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Phytochemistry 67 (2006) 62–69

PHYTOCHEMISTRY

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Ent-kaurane glycosides and sesquiterpene lactones of the hirsutinolide type from *Vernonia triflosculosa* ☆

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Received 7 July 2005; accepted 11 July 2005
Available online 29 August 2005

Dedicated in memory to Prof. Dr. M. Luckner.

Abstract

Investigation of the aerial parts of *Vernonia triflosculosa* afforded three hirsutinolides of which 8α-(4α-hydroxymethacryloyloxy)-10α-hydroxy-1,13-dimethoxy-hirsutinolide is new, three *ent*-kaurane diterpenes, among which the 19-[α-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranosyl] esters of 16β-hydroxy-*ent*-kauran-19-oic acid and of 16β,17-hydroxy-*ent*-kauran-19-oic acid are also new. Diterpenes are reported here for the first time in the large genus *Vernonia*. Their structures were elucidated using 1D and 2D NMR measurement as well as ESI, CIMS, and HRMS analysis. Two hirsutinolides were studied for their NF-κB DNA binding activity in HaCaT cells (a human cell line similar to keratinocytes) and for their inhibition on IL-8 production in HeLa cells.

Keywords: *Vernonia triflosculosa*; Asteraceae; Sesquiterpene lactones; Hirsutinolides; *ent*-Kaurane glycosides; Anti-inflammatory activity; NF-κB; IL-8

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1. Introduction

Several species of the large genus *Vernonia* (Asteraceae, tribe Vernonieae) are used in traditional medicine for the treatment of various ailments (Johri and Singh, 1997). In continuation of our search for natural compounds with antiinflammatory activity, we have investigated the aerial parts of *Vernonia triflosculosa* Kunth (syn. *Critoniopsis triflosculosa* (Kunth) H. Rob.) (Robinson, 1999; Anonymous, 2005). This understory tree can have a shrubby architecture (Enquist and Sullivan, 2001) and grows in Mexico, Guatemala, El Salvador, Honduras, Nicaragua, Costa Rica and Panama. Up to now, its roots have been investigated and a sesquiterpene lactone (SL) of the guaianolide type has been found (Bohlmann and Zdero, 1977). Additionally, a glaucolide has been reported from its aerial parts (Bohlmann and Zdero, 1988). This communication reports the isolation of one new and two known hirsutinolides, two new *ent*-kaurane glycosides, one known kaurane acid and two flavonoids. The hirsutinolides have been studied for their anti-inflammatory activity using the transcription factor NF-κB and IL-8 as molecular targets.

☆ Part VII in the series Phytochemical and Biological Studies of Costa Rican Asteraceae.

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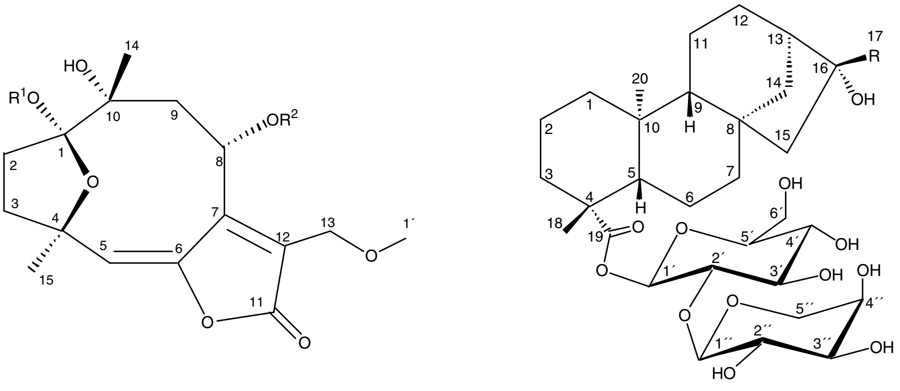
doi:10.1016/j.phytochem.2005.07.006



1 R_1=CH_3; R_2=4-OH-methacryloyl

2 R_1=H; R_2=4-OH-methacryloyl

3 R_1=H; R_2= methacryloyl

4 R=CH_3

5 R=CH_2OH

Fig. 1. Structures of the isolated compounds.

