A new kaurane diterpenoid from *Isodon inflexus*

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**ABSTRACT**

A new 7,20-epoxykauranediterpenoid, 15-acetyldemethylkamebacetal A (1) and six known kaurane diterpenoids (2–7) were isolated from the aerial parts of *Isodon inflexus* in nuclear transcription factor-κB (NF-κB)-dependent reporter gene assay-guided fractionation. Their chemical structures were determined on the basis of extensive spectroscopic analysis (UV, IR, MS, 1D- and 2D-NMR) and comparison with literature data. The isolated compounds were evaluated for their inhibitory effects on TNF-α-induced NF-κB activation, and all compounds exhibited NF-κB inhibitory activities with IC\textsubscript{50} values ranging from 1.91 to 20.15 μM.

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1. Introduction

The genus *Isodon* (Lamiaceae) is a rich source of diterpenoids, and many of them have various important bioactivities such as antibacterial, antitumour, anti-inflammatory and anti-feeding effects (Sun et al. 2006). Up to date, more than 600 diterpenoids have been identified from this genus since 1910, and most of them have *ent*-kaurane skeletons possessing highly oxygenated structures and show various biological activities (Hwang et al. 2001; Lee et al. 2002; Han et al. 2003; Yang et al. 2008; Liu et al. 2013; Chen et al. 2015). *Isodon inflexus* (Thunb.)
Kudo [synonym, *Rabdosia inflexa* (Thunb.) Hara] is a perennial shrub widely distributed in China, Korea and Japan. Previous phytochemical investigations on this plant resulted in the isolation of several ent-kaurane diterpenoids (Fujita et al. 1982; Takeda et al. 1989a, 1989b, 1990, 1993) and some of them exhibited cytotoxic activities and inhibitory effects on nitric oxide (NO) production (Lee et al. 2008; Xie et al. 2012).

Nuclear transcription factor-κB (NF-κB) plays a crucial role in the regulation of genes controlling the immune system, apoptosis, tumor cell growth and tissue differentiation. The activation of NF-κB is known to relate to multiple pathophysiological conditions such as cancer, arthritis, asthma, inflammatory bowel disease and other inflammatory conditions. Therefore, NF-κB and the signalling pathways that regulate its activity have become a focal point for intense drug discovery and development efforts (DiDonato et al. 2012).

In our ongoing research for NF-κB inhibitors from this plant, a new kaurane diterpenoid, 15-acetyldemethylkamebacetal A (1) and six known compounds, demethylkamebacetal A (2), excisanin A (3), rabdokunmin C (4), kamebakaurin (5), reniformin A (6) and isodomedin (7), were isolated from the methanol extract of *I. inflexus* (Figure 1). Herein, we present the isolation, structure elucidation and NF-κB inhibitory activities of these compounds.

**Figure 1.** Chemical structures of compounds 1–7.

**Table 1.** IC\(_{50}\) values of compounds 1–7 in the NF-κB activation.

| Compound | NF-κB activation |
|----------|------------------|
| 1        | 20.15 ± 0.95     |
| 2        | 7.36 ± 1.00      |
| 3        | 5.38 ± 0.99      |
| 4        | 17.86 ± 0.99     |
| 5        | 4.39 ± 0.95      |
| 6        | 2.88 ± 0.92      |
| 7        | 1.91 ± 0.91      |

Note: Results are expressed as mean ± SD of three replicates.
2. Results and discussion

