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Microbial transformation of isosteviol oxime and the inhibitory effects on NF-κB and AP-1 activation in LPS-stimulated macrophages ☆

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ABSTRACT

Microbial transformation of isosteviol oxime (*ent*-16-*E*-hydroxyiminobeyeran-19-oic acid) (**2**) with *Aspergillus niger* BCRC 32720 and *Absidia pseudocylindrospora* ATCC 24169 yielded several compounds. In addition to bioconverting the D-ring to lactone and lactam moieties, 4α-carboxy-13α-hydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone (**7**) and 4α-carboxy-13α-amino-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactam (**10**), one known compound, *ent*-1β,7α-dihydroxy-16-oxo-beyeran-19-oic acid (**6**), and five new compounds, *ent*-7α-hydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid (**3**), *ent*-1β,7α-dihydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid (**4**), *ent*-1β-hydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid (**5**), *ent*-8β-cyanomethyl-13-methyl-12-podocarpen-19-oic acid (**8**), and *ent*-8β-cyanomethyl-13-methyl-13-podocarpen-19-oic acid (**9**), were isolated from the microbial transformation of **2**. Elucidation of the structures of these isolated compounds was primarily based on 1D and 2D NMR, and HRESIMS data, and **3**–**5** were further confirmed by X-ray crystallographic analyses. Additionally, the inhibitory effects of all of these compounds were evaluated on NF-κB and AP-1 activation in LPS-stimulated RAW 264.7 macrophages. Among the compounds tested, **5** and **10** significantly inhibited NF-κB activation, with **5** showing equal potency to dexamethasone; **3** and **6**–**9** significantly inhibited AP-1 activation, particularly **8**, which showed more inhibitory activity than dexamethasone.

1. Introduction

Glucocorticoids (GCs) such as dexamethasone have long been used as effective immunosuppressive and anti-inflammatory agents.[1] The immunosuppressive and anti-inflammatory actions of glucocorticoid hormones are mediated by their trans-repression of nuclear factor-kappa B (NF-κB) and activator protein (AP)-1 transcription factors.[2–4] NF-κB is a pivotal mediator of the human immune response regulating the transcriptions of various proinflammatory and inflammatory mediators.[5] Activation of NF-κB was reported to induce the transcriptions of multiple proinflammatory mediators, such as inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and interleukin (IL)-1α, which are involved in the pathogenesis of inflammatory diseases.[6] AP-1 is a key mediator of cytokine signaling and is required for the activation of numerous proinflammatory genes,[7] a group of dimeric factors constituted by members of the Jun and Fos families of DNA-binding proteins.[8] Because GC therapy is still accompanied by a wide range of adverse effects,[9] the inhibition of NF-κB and/or AP-1 transcriptional activation may become an alternative attractive approach for developing novel immunoinflammatory agents.[10]

A microbe is a biocatalyst, which possesses a multi-enzyme system. Thus, microbial transformation is an important technique for structurally modifying natural and synthetic compounds due to its significant regio- and stereoselectivities.[11] Fungi are widely used in microbial transformation studies, since their versatile enzymatic reservoir allows them to modify a diverse array of molecules.[12] Isosteviol (**1**), with a rigid skeleton comprising four fused rings similar to the steroid skeleton, possesses many biological activities.[13] In general, natural products are very useful as template molecules for producing new drugs.[14] Small modifications in the chemical structure of a compound can modify its biological activities.[15] According to the literature, a number of biologically important

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properties of steroids are dependent upon structural features of the steroid D-ring.[16] Transformation of cyclic ketones into oximes by aqueous alkaline media, frequently used for preparing oximes of natural compounds, provides a way of changing functional groups, sizes, and the stereochemistry of the D-ring.[16] Thus, isosteviol oxime (*ent*-16-*E*-hydroxyiminobeyeran-19-oic acid) (**2**) was prepared by reacting **1** with hydroxylamine hydrochloride. The activity of **2** in preventing cholera toxin-induced intestinal fluid secretions was reported.[17] However, the microbial transformation of **2** has never been described in the literature. In order to obtain new functionalized analogues with new biological activities, microbial transformation of **2** was undertaken. A number of microorganisms were screened for their ability to biotransform **2**. *Aspergillus niger* BCRC 32720 and *Absidia pseudocylindrospora* ATCC 24169 were selected for preparative-scale fermentation because they reproducibly converted **2** into many metabolites. Because the skeleton of tetracyclic diterpenoids possesses similarities to steroids[18] and in a continuing search for potential anti-inflammatory agents,[13] the inhibitory effects of **1**–**10** on the expressions of NF-κB and AP-1 target genes in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages were also evaluated. This article deals with the production, isolation, structure elucidation, and biological evaluation of these compounds.