2. Results and discussion

The lipophilic extract of the aerial parts of *V. triflosculosa* was separated using column chromatography (CC) in addition to low-pressure chromatography. Bioguided fractionation using the agar plate diffusion assay with *Bacillus subtilis* led to isolation of the SLs **1**–**3** (structures see Fig. 1). These SLs exhibited antibacterial activity at a concentration of 100 µg per disk with a zone of inhibition of 19 mm for SL **1**, 17 for SL **2** and 20 for SL **3** (including the diameter of the bore 7 mm). Bactrim was used as a control (35 mm at a concentration of 25 µg per disk). Structure elucidation was based on mass (EI, CI) and 1D and 2D NMR measurements and led to identification of the known hirsutinolides 8α-(4-hydroxymethacryloyloxy)-10α-hydroxy-13-methoxy-hirsutinolide (**2**) (Ahmed et al., 1991) and 8α-(methacryloyloxy)-10α-hydroxy-13-methoxy-hirsutinolide (**3**) (Budesinsky et al., 1994). Although compound **2** has been mentioned in the literature (Ahmed et al., 1991), no NMR data exist. Therefore, complete ^1H and ^{13}C NMR data are given (see Tables 1 and 2). In addition, ^{13}C NMR data of SL **3** are reported for the first time (see Table 2).

The molecular formula of compound **1**, $C_{21}H_{28}O_9$, followed from its HREIMS which showed an $[M]^+$ at *m*/*z* 424.174727. Consequently, the ESIMS exhibited pseudomolecular ions at *m*/*z* 463 $[M+K]^+$ and 447 $[M+Na]^+$. ^1H and ^{13}C NMR spectra taken in $CDCl_3$ agreed well with those from SL **2** indicating the occurrence of a SL of the hirsutinolide type with an 4α-hydroxymethacryloyloxy group at C-8 (see Tables 1 and 2). However, an additional signal, attributed to a further methoxy group, was observed at δ_C 50.9/δ_H 3.49. Its position remained unclear because these signals did not have correlations with other signals in the gHMQCR spectrum. Moreover, compound **1** was degraded in the slightly acidic $CDCl_3$ solution. NMR measurements in C_6D_6 solved these problems. The signals of both methoxy groups were better separated in the ^1H NMR spectrum. The correlation between one methoxy group at δ_H 3.45 and C-1 (δ_C 111.9), and the other at δ_H 3.01 and C-13 (δ_C 59.1), in the gHMQCR spectrum unambiguously confirmed the positions of the methoxy group at C-1 and C-13. Assignment of the stereochemistry was confirmed by a NOESY experiment (see Fig. 2). Thus, compound **1** is 8α-(4α-hydroxymethacryloyloxy)-10α-hydroxy-1,13-dimethoxy-hirsutinolide, which is new.

It has been proposed that hirsutinolides are formed from glaucolides by silicagel catalyzed rearrangement in methanolic or ethanolic solution (Martinez-Vazquez et al., 1992). However, in *V. triflosculosa* the isolated hirsutinolides are probably native, because they were already detected by TLC before the fractions containing them were exposed to silicagel and methanol. Furthermore, no silicagel was used in the isolation of compound **2**. There is also another example in the literature in which the isolation of hirsutinolides is reported in the absence of MeOH or EtOH (Budesinsky et al., 1994).