Compound 1 was obtained as a white amorphous powder with $\alpha$-value of 26.0 (c = 0.02, MeOH) and had a molecular formula of $C_{22}H_{32}O_6$ as determined by HR-ESI-MS $m/z$ 375.2173 ($M + H - H_2O$) (calcd for $C_{22}H_{31}O_5$, 375.2172). The IR spectrum showed absorption bands due to hydroxyl groups (3392 cm$^{-1}$) and an exocyclic methylene moiety (1739 and 1671 cm$^{-1}$). All protons and related carbons were assigned according to the DEPT, HSQC and HMBC experiments, which provided information on the characteristic signals of three methines ($\delta_C$ 49.8, 47.3 and 46.4 due to C-5, 9 and 13), three quaternary carbons ($\delta_C$ 34.8, 53.4 and 43.6 assignable to C-4, 8 and 10), two methyls ($\delta_C$ 32.8 and 21.1 attributable to C-18 and 19), an oxygenated methine ($\delta_C$ 69.8 assigned as C-7) and a hemiacetal methine carbon ($\delta_C$ 95.6 assigned as C-20), indicating 1 would be a 7,20-epoxy-kaurane diterpenoid (Han et al. 2003). The epoxy hemiacetal linkage between C-7 and C-20 was suggested by the HMBC spectrum of 1, in which the H-20 ($\delta_H$ 5.77) was coupled to C-9 ($\delta_C$ 47.3), C-7 ($\delta_C$ 69.8), C-5 ($\delta_C$ 49.8) and C-10 ($\delta_C$ 43.6), and the relative configuration at C-20 was determined as $S$ by the downfield shift of C-11 due to the $\delta$-syn-axial effect between 20–OH and C-11 (Huang et al. 1989), which was supported by the NOESY correlation of H-20 with Me-19. Comparison of the 1H- and 13C-NMR spectral data of 1 with those of 2 showed only two differences: two newly observed carbon signals at $\delta_C$ 172.6 and 21.5 arised from an acetyl group, and upfield shift of the signal at $\delta_C$ 207.6 of 2 to $\delta_C$ 76.9 (Table S1). This was confirmed by the maximum absorption wavelength at 204 nm in UV spectrum and HMBC correlations of H-15 ($\delta_H$ 5.59) with C-7 ($\delta_C$ 69.8), C-9 ($\delta_C$ 47.3), C-14 ($\delta_C$ 73.5) and carbonyl carbon ($\delta_C$ 172.6). The $\beta$-orientation of OAc-15 was determined by the upfield shift of C-9 ($\Delta-5.6$ ppm) because of the $\gamma$-steric compression effect between OAc-$\beta$ and H-9$\beta$ (Zhao et al. 1997), which was supported by the NOESY correlation of OAc-15 with H-9$\beta$. The $\alpha$-orientation of 1-OH was indicated by the large coupling constants of H-1$\beta$ with H-2$\alpha$ ($J = 11.2$) and H-2$\beta$ ($J = 5.5$ Hz) (Takeda et al. 1994). The substituents at C-7 and C-14 possessed $\alpha$- and $\beta$-orientations according to the observed NOESY correlations of H-7$\beta$ with H-5$\beta$ and of H-14$\alpha$ with H-11$\alpha$ and H-20. Consequently, compound 1 was elucidated as 1$\alpha$,14$\beta$,20-trihydroxy-15$\beta$-acetoxy-7$\alpha$,20-epoxy-kaur-16-ene.

Six known diterpenoids were identified as demethylkamebacetal A (2) (Takeda, Ichihara, Fujita et al. 1989), excisanin A (3) (Sun et al. 1981), rabdokunmin C (4) (Zhang & Sun 1989), kamebakaurin (5) (Takeda et al. 1987), reniformin A (6) (Wang et al. 1986) and isodomedin (7) (Li & Chen 1990) by comparing their spectral data with those reported in the literature. All the isolates were examined for their dose-response effect on the TNF-$\alpha$-induced NF-$\kappa$B activation using the NF-$\kappa$B mediated reporter gene assay system. HeLa cells, which were transiently transfected with a NF-$\kappa$B-dependent reporter gene construct, were stimulated with TNF-$\alpha$ in the presence of various concentrations of compounds, and then the expression of reporter gene luciferase activity was measured. As a result, compounds 1–7 all showed inhibitory activity in the reporter gene expression, with IC$_{50}$ values of 20.15, 7.36, 5.38, 17.86, 4.39, 2.88 and 1.91 $\mu$M, respectively (Table 1).