2. Results and discussion

2.1. Bioconversion of isosteviol oxime (2) by *Aspergillus niger* BCRC 32720 and *Absidia pseudocylindrospora* ATCC 24169

Isosteviol oxime (**2**) was prepared as previously reported[19] and identified by 1D and 2D NMR spectroscopy, and HRESIMS. The bioconversion of **2** by *Asp. niger* and *Abs. pseudocylindrospora* reproducibly produced diverse products. Thus, they were selected for the preparative-scale biotransformation of **2**. Incubation of **2** with *Asp. niger* for 6 days led to the formation of *ent*-7α-hydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid (**3**), *ent*-1β,7α-dihydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid (**4**), *ent*-1β-hydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid (**5**), *ent*-1β,7α-dihydroxy-16-oxobeyeran-19-oic acid (**6**), 4α-carboxy-13α-hydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone (**7**), *ent*-8β-cyanomethyl-13-methyl-12-podocarpen-19-oic acid (**8**), *ent*-8β-cyanomethyl-13-methyl-13-podocarpen-19-oic acid (**9**), and 4α-carboxy-13α-amino-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactam (**10**) (Fig. 1). Incubation of **2** with *Abs. pseudocylindrospora* for 6 days yielded **3**, **4**, and **6**. Among these, **3**–**5**, **8**, and **9** are new compounds, whereas **6**, **7**, and **10** have previously been reported.[13,20,21] The structures of these isolated compounds were elucidated through analysis of 1D and 2D NMR spectroscopic data, with **3**–**5** being further confirmed by X-ray crystallographic studies.

Compound **3** displayed $[M+H]^+$ at *m*/*z* 350.2309 on HRESIMS, corresponding to the molecular formula $C_{20}H_{31}NO_4$, consistent with its ^{13}C NMR and DEPT spectra. The ^1H and ^{13}C NMR spectra indicated 20 carbons attributable to six quaternary carbons (including one carbonyl carbon), three CH_3, eight CH_2, and three CH (including one oxymethine). These data suggested that **3** contains one more oxygen atom than **2**. Analyses of the HSQC and HMBC spectra of **3** revealed that the new proton resonance at δ 4.01 showed connectivities with C-5 (δ 48.1), C-9 (δ 50.1), and C-15 (δ 37.4). Thus, the additional OH group in **3** occurred at C-7. The orientation of the OH group at C-7 followed from the multiplicity of the H-7 signal in the ^1H NMR spectrum, a broad singlet signal, indicating that H-7 was α-oriented.[22] Furthermore, the structure of **3** was confirmed by an X-ray crystallographic experiment (Fig. 2). Thus, **3** was characterized as *ent*-7α-hydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid.

Compound **4** had the molecular formula of $C_{20}H_{31}NO_5$, as evidenced by the HRESIMS [*m*/*z* 366.2313 $(M+H)^+$] and NMR spectra. The ^{13}C NMR and DEPT spectra of **4** possessed similar features as **2** except for the disappearance of two CH_2 signals and the presence of two new CH resonances at δ 82.1 and 76.6. This suggests that **4** is a dihydroxylated metabolite of **2**. In the HMBC spectrum, the proton resonance at δ 3.92 showed connectivities with CH_3-20 (δ 10.4), C-2 (δ 31.4), C-10 (δ 45.3), and C-9 (δ 51.1). Thus, one of

