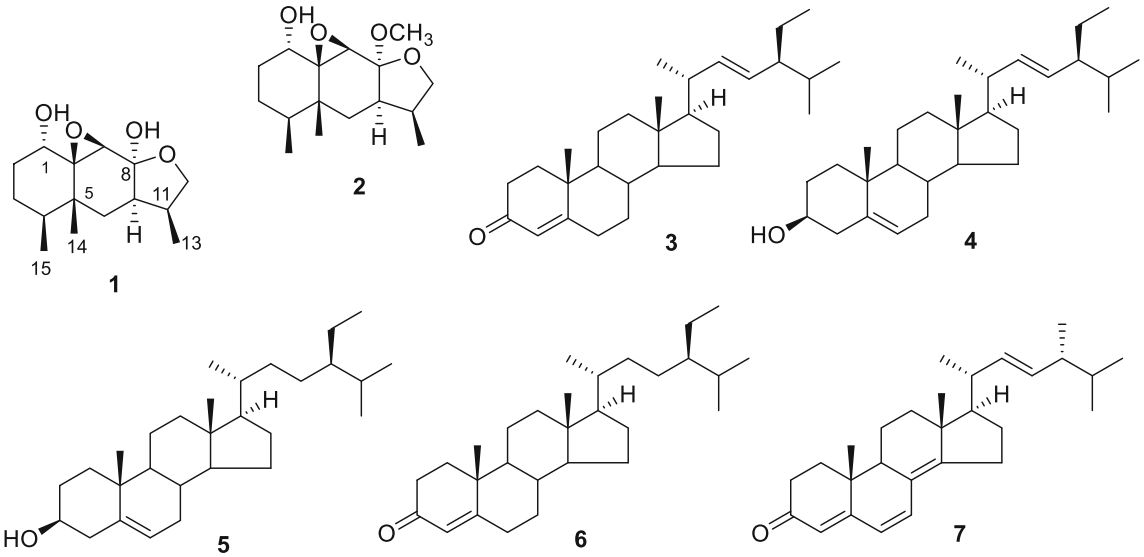

Fig. 1 Structures of compounds **1–7** from *A. truncatum*

cultivated at 28 °C for 28 days in four 500 mL Erlenmeyer flasks each containing 80 g of rice and 120 mL of water. Fungal strains were cultured by transferring actively growing edges to a new potato dextrose agar (PDA, Difco).

Fermentation, extraction and isolation

The fermentation was performed in Erlenmeyer flasks (20 × 500 mL) on solid rice medium containing 80 g of rice, 2.0 g of sea salt, and 80 mL of demineralized water. After autoclaving at 121 °C for 20 min and then cooling to room temperature, each flask was inoculated and then incubated at 28 °C under static conditions. After 30 days, the fermentation was stopped by adding 500 mL of EtOAc to each flask. The extraction was completed after the flasks had been shaken on a laboratory shaker at 150 rpm for 2 h.

The EtOAc solution were then evaporated under reduced pressure at 45 °C to give EtOAc extract (28.5 g). The EtOAc extract (26.0 g) was subjected to silica gel (5 × 30 cm) column chromatography with a gradient of hexane–EtOAc-MeOH (30:1:0, 10:1:0, 4:1:0, 2:1:0; 1.5:1:0.12, 1:1:0.2, 0:5:1, 0:0:1; 1.5 L for each step) to give 8 fractions (Fr. 1A–1H). The fraction 1B (3.3 g) was separated using an YMC (2.0 × 80 cm) column chromatography with a MeOH-acetone-H_2O (0.2:0.2:1, 0.5:0.5:1, 1:1:1, 2:2:1, 4:4:1, 8:8:1; 1.0 L for each step) elution solvent to give 16 fractions (Fr. 1B1–1B16). The fraction 1B8 (35.0 mg) was separated using a silica gel (1 × 80 cm) column chromatography with a hexane–EtOAc (19:1; 900 mL) elution solvent to give compound **7** (8.0 mg). The fraction 1B11 (60.0 mg) was separated using a silica gel (1 × 80 cm) column chromatography with a hexane–EtOAc (20:1; 1.0 L) elution solvent to give compounds **3** (25.0 mg) and **4** (2.8 mg). The fraction 1B13 (210.0 mg) was separated using a silica gel (1 × 80 cm) column chromatography with a hexane–EtOAc (22:1; 1.0 L) elution solvent to give compounds **5** (11.7 mg) and **6** (8.2 mg). The fraction 1E (0.7 g) was separated using an YMC (1.0 × 80 cm) column chromatography with a MeOH-acetone-H_2O (0.2:0.2:1, 0.35:0.35:1, 0.5:0.5:1, 0.75:0.75:1, 1:1:1; 1.0 L for each step) elution solvent to give 8 fractions (Fr. 1E1–1E8). The fraction 1E3 (25.0 mg) was separated using a silica gel (1 × 80 cm) column chromatography with a hexane–acetone (7:1, 5.5:1; 1.2 L) elution solvent to give compound **1** (6.5 mg). The fraction 1E6 (80.0 mg) was separated using a silica gel (1 × 80 cm) column chromatography with a hexane–acetone (11:1; 1.0 L) elution solvent to give compound **2** (4.6 mg).