The active compounds 2–7 were kaurane diterpenes containing an active centre (cyclopentanone conjugated with an exomethylene group), which can react by Michael-type reaction with biological nucleophile, especially sulfhydryl group of cysteine residue in the target proteins involved in the NF-$\kappa$B signalling pathway (Lee et al. 2002; Leung et al. 2006). However, compound 1, which contains only the exomethylene group without a conjugated carbonyl group, also exhibited NF-$\kappa$B inhibitory activity. It would be interesting to examine
whether the structural difference of Michael acceptor in the diterpenes would compromise
the target cysteine for the covalent modification in the various target proteins involved in
the NF-κB signalling pathway.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured using a JASCO DIP-370 (Tokyo, Japan) digital polarimeter.
The IR spectra were recorded from KBr pellets on the IRPrestige-21 spectrometer (Shimadzu
Ltd, Kyoto, Japan), and UV spectra on a Dynamica Halo DB-20 UV/Visible double beam spec-
trophotometer (Dynamica Pty Ltd, Prahran, Victoria, Australia). The NMR spectra were meas-
ured on Varian Unity Inova 400 spectrometer (Palo Alto, USA), and chemical shifts were
reported in ppm downfield from TMS. The ESI-MS and HREI-MS spectra were recorded Bruker
microTOF Q II mass spectrometer (Bruker Daltonics, Fremont, CA, USA). Column chroma-
tography was carried out on silica gel (40–63 μm, Merck), Lichroprep RP-18 gel (40–63 μm,
Merck) and Sephadex LH-20. TLC was performed on the plates precoated with silica gel 60
F_{254} (Merck, Germany) and RP-18 F_{254s} (Merck, Germany). Preparative HPLC was performed
on a YMC-Pack ODS column (20 × 250 mm, YMC, Kyoto, Japan) equipped with a Waters 600
pump, 600 Controller and 486 Tunable Absorbance Detector (Waters, Milford, MA, USA).

3.2. Plant material

The aerial parts of I. inflexus were collected at Jeju, Korea, in September 2006, and identified
by Dr Young Ho Kim at Chungnam National University. A voucher specimen (No. 060908)
was deposited at Korea Research Institute of Bioscience and Biotechnology.

3.3. Extraction and isolation

The air-dried aerial parts of I. inflexus (3 kg) were extracted three times with MeOH at room
temperature and the combined MeOH solution was concentrated under reduced pressure
to give a residue (109.5 g), which was suspended in H₂O and then successively partitioned
with CH₂Cl₂. The concentrated CH₂Cl₂ fraction (50.2 g) was loaded on a silica gel column and
eluted with n-hexane/EtOAc in a gradient mode to give 10 fractions (CXF1-10). The fraction
CXF7 (18.3 g) was chromatographed on a silica gel column and eluted with n-hexane/EtOAc
in a gradient mode to give seven fractions (CXF71-7). The fraction CXF74 was subjected to
Sephadex LH-20 column chromatography and eluted with CH₂Cl₂–MeOH (1:1) to give five
fractions (CXF741-5), and the fraction CXF743 was purified by prep-HPLC (MeOH:H₂O = 2:3)
to give 1 (13 mg) and 2 (29 mg). The fraction CXF75 was loaded on a RP-18 column and
eluted with MeOH–H₂O (1:4→4:1) to yield 3 (100 mg) and 4 (3.5 mg). The fraction CXF76 was
refractionated by repeated Sephadex LH-20 column chromatography and then purified by
prep-HPLC (MeOH:H₂O = 3:4) to give 5 (50 mg), 6 (19 mg) and 7 (9 mg).