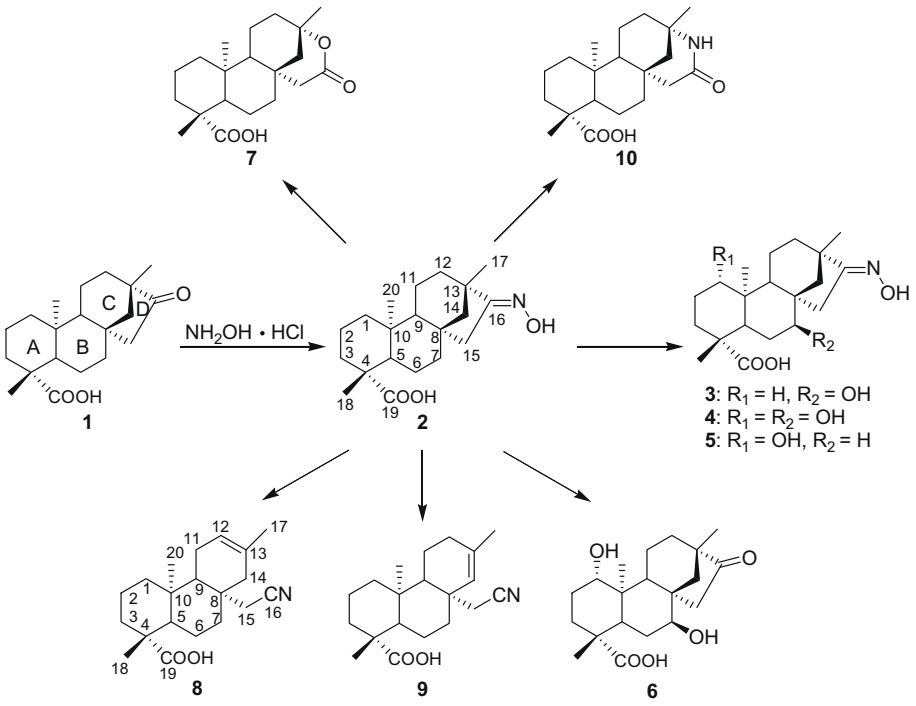

Figure 1. Structures of compounds **1**–**10**.

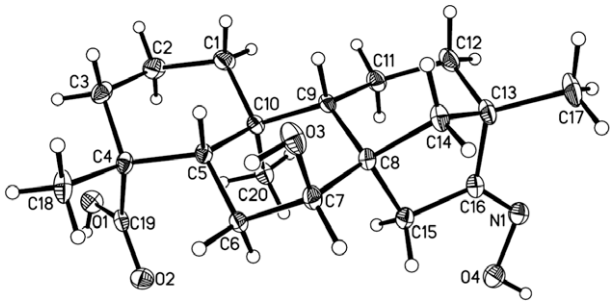

Figure 2. Perspective drawing of the X-ray structure of **3**.

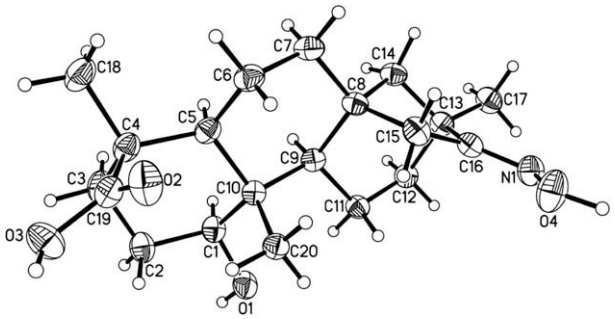

Figure 4. Perspective drawing of the X-ray structure of **5**.

the OH groups is at C-1. An α-orientation of the OH group was established by NOE correlations of δ_H 3.92 with H-2β and H-5β. The location of the second OH group at C-7 was deduced by 2D NMR data. In the HMBC spectrum, the proton resonance at δ_H 4.02 showed connectivities with C-15 (δ 37.9), C-8 (δ 47.2), C-5 (δ 46.8), and C-9 (δ 51.1). This indicated that the OH group is at C-7. H-7 resonated as a broad singlet at δ 4.02 indicating that the proton was α-oriented.[22] An X-ray crystallographic analysis was further performed to confirm the structure of **4** (Fig. 3). Thus, **4** was characterized as *ent*-1β,7α-dihydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid.

The HRESIMS of **5** displayed a quasi-molecular ion $[M+H]^+$ at *m*/*z* 350.2366, which indicated a molecular formula of $C_{20}H_{31}NO_4$ in combination with the ^1H and ^{13}C NMR data. The ^{13}C NMR and DEPT spectra revealed 20 signals due to six quaternary, three CH_3, eight CH_2, and three CH carbons. The ^1H and ^{13}C NMR spectra, when compared to **2**, showed new peaks at δ_H 3.72 and δ_C 81.9, respectively, indicating hydroxylation at a CH_2 carbon. The HMBC spectrum showed correlations of δ_C 81.9 with CH_3-20 (δ 1.47), H-2 (δ 2.60), H-3 (δ 2.50), and H-5 (δ 1.19). This indicated that an OH group had been introduced at C-1. In the COSY spectrum, the methine proton at C-1 (δ 3.72) resonated as a doublet of doublets (J = 11.0, 4.0 Hz) due to coupling with the protons of the neighboring C-2. This indicated that the proton is in a β-orientation.[22] Additionally, a single-crystal X-ray crystallographic experiment was used to confirm the structure of **5** (Fig. 4). Thus, **5** was established as *ent*-1β-hydroxy-16-*E*-hydroxyiminobeyeran-19-oic acid.