Compound **4** showed an $[M-H]^+$ peak at *m*/*z* 613 as well as a signal at *m*/*z* 1227, which is formed by dimerization, in the ESI MS (negative mode). The spectrum of the positive ions exhibited signals from the sodium adduct at *m*/*z* 637 and after dimerization at *m*/*z* 1251 $[(2\times M)+Na]^+$. Accordingly, the molecular formula $C_{31}H_{50}O_{12}$ was deduced from the HRESIMS. ^1H and ^{13}C NMR spectral data were in close agreement with those reported for 16β-hydroxy-*ent*-kauran-19-oic acid except for the chemical shift of the carboxyl group which was shifted upfield due to substitution, such as

Table 1
^{1}H NMR data for SLs **1** ($CDCl_3$ and C_6D_6) and **2** in $CDCl_3$ (300 MHz)

Position	**1**		**2**
	^{1}H ($CDCl_3$) δ/ppm (H; mult; J/Hz)	^{1}H (C_6D_6) δ/ppm (H; mult; J/Hz)	^{1}H ($CDCl_3$) δ/ppm (H; mult; J/Hz)
2α	1.87–2.005 (2H; *m*)	1.23 (1H; *m*)	1.80–2.00 (2H; *m*)
2β		1.45 (1H; *m*)	
3α	2.44 (1H; *ddd*; 6.9, 12.6, 12.6)	1.77 (1H; *m*)	2.44 (1H; *ddd*; 6.9, 12.6, 12.6)
3β	2.14 (1H; *m*)	1.29 (1H; *m*)	2.13 (1H; *m*)
5	5.86 (1H; *s*)	5.36 (1H; *s*)	5.85 (1H; *s*)
8	6.68 (1H; *br d*; 10.8)	6.64 (1H; *br d*; 10.3)	6.67 (1H; *br d*; 10.8)
9α	2.63 (1H; *dd*; 10.8, 16.0)	2.18 (1H; *dd*; 10.3, 15.9)	2.63 (1H; *dd*; 10.8, 16.0)
9β	2.14 (1H; *br d*; 16.0)	1.75 (1H; *br d*; 15.9)	2.13 (1H; *br d*; 16.0)
13a	4.29 (1H; *d*; 12.2)	4.25 (1H; *d*; 12.1)	4.28 (1H; *d*; 12.3)
13b	4.52 (1H; *d*; 12.2)	4.45 (1H; *d*; 12.1)	4.52 (1H; *d*; 12.3)
14	1.24 (3H; *s*)	1.02 (3H; *s*)	1.24 (3H; *s*)
15	1.62 (3H; *s*)	1.20 (3H; *s*)	1.61 (3H; *s*)
3′a	5.82 (1H; *br s*)	5.27 (1H; *br s*)	5.81 (1H; *br s*)
3′b	6.38 (1H; *br s*)	6.25 (1H; *br s*)	6.37 (1H; *br s*)
4′a	4.24 (1H; *d*; 13.0)	4.01 (1H; *d*; 13.2)	4.23 (1H; *d*; 12.9)
4′b	4.39 (1H; *d*; 13.0)	4.19 (1H; *d*; 13.2)	4.39 (1H; *d*; 12.9)
1″	3.42 (3H; *s*)	3.01 (3H; *s*)	3.42 (3H; *s*)
1‴	3.49 (3H; *s*)	3.45 (3H; *s*)	

Table 2
^{13}C NMR data and gHMQCR correlations for SLs **1**–**3** at 75 MHz in $CDCl_3$^{a} or C_6D_6^{b}