Annulohpoxylotol A (**1**): white powder; $[\alpha]_D^{25}$: –20.8 (*c* 0.1, MeOH); IR (KBr): ν_{max} 3390, 1620, 1425 cm^{-1}; ^1H NMR (methanol-d_4, 600 MHz) and ^{13}C NMR data (methanol-d_4, 150 MHz), see Table 1; HR-ESI–MS: *m/z* 291.1574 $[M + Na]^+$ (calcd. for 291.1567).

Annulohpoxylotol B (**2**): white powder; $[\alpha]_D^{25}$: –35.2 (*c* 0.15, MeOH); IR (KBr): ν_{max} 3410, 1610, 1445 cm^{-1}; ^1H NMR (chloroform-*d*, 600 MHz) and ^{13}C NMR data (chloroform-*d*, 150 MHz), see Table 1; HR-ESI–MS: *m/z* 305.1728 $[M + Na]^+$ (calcd. for 305.1723).

Preparation of mosher ester of 1

Compound **1** (3.0 mg) was dissolved in $CHCl_3$ (0.5 mL) under argon. DMAP (1 mg), DIEA (35 μL), and (*S*)/(*R*)-MTPA-Cl (20 μL) were then added, and the reaction mixture was stirred for 12 h. The reaction mixture was diluted with excess $CHCl_3$ and the organic layer was washed with brine and water and concentrated to dryness under vacuum, and MTPA derivatives were purified by semi-preparative HPLC using C18 column (Phenomenex Luna C_{18}, 150 mm × 21.2 mm), eluting with a gradient solvent system composed of H_2O and MeOH (45–100% MeOH).

(*R*)-MTPA ester of **1** (**1a**): ^1H NMR (300 MHz, $CDCl_3$) δ_H 4.67 (br s, 1H, H-1), 4.60 (m, 1H, H-12), 4.41 (m, 1H, H-12), 3.33 (m, 1H, H-9), 3.10 (m, 1H, H-11), 2.40 (m, 1H, H-7), 1.98 (m, 1H, H-2), 1.88 (m, 1H, H-2), 1.62 (m, 1H, H-3), 1.55 (m, 1H, H-4), 1.44 (m, 1H, H-6), 1.41 (m, 1H, H-3), 1.30 (m, 1H, H-6), 0.84 (s, 3H, H-14), 0.82 (d, $J = 6.8$ Hz, 3H, H-15), 0.80 (d, $J = 6.6$ Hz, 3H, H-13).

(*S*)-MTPA ester of **1** (**1b**): ^1H NMR (300 MHz, $CDCl_3$) δ_H 4.69 (br s, 1H, H-1), 4.63 (m, 1H, H-12), 4.43 (m, 1H, H-12), 3.40 (m, 1H, H-9), 3.12 (m, 1H, H-11), 2.49 (m, 1H, H-7), 1.95 (m, 1H, H-2), 1.86 (m, 1H, H-2), 1.65 (m, 1H, H-3), 1.53 (m, 1H, H-4 1.45), (m, 1H, H-3), 1.40 (m, 1H, H-6), 1.27 (m, 1H, H-6), 0.88 (s, 3H, H-14), 0.82 (d, $J = 6.8$ Hz, 3H, H-15), 0.81 (d, $J = 6.6$ Hz, 3H, H-13).

Cell toxicity assay

Cell-Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) was used to analyze the effect of compounds on cell toxicity according to the manufacturer's instructions. Cells were cultured overnight in 96-well plate (~1 × 104 cells/well). Cell toxicity was assessed after the addition of compounds on dose-dependent manner. After 24 h of treatment, 10 μL of the CCK-8 solution was added to triplicate wells, and incubated for 1 h. Absorbance was measured at 450 nm to determine viable cell numbers in wells.