Compounds **8** and **9** displayed $[M-H]^-$ at *m*/*z* 314.2112 and 314.2118 (calcd for $C_{20}H_{28}NO_2$ 314.2120), respectively, in the negative-mode HRESIMS, corresponding to the molecular formula $C_{20}H_{29}NO_2$, consistent with the ^{13}C NMR and DEPT spectra. The ^{13}C NMR and DEPT data of **8** and **9** displayed 20 carbons attributable to six quaternary carbons (including one cyano and one carbonyl carbon), three CH_3, eight CH_2, and three CH carbons. Comparison of the HSQC and HMBC data of **8** and **9** with that of **2** revealed that no additional OH group had been introduced. In the HSQC and HMBC spectra of **8** and **9**, the proton resonance at δ 5.35 (δ_C 121.3) showed connectivities with δ 24.0 (C-17), 52.4 (C-9), and 46.6 (C-14) in **8**; the proton resonance at δ 5.51 (δ_C 131.7) showed connectivities with δ 23.7 (C-17), 32.3 (C-12), 37.5 (C-8), and 39.0 (C-7) in **9**. These suggested the presence of double bonds at C-12 in **8**, and C-13 in **9**. No connectivity was found between δ_C 120.1 and δ 5.35 (H-12), 1.81 (H-14), or 1.65 (H-17) in **8** or between δ_C 120.2 and δ 1.92 (H-12), 5.51 (H-14), or 1.62 (H-17) in **9**, suggesting that the bond between C-16 and C-13 had been cleaved. Accordingly, an abnormal Beckmann rearrangement of **2** to yield ring-cleaved carbonitrile products of **8** and **9** had occurred.[16] Thus, **8** and **9** were characterized as *ent*-8β-cyanomethyl-13-methyl-12-podocarpen-19-oic acid and *ent*-8β-cyanomethyl-13-methyl-13-podocarpen-19-oic acid, respectively.

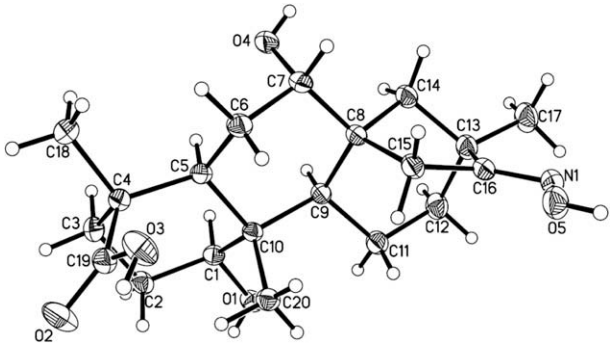

Figure 3. Perspective drawing of the X-ray structure of **4**.

2.2. Biological evaluation

The inhibitory effects of compounds **1**–**10** on both NF-κB and AP-1 activations were evaluated in LPS-stimulated RAW 264.7 macrophages using an NF-κB- or AP-1-mediated luciferase reporter gene assay. Among the compounds tested, **5** and **10** significantly inhibited NF-κB activation, with **5** showing equal potency to dexamethasone. On the other hand, **3** and **6**–**9** showed significant inhibition of AP-1 activation, particularly **8**, which showed greater inhibitory activity than dexamethasone (Tables 3 and 4).

3. Conclusion

In summary, the two selected filamentous fungi possess the ability to perform hydroxylation (**3**–**6**), Beckmann rearrangement (**10**), and an abnormal Beckmann rearrangement (**8** and **9**). According to the literature,[21] the formation of lactone **7** might be from the introduction an OH group into nitrile carbocation and subsequently cyclization to yield imidate, which was hydrolyzed to **7**. On the other hand, the results also suggested that substrate **2** might be hydrolyzed to isosteviol (**1**) first, and then converted to the **6** and **7** by hydroxylation and Baeyer–Villiger reaction, respectively. Although substrate **2** can be transformed into lactone and lactam derivatives under concentrated hydrochloric acid in an ampoule at 180 °C,[21] this is the first report to yield these two products by microbes. *Asp. niger* also has the abilities to yield ring-cleaved carbonitrile products and also to cause stereoselective hydroxylation at the 1α- and/or 7β-positions. Results obtained from the inhibitory effects on NF-κB or AP-1 activation in LPS-stimulated RAW 264.7 macrophages showed that **5** possessing 1α-OH on substrate **2** exhibited an equal potency of inhibition toward NF-κB activation as dexamethasone; **8**, with a ring-cleaved carbonitrile, exhibited more-significant inhibition toward AP-1 activation than dexamethasone. Thus, the suppression of NF-κB or AP-1 activation in LPS-stimulated RAW 264.7 macrophages by compounds **5** and **8** may have value for protection against inflammation. This study also demonstrates the use of microbial transformation techniques