C	**1**				**2**		**3**
	δ_C^{a}	gHMQCR^{a} (^{13}C–^{1}H)	δ_C^{b}	gHMQCR^{b} (^{13}C–^{1}H)	δ_C^{a}	gHMQCR^{a} (^{13}C–^{1}H)	δ_C^{a}
1	108.9	9α, 14	111.9	9α, 14, 1‴	108.9	9α, 14	108.6
2	32.1	3α	33.9	3α	32.1	3α	31.8
3	37.9	15	38.3	15	37.9	15	38.1
4	82.5	3α, 5, 15	83.9	2β, 5, 15	82.5	3α, 5, 15	82.1
5	125.4	15	124.4	3α, 15	125.4	3β, 15	125.7
6	144.1	5	143.9	5	144.1	5	144.1
7	150.4	5, 9α, 9β, 13a, 13b	150.8	5, 9α, 13a, 13b	150.4	5, 9α, 9β, 13a, 13b	150.6
8	65.5	9α	65.5	9α	65.5	9α	66.2
9	38.1	14	40.5	14	38.1	14	38.1
10	77.6	9α, 14	79.4	9α, 14	77.6	9α, 14	78.1
11	133.1	13a, 13b	129.3	13a, 13b	133.1	13a, 13b	133.2
12	167.3	13a, 13b	167.6	13a, 13b	167.3	13a, 13b	167.5
13	63.6	1″	64.3	1″	63.6	1″	63.6
14	26.3	9β	27.1	9α	26.3		25.5
15	29.0	3α	28.1	3α	29.0	3α	29.0
1′	165.0	3′a, 3′b, 4′a, 4′b	165.6	3′a, 3′b, 4′a, 4′b	165.0	3′a, 3′b, 4′a, 4′b	165.8
2′	139.0	3′b, 4′a, 4′b	140.8	3′b, 4′a, 4′b	139.0	3′b, 4′a, 4′b	135.9
3′	129.4	4′a, 4′b	127.7	4′a, 4′b	129.4	4′a, 4′b	127.1
4′	62.5	3′a, 3′b	62.8	3′a, 3′b	62.5	3′a, 3′b	18.1
1″	59.0	13a, 13b	59.1	13a, 13b	59.0	13a, 13b	59.0
1‴	50.9		52.4				

esterification by a sugar moiety (Hui et al., 1990; Martin et al., 1997). Nomenclature is done according to the recommendations of the IUPAC rules F-6.4 (Moss, 1989). Signals of anomeric protons at δ 5.17 (7.3 Hz) and at δ 6.35 (J 7.5 Hz) typical of an ester-linked β-hexose suggested that two different types of glycosidic linkage were present (Harinantenaina et al., 2002a). ^{1}H and ^{13}C NMR data revealed the occurrence of β-glucopyranose and α-arabinopyranose as sugar residues (Harinantenaina et al., 2002b). β-Glucose was directly bound to the carboxyl group at C-19 proven by the correlation of C-19 (δ 176.3) with the anomeric proton (H-1′ δ 6.35) in the gHMQCR spectrum. Moreover, long-range correlations were shown between C-2 of glucose (δ 83.4) and the anomeric proton of α-arabinose (δ 5.17), by which the attachment of the terminal arabinopyranosyl unit was assigned to the hydroxyl group at C-2. Assignment of all sugar signals followed from gHSQCR, gHMQCR and 1D NOE experiments (see Table 3). From the above data, the structure of **4** was unambigu-

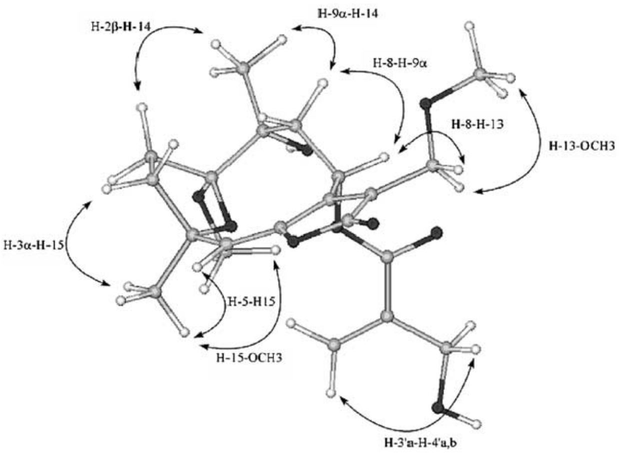


Fig. 2. NOE correlations of SL **1**.

ously concluded to be 16β-hydroxy-*ent*-kauran-19-oic 19-[α-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranosyl] ester (Fig. 1) which is described here for the first time.