3.3.1. 15-Acetyldemethylkamebacetal A (1)
White amorphous powder; [α]_{D}^{26} = −60.0 (c = 0.02, MeOH); UV (MeOH) λ_{max} (log ε) nm: 204
(3.31); IR (KBr) ν_{max} cm⁻¹: 3392, 2929, 2866, 1739, 1671, 1454, 1371, 1238, 1087, 1031; ¹H-NMR
(400 MHz, CD₃OD) and ¹³C-NMR (100 MHz, CD₃OD), see Table S1; positive ESI-MS m/z 415.5
[M + Na]^+, negative ESI-MS m/z 391.5 [M–H]^–; HR-ESI-MS m/z 375.2173 [M + H–H2O]^+ (Calcd for C22H31O5, 375.2172).

3.4. NF-κB activity assay

A pNF-κB-Luc plasmid for NF-κB luciferase reporter assay was obtained from Stratagene (LaJolla, CA, USA). Transfections were performed using Lipofectamine2000 according to the manufacturer’s protocol. NF-κB-dependent luciferase activity was measured using the Dual Luciferase Reporter Assay system. Briefly, HeLa cells (1 × 10^5 cells/well) were seeded in a 96-well plate for 24 h. The cells were then transfected with plasmids for each well and then incubated for a transfection period of 48 h. After that, the cell culture medium was removed and replaced with fresh medium containing various concentrations of compounds and TNF-α for 8 h. Luciferase activity was determined in Microlumat plus luminometer (EG&G Berthold, BadWildbad, Germany) by injecting 100 μL of assay buffer containing luciferin and measuring light emission for 10 s. Co-transfection with pRL-CMV (Promega, Madison, WI, USA), which expresses Renilla luciferase, was performed to enable normalisation of data for transfection efficiency (Lee et al. 2006).

4. Conclusions

In this study, a new kaurane diterpenoid, 15-acetyldemethylkamebacetal A (1) and six known compounds (2–7) were isolated from the aerial parts of I. inflexus. Their structures were elucidated by spectroscopic analysis including UV, IR, MS, 1D- and 2D-NMR experiments and comparison with literature values. Bioassay results showed that compounds 1–7 all exhibited NF-κB inhibitory activity in a dose-dependent manner with IC_{50} values ranging from 1.91 to 20.15 μM.

Supplementary material

Supplementary material relating to this article is available online, alongside Table S1 and Figures S1–S10.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Chen L, Zhan R, Jiang J, Zhang Y, Dong Y, Chen Y. 2015. A new ent-kaurane diterpenoid from Ixora amplexicaulis. Nat Prod Res. 1–5. doi:10.1080/14786419.2015.1039000.

DiDonato JA, Mercurio F, Karin M. 2012. NF-κB and the link between inflammation and cancer. Immunol Rev. 246:379–400.
Fujita T, Takeda Y, Yuasa E, Okamura A, Shingu T, Yokoi T. 1982. Structure of inflexinol, a new cytotoxic diterpene from *Rabdosia inflexa*. Phytochemistry. 21:903–905.

Han QB, Li ML, Li SH, Mou YK, Lin ZW, Sun HD. 2003. *Ent*-kaurenane Diterpenoids from *Isodon rubescens var. luschanensis*. Chem Pharm Bull. 51:790–793.

Huang H, Xu Y, Sun H. 1989. Diterpenoids from *Rabdosia coetsoides*. Phytochemistry. 28:2753–2757.

Hwang BY, Lee JH, Koo TH, Kim HS, Hong YS, Ro JS, Lee KS, Lee JJ. 2001. Kaurane diterpenes from *Isodon japonicas* inhibit nitric oxide and prostaglandin E2 production and NF-κB activation in LPS-stimulated macrophage RAW264.7 cells. Planta Med. 67:406–410.

Lee C, Hong SS, Han XH, Jin Q, Li D, Kim TO, Kim HK, Lee J, Kwon SH, Kim YB, et al. 2008. A new abietane diterpenoid from *Isodon inflexus*. Arch Pharmacal Res. 31:1381–1384.

Lee JH, Koo TH, Hwang BY, Lee JJ. 2002. Kaurane diterpene, kamebakaurin inhibits NF-κB by directly targeting the DNA-binding activity of p50 and blocks the expression of antiapoptotic NF-κB target genes. J Biol Chem. 277:18411–18420.