Table 1
^{1}H NMR chemical shifts of compounds **2**–**5**, **8**, and **9** (C_5D_5N, δ values in ppm)[a,b]

Position	2	3	4	5	8	9
1	1.53–1.73 m[c]	1.74–1.80 m[c]	β3.92 dd (11.3, 4.5)	β3.72 dd (11.0, 4.0)	1.61 m	1.70 d (13.0)
	0.86 td (12.0, 4.0)	1.07 td (13.2, 3.9)			0.85 m	0.82 td (13.0, 4.0)
2	2.11–2.23 m[c]	2.26 m	2.65–2.75 m[c]	2.60 m	2.14–2.25 m[c]	2.22–2.33 m[c]
	1.37–1.50 m[c]	1.48–1.56 m[c]	1.95–2.02 m[c]	2.00 m	1.48 br d (14.0)	1.53 m
3	2.43 br d (12.8)	2.50–2.61 m[c]	2.54–2.57 m[c]	2.50 d (13.2)	2.41–2.45 m[c]	2.44 d (13.0)
	1.01–1.07 m[c]	1.18 dd (13.3, 4.0)	1.42 dd (13.6, 4.0)	1.28 dd (13.2, 3.5)	1.04–1.21 m[c]	1.05–1.13 m[c]
5β	1.12 m	2.38 d (1.9)	2.54–2.57 m[c]	1.19 br d (11.6)	1.04–1.21 m[c]	1.05–1.13 m[c]
6	2.11–2.23 m[c]	2.50–2.61 m[c]	2.65–2.75 m[c]	2.32 m	2.14–2.25 m[c]	2.22–2.33 m[c]
	2.02 m		2.54–2.57 m[c]	2.10 d (12.6)	1.95–2.04 m[c]	2.02 m
7	1.53–1.73 m[c]	α4.01 br s	α4.02 br s	1.64 d (13.0)	2.14–2.25 m[c]	2.13 dt (16.2, 2.85)
	1.37–1.50 m[c]			1.48–1.62 m[c]	1.04–1.21 m[c]	1.23–1.32 m[c]
9β	1.01–1.07 m[c]	1.90 m	2.35 dd (12.3, 4.3)	1.48–1.62 m[c]	1.26 dd (12.5, 5.1)	1.05–1.13 m[c]
11	1.53–1.73 m[c]	1.68 m	3.28 m	3.23 m	1.95–2.04 m[c]	1.64 m
	1.37–1.50 m[c]	1.48–1.56 m[c]	1.95–2.02 m[c]	1.80–1.88 m[c]	1.67 m	1.23–1.32 m[c]
12	1.53–1.73 m[c]	1.74–1.80 m[c]	1.80–1.86 m[c]	1.80–1.88 m[c]	5.35 s	1.92 m
	1.37–1.50 m[c]	1.48–1.56 m[c]	1.66 td (12.5, 5.0)	1.48–1.62 m[c]		
14	1.37–1.50 m[c]	1.99 dd (11.3, 2.8)	2.09 dd (11.3, 2.6)	1.48–1.62 m[c]	2.14–2.25 m[c]	5.51 s
	1.22 dd (10.8, 2.8)	1.74–1.80 m[c]	1.80–1.86 m[c]	1.36 dd (11.0, 2.5)	1.81 br d (17.2)	
15	3.32 dd (18.4, 2.8)	3.40 dd (18.5, 2.8)	3.55 dd (18.3, 2.6)	3.52 dd (18.0, 2.5)	2.73 dd (16.7, 1.25)	2.90 d (16.2)
	2.19 br d (18.4)	2.36 d (18.5)	2.45 d (18.3)	2.31 d (18.0)	2.41–2.45 m[c]	2.53 d (16.2)
17	1.28 s	1.37 s	1.37 s	1.32 s	1.65 s	1.62 s
18-CH_3	1.35 s	1.46 s	1.48 s	1.39 s	1.35 s	1.34 s
20-CH_3	1.09 s	1.23 s	1.56 s	1.47 s	0.96 s	0.94 s
N-*OH*	12.22 s	12.32 s	12.27 s	12.36 s		

[a] Assignments based on DEPT, HSQC, and HMBC.
[b] Signal multiplicity and coupling constants (Hz) are in parentheses.
[c] Overlapping signals.