NF-κB luciferase assay

293T cells were maintained in Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum

Table 1 The ^{1}H and ^{13}C NMR spectroscopic data of compounds **1** and **2**

	1[a]		**2**[b]	
	δ_C[c]	δ_H[d] (*J* in Hz)	δ_C[c]	δ_H[d] (*J* in Hz)
1	75.1	3.38 brs	74.5	3.43 brs
2	32.9	1.69 m[e]	31.2	1.62 m[e]
		1.85 m[e]		1.90 m[e]
3	26.2	1.38 m[e]	24.9	1.37 m[e]
		1.83 m[e]		1.78 m[e]
4	36.5	1.78 m[e]	35.2	1.73 m[e]
5	38.2	–	36.8	–
6	28.1	1.04 t (14.4)	26.9	1.01 t (14.1)
		1.20 dd (14.1, 4.7)		1.15 dd (14.1,4.5)
7	43.1	1.93 ddd (14.6, 7.3, 4.8)	41.3	1.89 ddd (14.0, 6.4, 2.9)
8	103.4	–	104.8	–
9	64.0	2.87 s	59.6	2.98 s
10	67.7	–	66.3	–
11	34.8	2.74 ddd (14.6, 10.5, 7.4)	33.5	2.66 ddd (14.0, 9.6, 8.3)
12	74.6	3.40 dd (10.0, 8.0)	74.3	3.50 dd (10.2, 8.2)
		4.07 t (8.0)		4.06 t (8.2)
13	11.5	0.93 d (7.0)	11.6	0.90 d (7.0)
14	19.9	1.14 s	19.4	1.13 s
15	15.1	0.88 d (6.3)	14.8	0.85 d (6.6)
OMe			49.0	3.31 s

Assignments were done by HMQC and HMBC experiments; *J* values (Hz) are in parentheses

[a] Measured in methanol-d_4

[b] Measured in chloroform-*d*

[c] 150 MHz

[d] 600 MHz

[e] Overlapped

(FBS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37 °C and 5% CO_2. The luciferase vector was first transfected into 293T cells. After a limited amount of time, the cells were lysed, and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg^{2+} and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2×10^5 cells per well in a 12-well plate and grown. After 24 h, cells were transfected with inducible NF-κB firefly luciferase reporter and constitutively expressing Renilla reporter. After 24 h of transfection, medium was changed to assay medium (Opti-MEM + 0.5% FBS + 0.1 mM NEAA + 1 mM sodium pyruvate + 100 units/mL penicillin + 10 μg/mL streptomycin) and cells were pretreated for 1 h with either vehicle (1% DMSO in water) and compounds, followed by 1 h of treatment with 20 ng/mL TNFα for 23 h. Unstimulated 293T cells were used as a negative control (−), apigenin was used as a positive control. Dual Luciferase assay was performed 48 h after transfection, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization.

Results and discussion

Compound **1** was isolated as a white powder. The molecular formula was established as $C_{15}H_{24}O_4$ by HR-ESI–MS (*m/z* 291.1574 ($[M + Na]^+$); calcd. 291.1567). The ^{1}H NMR spectrum of compound **1** (Table 1) revealed oxygenated methine proton signals at δ_H 2.87 (s, H-9) and 3.38 (brs, H-1), oxygenated methylene proton signals at δ_H 3.40 (dd, $J = 10.0$, 8.0 Hz, H-12a) and 4.07 (t, $J = 8.0$ Hz, H-12b), methine group signals at δ_H 1.78 (m, H-4), δ_H 1.93 (ddd, $J = 14.6$, 7.3, 4.8 Hz, H-7), and 2.74 (ddd, $J = 14.6$, 10.5, 7.4 Hz, H-11), methylene group signals at δ_H 1.04 (t, $J = 14.4$ Hz, H-6a), 1.20 (dd, $J = 14.1$, 4.7 Hz, H-6b), 1.38 (m, H-3a), 1.83 (m, H-3b), 1.69 (m, H-2a), and 1.85 (m, H-2b), and methyl group signals at δ_H 0.88 (d, $J = 6.3$ Hz, H-15), 0.93 (d, $J = 7.0$ Hz, H-13), and 1.14

(s, H-14). The ^{13}C NMR spectrum (Table 1) showed a characteristic acetal carbon at δ_C 103.4 (C-8). Oxygenated carbon signals were at δ_C 64.0 (C-9), 67.7 (C-10), 74.6 (C-12), and 75.1 (C-1), with a quaternary carbon at δ_C 38.2 (C-5), methylene groups at δ_C 26.2 (C-3), 28.1 (C-6), and 32.9 (C-2), methine groups at δ_C 34.8 (C-11), 36.5 (C-4), and 43.1 (C-7), and methyl groups at δ_C 11.5 (C-13), 15.1 (C-15), and 19.9 (C-14). These observations indicated that **1** was an eremophilane-type sesquiterpenoid (Li et al. 2003). In the ^1H-^1H COSY spectrum (Fig. 2), correlations between the oxygenated methane group (H-1) and methylene group (H-2), together with the spin system from H-2 through H-3 to H-4 and continuing to H-15, established the segment –OCH(1)–CH_2(2)–CH_2(3)–CH-(4)–CH_3(15). In addition, correlations between the oxygenated methylene group (H-12) and methine group (H-11), together with the spin system from H-11 through H-7 to H-6, and the correlation between H-11 and H-13, established the segment –OCH_2(12)–CH(11)–[CH_3(13)]–CH(7)–CH_2(6).