Lee JH, Koo TH, Yoon H, Jung HS, Jin HZ, Lee K, Hong Y-S, Lee JJ. 2006. Inhibition of NF-κB activation through targeting IkB kinase by celestrol, a quinone methide triterpenoid. Biochem Pharmacol. 72:1311–1321.

Leung CH, Grill SP, Lam W, Gao W, Sun HD, Cheng YC. 2006. Eriocalyxin B inhibits nuclear factor-κB activation by interfering with the binding of both p65 and p50 to the response element in a noncompetitive manner. Mol Pharmacol. 70:1946–1955.

Li Y, Chen Y. 1990. Diterpenoids from *Rabdosia pseudo-irrorata*. J Nat Prod. 53:841–844.

Liu W, Fan BL, Guo HY, Yuan YL, Liang HJ, Bai SP. 2013. Isolation and identification of a new *ent*-kaurenane diterpenoid from the leaves of *Isodon japonica*. Nat Prod Res. 27:1388–1392.

Sun H, Sun X, Lin Z, Xu Y, Minammi Y, Maranka T, Fujita T. 1981. Excisanin A and B, new diterpenoids from *Rabdosia excise*. Chem Lett. 6:753–756.

Sun HD, Huang SX, Han QB. 2006. Diterpenoids from *Isodon* species and their biological activities. Nat Prod Rep. 23:673–698.

Takeda Y, Ichihara T, Fujita T, Ueno A. 1989. *Ent*-kaurenoids from *Rabdosia umbrosa*. Phytochemistry. 28:1691–1694.

Takeda Y, Ichihara T, Takaishi Y, Fujita T. 1987. Structural elucidation of new diterpenoids isolated from *Rabdosia umbrosa var. leucanthera* f. Kameba. J Chem Soc, Perkin Trans. 1:2403–2409.

Takeda Y, Ichihara T, Yamasaki K, Otsuka H. 1989a. Diterpenoids from *Rabdosia inflexa*. Phytochemistry. 28:2423–2426.

Takeda Y, Ichihara T, Yamasaki K, Otsuka H. 1989b. *Inflexarabdonins* A and B, diterpenoids from *Rabdosia inflexa*. Phytochemistry. 28:851–854.

Takeda Y, Ichihara T, Yamasaki K, Otsuka H. 1990. Structure elucidation of inflexarabdonins G and H: two new diterpenoids from *Rabdosia inflexa*. Planta Med. 56:281–283.

Takeda Y, Ichihara T, Yamasaki K, Otsuka H, Utsumi H. 1993. *Inflexarabdonins* I, J and K, *ent*-kaurenoids from *Rabdosia inflexa*. Phytochemistry. 32:145–150.

Takeda Y, Takeda KI, Fujita T, Sun H, Minami Y. 1994. *Rabdoternins* DG, *ent*-7β, 20-epoxykaurenes from *Rabdosia ternifolia*. Phytochemistry. 35:1513–1516.

Wang XR, Wang ZQ, Dong JG, Wang XW. 1986. The chemical structures of reniformin A, B and C. Acta Bot Sin. 28:292–298.

Xie WD, Li X, Zhao JH, Liu YH, Row KH. 2012. Abietane diterpenoids from *Isodon infulus*. Phytochemistry. 81:153–158.

Yang LB, Yang J, Li LM, Lei C, Zhao Y, Huang SX, Xiao WL, Han QB, Pu JX, Sun HD. 2008. Symmetric and asymmetric *ent*-kaurenane dimers isolated from *Isodon japonicus*. Tetrahedron Lett. 49:3574–3577.

Zhang H, Sun H. 1989. Diterpenoids from *Rabdosia kunmingensis*. Phytochemistry. 28:3405–3409.

Zheng QS, Tian J, Yue JM, Lin ZW, Sun HD. 1997. *Ent*-kaurenane diterpenoids from *Isodon angustifolius var. glabrescens*. J Nat Prod. 60:1075–1081.