Table 2
^{13}C NMR chemical shifts of compounds **2**–**5**, **8**, and **9** (C_5D_5N, δ values in ppm)[a]

Carbon no.	2	3	4	5	8	9
1	40.2	40.8	82.1	81.9	40.6	40.4
2	19.6	20.3	31.4	31.3	20.2	20.1
3	38.6	39.2	37.5	37.3	38.9	39.1
4	43.8	44.0	43.8	44.1	44.3	44.4
5	57.1	48.1	46.8	56.8	57.6	57.6
6	22.4	31.2	31.1	23.1	21.0	21.0
7	41.4	76.2	76.6	42.7	39.9	39.0
8	40.8	46.3	47.2	42.2	35.9	37.5
9	55.2	50.1	51.1	56.6	52.4	55.2
10	38.5	39.0	45.3	45.3	38.2	38.2
11	20.8	21.1	24.7	24.9	23.0	18.3
12	40.0	40.5	41.3	41.2	121.3	32.3
13	43.5	44.1	44.3	44.2	131.4	134.8
14	56.8	54.0	54.4	57.7	46.6	131.7
15	37.6	37.4	37.9	38.8	20.8	25.1
16	167.6	168.1	168.4	168.6	120.1	120.2
17	23.0	23.8	23.6	23.4	24.0	23.7
18	29.4	29.9	29.8	29.8	29.8	29.6
19	180.1	181.1	181.0	180.5	180.4	180.3
20	13.7	14.2	10.4	10.4	14.2	14.7

[a] Assignments are based on DEPT, HSQC, and HMBC.

Table 3
Data of compounds **1–10** on an NF-κB-mediated luciferase reporter gene assay[a]

Compound	Luciferase activity
1	0.32 ± 0.12
2	0.15 ± 0.07
3	0.16 ± 0.05
4	0.26 ± 0.24
5	0.13 ± 0.03*
6	0.16 ± 0.06
7	0.20 ± 0.04
8	0.18 ± 0.05
9	0.22 ± 0.06
10	0.15 ± 0.03*
Control	0.21 ± 0.04
Dex	0.13 ± 0.04*

[a] The concentration of each test compound was 10 μM. All firefly luciferase activities were normalized to *Renilla* luciferase activity. The data were expressed as multiples of luciferase activity compared to the no-treatment (control) group. Dexamethasone (Dex) is the reference compound. Each value is the average of the firefly/*Renilla* luciferase ratio and presented as the mean ± SEM ($n = 3$).
* Significantly different equals $p < 0.05$, using Student's t-test for paired samples.

as a useful means for chemists to prepare diverse new derivatives of diterpenoids.

4. Experimental

4.1. General

Melting points were determined using a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO DIP-1020 digital polarimeter. ^{1}H, ^{13}C NMR, DEPT, and 2D NMR spectra were recorded on a Bruker AVANCE DRX 500 spectrometer. Chemical shifts are reported in parts per million (ppm) with respect to the corresponding solvent as the internal standard, and coupling constants (J) are in hertz (Hz). Low- and high-resolution ESI mass spectra were recorded using a VG Platform Electrospray ESI/MS spectrometer. Column chromatography (CC) was performed with Kieselgel silica (70–230 and 230–400 mesh, Merck, Darmstadt, Germany). HPLC was performed on a Hitachi L-2130 (Tokyo, Japan) using a Betasil Silica-100 column (250 × 10 mm, 5 μm, at a flow rate of 2 mL/min) (Thermo Scientific, Waltham, MA, USA) equipped with a refractometer detector (Hitachi L-2490). X-ray single-crystal diffraction was measured on a Siemens SMART CCD XRD. Fractions were monitored by TLC (Merck 1.05554), and spots were visualized by heating Si gel plates sprayed with 10% H_2SO_4.

4.2. Substrate

Isosteviol oxime (**2**) was prepared as previously reported.[19] Signals for the proton and carbon of **2** were initially assigned by 1D and 2D NMR, and HRESIMS data.