Further interpretation of the HMBC spectrum showed the correlations of H-1/C-3, C-5, C-9, and C-10; H-6/C-10; and H7/C-9, suggesting that the epoxy moiety involved C-9 and C-10. The correlations of H-6, H-11, and H-12/C-8 indicated that the acetal carbon was C-8. Furthermore, three methyl groups were located at C-4, C-5, and C-11 via the HMBC correlations of H-13/C-7, C-11, and C-12; H-14/C-4, C-5, C-6, and C-10; and H-15/C-3, C-4, and C-5 (Fig. 2). Therefore, assignment of the planar structure of **1** was completed.

The relative configuration of **1** was elucidated by analyzing the partial NOESY data and comparing the chemical shifts and coupling constants with those of cuspidatol (Kawamura et al. 2000) cryptosphaerolide (Yang et al. 2011), and dihydroberkleasmin A (Oh et al. 2010). The very similar carbon and proton chemical shifts led to the deduction that **1** had the same relative stereochemistry of C-4, C-5, C-7, C-8, C-9, C-10, and C-11 as cuspidatol. Considering the biosynthetic relationship of eremophilane-type sesquiterpenoids, such as cuspidatol and dihydroberkleasmin A, CH_3-13, CH_3-14, and CH_3-15 were deemed to be β-oriented (Moriyama and Takahashi 1976; Chen et al. 2014). The observation of NOE interactions between H-7/H-11 and H-2α/H-9 suggested the α-oriented configuration of H-7 and H-9. The observation of NOE interactions between H-1/H-3β and H_3-15, and H-3β/H_3-14 and H-6β, together with the coupling constants of H-1 (δ_H 3.38, brs), suggested the β-oriented configuration of H-1. Evidence supporting the proposed confirmation of the

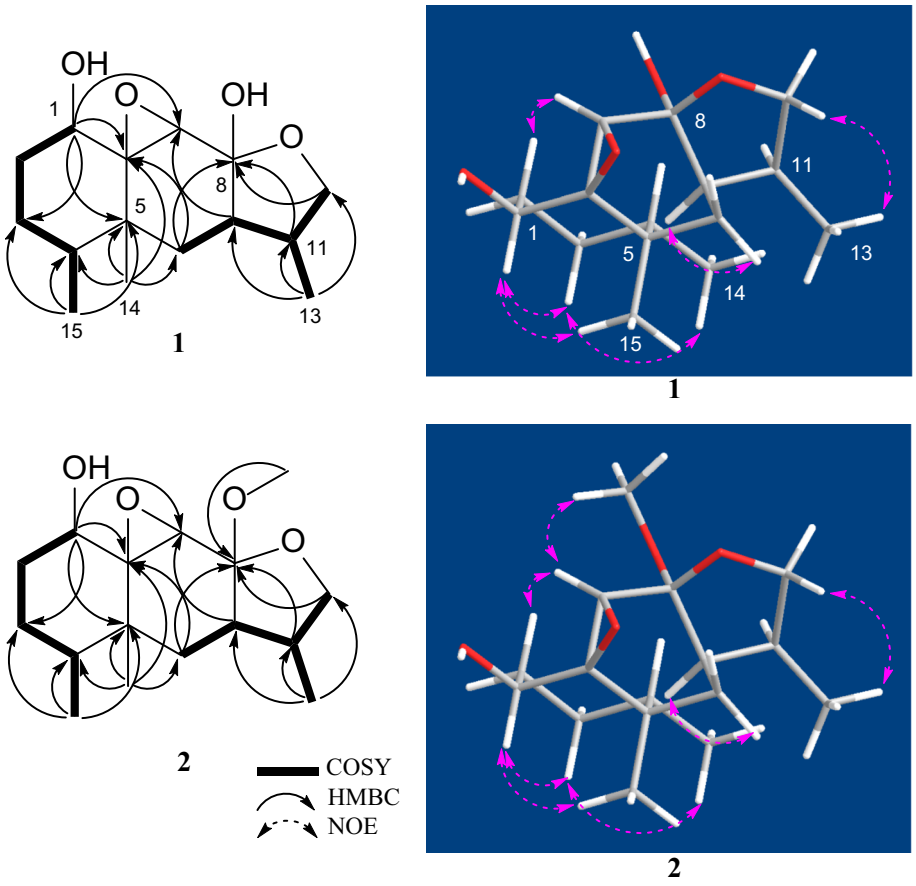

Fig. 2 ^1H-^1H COSY, key HMBC, and key NOE correlations of compounds **1** and **2**