Table 4
Data of compounds **1–10** on an AP-1-mediated luciferase reporter gene assay[a]

Compound	Luciferase activity
1	3.41 ± 0.60
2	4.87 ± 2.15
3	2.69 ± 0.45*
4	3.37 ± 0.70
5	3.52 ± 0.85
6	2.98 ± 0.35*
7	2.38 ± 0.18*
8	2.22 ± 0.35*
9	2.85 ± 0.48*
10	3.19 ± 0.72
Control	3.94 ± 0.53
Dex	2.33 ± 0.36*

[a] The concentration of each test compound was 10 μM. All firefly luciferase activities were normalized to *Renilla* luciferase activity. The data were expressed as multiples of luciferase activity compared to the no-treatment (control) group. Dexamethasone (Dex) is the reference compound. Each value is the average of the firefly/*Renilla* luciferase ratio and presented as the mean ± SEM (n = 3).
* Significantly different equals p <0.05, using Student's t-test for paired samples.

4.3. Microorganisms, incubation, and screening procedures

Twenty-one microorganisms including 10 genera (number of species): *Aspergillus* (four), *Absidia* (one), *Bacillus* (one), *Beauveria* (two), *Cunninghamella* (four), *Mortierella* (one), *Mucor* (two), *Nocardia* (two), *Pseudomonas* (two), and *Streptomyces* (two), obtained from the Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA, USA and Bioresources Collection and Research Center, Hsinchu, Taiwan, were used for the preliminary screening of **2**. The fermentation protocol for screening was identical to that described previously.[23] Culture controls and a substrate control were both run. Eight compounds were reproducibly produced by *Asp. niger* and *Abs. pseudocylindrospora*.

4.4. Microbial transformation of 2 by *Asp. niger* and *Abs. pseudocylindrospora*

Using 24-h-old stage II cultures, a solution of **2** (1.00 g dissolved in 10 mL DMF) was evenly distributed among 100 flasks containing stage II cultures. Substrate-containing cultures were incubated for 144 h. Extraction as previously described[23] produced 4.4 g of black oil and 3.6 g of brown oil after respective bioconversions by *Asp. niger* and *Abs. pseudocylindrospora*. The crude residue from *Asp. niger* (4.4 g) was subjected to silica gel CC (70–230 mesh, 5 × 90 cm). In total, six fractions were eluted with mixtures of CH_2Cl_2–MeOH (800 mL each of 20:1, 15:1, and 10:1). The elutes were monitored using TLC. Three fractions (1–3) were obtained on the basis of similar TLC profiles. Further chromatography of the fraction 3 (1.02 g) over silica gel (230–400 mesh, 3 × 50 cm) eluted with CH_2Cl_2–isopropanol (160 mL each of 12:1, 10:1, and 8:1) yielded four fractions (3-1–3-4). Compound **2** of 760 mg was recovered from fraction 3-2 (780 mg). After recrystallization of fractions 3-3 (106 mg) and 3-4 (102 mg) with CH_3OH, 98 mg of **3** and 96 mg of **6** were obtained as white crystals and a white powder, respectively. Fraction 4 (85 mg) was chromatographed over silica gel (*n*-hexane–EtOAc with increasing polarity) to give 71 mg of a white solid. The white solid was further purified by HPLC on a semi-preparative column to obtain **7** (20 mg), **8** (32 mg), **9** (10 mg), and **10** (7 mg). Fraction 5 (108 mg) was subjected to repeated semi-preparative HPLC separation (CH_2Cl_2–isopropanol, 9:1) to give **4** (80 mg) and **5** (16 mg). The crude residue from *Abs. pseudocylindrospora* (3.6 g) was purified by CC over silica gel using mixtures of *n*-hexane, EtOAc, CH_2Cl_2, and MeOH with increasing polarity to obtain four fractions (1–4). Fraction 3 (610 mg) was chromatographed over silica gel (CH_2Cl_2–MeOH with increasing polarity) to give 452 mg of a white solid. The white solid was further purified by HPLC on a semi-preparative column to give **6** (210 mg) and **3** (180 mg). Fraction 4 (35 mg) was subjected to repeated semi-preparative HPLC separation (CH_2Cl_2–isopropanol, 9:1) to give **4** (17 mg). Fraction 2 (810 mg) was applied to a silica gel column (230–400 mesh, 2 × 55 cm) eluted with *n*-hexane–EtOAc (2:1) to give two fractions (2-1 and 2-2). Fraction 2-2 (760 mg) was recrystallized with EtOAc and MeOH, and 710 mg of **2** was obtained as white crystals.

4.4.1. Compound 3

White crystals, mp 214–216 °C; $[\alpha]_D^{25}$ –48.7 (c 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS m/z calcd for $C_{20}H_{32}NO_4$ $[M+H]^+$ 350.2331, found 350.2309.

4.4.1.1. X-ray crystallographic data of 3. $C_{20}H_{31}NO_4$, M 349.46, monoclinic, $P2_1$, a 8.64800 (10) Å, b 8.89210 (10) Å, c 11.69010 (10) Å, V 898.030 (16) Å^3; Z 2, D_{calcd} 1.292 g cm^{-3}, F(0 0 0) 380, λ (Mo Kα) 0.71073 Å, T 295(2) K, 7082 reflection collected. Final GooF 1.093, final R indices R_1 0.0312, wR_2 0.0864, 238 parameters, μ 0.089 mm^{-1}, R indices based on 3957 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **3** have been deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 722590.[24]

4.4.2. Compound 4

White crystals, mp 217–219 °C; $[\alpha]_D^{25}$ +30.8 (c 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS m/z $C_{20}H_{32}NO_5$ $[M+H]^+$ calcd for 366.2280, found 366.2313.

4.4.2.1. X-ray crystallographic data of 4. $C_{20}H_{31}NO_5$, M 365.46, monoclinic, $P2_1$, a 7.053 Å, b 18.44180 (10) Å, c 7.649 Å, V 956.783 (5) Å^3; Z 2, D_{calcd} 1.269 g cm^{-3}, F(0 0 0) 396, λ (Mo Kα) 1.54178 Å, T 295(2) K, 31,108 reflection collected. Final GooF 1.061, final R indices R_1 0.0299, wR_2 0.0832, 252 parameters, μ 0.734 mm^{-1}, R indices based on 3336 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **4** have been deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 722592.[24]

4.4.3. Compound 5

White crystals, mp 161–163 °C; $[\alpha]_D^{25}$ –48.2 (c 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS m/z $C_{20}H_{32}NO_4$ $[M+H]^+$ calcd for 350.2331, found 350.2366.

4.4.3.1. X-ray crystallographic data of 5. $C_{20}H_{32}NO_{4.50}$, M 358.47, orthorhombic, $P2_12_12_1$, a 11.86090 (10) Å, b 14.08840 (10) Å, c 23.35940 (10) Å, V 3903.38 (5) Å^3; Z 8, D_{calcd} 1.220 g cm^{-3}, F(0 0 0) 1560, λ (Mo Kα) 1.54178 Å, T 295(2) K, 68,493 reflection collected. Final GooF 1.029, final R indices R_1 0.0346, wR_2 0.0975, 493 parameters, μ 0.689 mm^{-1}, R indices based on 7109 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **5** have been deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 722591.[24]

4.4.4. Compound 8

White powder; $[\alpha]_D^{25}$ –76.5 (c 0.8, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS m/z $C_{20}H_{28}NO_2$ $[M-H]^-$ calcd for 314.2120, found 314.2112.

4.4.5. Compound 9

White powder; $[\alpha]_D^{25}$ +40.0 (c 0.8, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS m/z $C_{20}H_{28}NO_2$ $[M-H]^-$ calcd for 314.2120, found 314.2118.

4.5. Transfection procedures and reporter gene assays

Twenty-four hours before transfection, about 1×10^5 mouse RAW 264.7 macrophage cells per well were seeded in 96-well white plates. The reporter plasmid, pAP-1-Luc or pNF-κB-Luc plasmid together with an internal control plasmid, pGL-*hRluc*, were transfected into RAW 264.7 cells using lipofectamine plus (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. At 48 h post-transfection, lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4) (Sigma, St. Louis, MO, USA) at a final concentration of 100 ng/mL was added to the transfected cells for 6 h. After LPS stimulation, a final concentration of 10 μM of each test compound including the reference compound, dexamethasone (Sigma), in DMSO was added to the cells. Cells were harvested 24 h after treatment, and the reporter activities of firefly luciferase expressed from pAP-1-Luc or pNF-κB-Luc, and *Renilla* luciferase from pGL-*hRluc* were assayed in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA) using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

4.5.1. Statistical analysis

Data are from at least three individual experiments. Average firefly/*Renilla* luciferase ratios were analyzed by two-tailed Student's *t*-test for paired samples. Significance was accepted at $p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.07.029.

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24. CCDC 722590, 722591, and 722592 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033; e-mail: data_request@ccdc.cam.ac.uk).