Table 2 Inhibitory effects of compounds **1**–**7** on the TNFα-induced NF-κB transcriptional activity in 293T cells

Compound	IC_{50} (μM)[a]
1	7.11 ± 0.34
2	19.24 ± 1.13
3	>50
4	>50
5	>50
6	>50
7	17.51 ± 3.01
Apigenin[b]	1.60 ± 0.28

[a] The values are means ± SDs (n = 3)

[b] Positive control

tetracyclic ring system was obtained from interpretation of the NOESY data of cuspidatol (Fig. 2) (Chen et al. 2014). A NOE correlation between H-9 and H_3-14 led to assignment of the C-5/C-10 ring fusion as *cis* (Oh et al. 2010). The modification of **1** with each of *S*- and *R*-MTPA-Cl resulted in a pair of MTPA esters, which led to the assignment of the $\Delta\delta_{S\text{-}R}$ values that were calculated to be negative for H-2 (−0.02; −0.03), while those of H-3 (+0.03; +0.04), and H-9 (+0.07) were positive negative. It is clearly indicated that the absolute configurations of C-1, C-3, and C-8 were appropriate to *S*, respectively, allowing assignment of the absolute configuration of the remaining stereocenters in the fused ring system as 1*S*, 4*S*, 5*R*, 7*R*, 8*S*, 9*R*, 10*S*. Therefore, the structure of **1** was established and the compound was named annulohpoxylotol A.

The molecular formula of annulohpoxylotol B (**2**), a white powder, was assigned as $C_{16}H_{26}O_4$ by HR-ESI–MS (*m/z* 305.1728 ($[M + Na]^+$); calcd. 305.1723). Both the 1D and 2D NMR signals were similar to those of annulohpoxylotol A (**1**) (Table 1). A difference was found in the methoxy group signal (δ_H 3.31, δ_C 49.0), which is located at C-8, through HMBC correlation between H-OMe and C-8 (Fig. 2). Consequently, annulohpoxylotol B (**2**) was elucidated as shown in Fig. 1.

The other five sterols were identified as stigmasta-4,22-dien-3-one (**3**; Georges, et al. 2006), β-stigmasterol (**4**; Sultana and Khalid 2010), β-sitosterol (**5**; Sultana and Khalid 2010), β-sitostenone (**6**; Sultana and Khalid 2010), and ergone (**7**; Kwon et al. 2012).

The NF-κB inhibitory activities of compounds **1**–**7** were evaluated through inhibition of tumor necrosis factor-alpha (TNF-α)-induced NF-κB in a luciferase reporter assay. Cell viability was measured using a Cell Counting Kit (*CCK*)-*8*. The results showed that compounds **1**–**7** did not exhibit significant cytotoxicity in 293T cells at the concentrations tested, whereas compounds **5**–**7** showed moderate cytotoxicity (data not shown). Compared to untreated cells, human embryonic kidney 293T cells treated with 10 ng/mL TNF-α showed increased transcriptional activity. The compounds were pretreated with transfected 293T cells at various concentrations (0.2, 1, 5, 10, 25, and 50 μM), and then stimulated with TNF-α. Apigenin was used as a positive control.

The results showed that annulohpoxylotol A (**1**) significantly inhibited of NF-κB activation in a dose-dependent manner, with an IC_{50} of 7.11 ± 0.34 μM. Annulohpoxylotol B (**2**) and ergone (**7**) moderately inhibited NF-κB transcriptional activity, with IC_{50} values of 19.24 and 17.51 μM, respectively (Table 2). In comparison, the other compounds were inactive at the indicated concentrations (IC_{50} > 50 μM). A comparison of compounds **1** and **2** indicated that the hydroxyl group located at C-8 plays an important role. The metabolites of *A. truncatum*, such as truncaquinones, have antibacterial activity. To the best of our knowledge, however, this is the first report on the sesquiterpenoid and sterol components of *A. truncatum* and their effects on NF-κB inhibition. These results may provide a scientific basis for the development of novel anti-inflammatory agents.

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