Compound **5** was assigned the molecular formulae $C_{31}H_{50}O_{13}$ followed from its HRESIMS and also confirmed by its ESIMS taken in the positive and negative mode. ^1H and ^{13}C NMR data were similar to those of diterpene **4** differing only in the substitution at C-16 (see Table 3). The signals for the methyl group at δ_H 1.58/δ_C 25.6 were missing and replaced by methylene proton resonances at δ_H 4.06 and 4.15/δ_C 67.0 indicating a C-17-CH_2OH group. Consequently, chemical shifts for the adjacent C-13 and C-15 were shifted upfield and those of the respective protons H-13 and H-15a downfield. Furthermore, the signal assignable to C-16 showed a downfield shift. Hence, **5** was the new 16β,17-dihydroxy-*ent*-kauran-19-oic 19-[α-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranosyl] ester (Fig. 1).

Compound **7** was identified as the aglycone, 16β,17-dihydroxy-*ent*-kauran-19-oic acid by comparison of its MS as well as its ^1H and ^{13}C NMR data (Duan et al., 1999; Hui et al., 1990).

The isolation of these *ent*-kaurane diterpenes serve special interest. Whereas hirsutinolides are common constituents within the subtribe Vernoniinae, diterpenes have been rarely found in the whole tribe Vernonieae. Actually, there are three reports on diterpenes in the literature (Zdero and Bohlmann, 1988; Borkosky et al., 1995; Krishna et al., 1998). Further phytochemical studies will show whether diterpenes occur more often within the large tribe Vernonieae (consisting of about 1500 species).

SLs are not only interesting taxonomical markers, but have also received considerable attention due to their antiinflammatory activity (Hall et al., 1979). We and others have previously shown that the antiinflammatory effect can be partly explained by the inhibition of the transcription factor NF-κB, probably by alkylating p65 at cysteine 38 (Garcia-Pineres et al., 2001). NF-κB is a central mediator of the human immune response and regulates the transcription of genes encoding various inflammatory and proinflammatory cytokines, adhesion molecules and inflammatory enzymes like iNOS, COX-2 or 5-LOX (Barnes and Karin, 1997). A quantitative structure–activity relationship study recently undertaken has revealed that a strong NF-κB inhibitory activity of SLs mostly correlates with the occurrence of an α-methylene-γ-butyrolactone moiety (Siedle et al., 2004). For this reason, it was interesting to investigate whether the isolated hirsutinolides possessing an endocylic α,β-unsaturated butyrolactone show a similar strong inhibitory effect on DNA binding of the transcription factor NF-κB. Whereas in the past Jurkat T cells, RAW 264.7 or 293 epithelial cells were used, this time the effect of SLs on HaCaT cells (a transformed human epithelial cell line from adult skin and similar to normal keratinocytes) was studied (Boukamp et al., 1988).

For this study, HaCaT cells were incubated with SL **2** or **3** at various concentrations for one hour and subsequently stimulated with TNF-α for a further one hour. Total protein extracts were prepared and analysed for NF-κB DNA binding activity in an electrophoretic mobility shift assay (EMSA) (see Fig. 3). Stimulation with TNF-α induced one novel DNA binding activity in HaCaT cells (Fig. 3, lane 2). Antibody reactivity and competition assays identified this complex as an NF-κB p50/p65 heterodimer (data not shown, see also Pahl and Baeuerle, 1995). Both SLs completely prevented NF-κB activation at concentrations of 20 μM (SL **2**) or 10 μM (SL **3**) (see Fig. 3, lane 8 or 7). The SL parthenolide was used as a positive control and inhibited NF-κB DNA binding at a concentration of 20 μM (data not shown). SL **2** was also studied in Jurkat T cells under the same conditions (data not shown). Once more it was necessary to use a 20 μM concentration for a complete inhibition of NF-κB DNA binding. These results show that both SLs which do not contain an exocyclic methylene group conjugated to the lactone carbonyl, possess a strong inhibitory activity. This may probably be due to the extended dienone system in their molecules. Altogether, this study confirms again that an α,β-unsaturated acyl residue can also be involved in the NF-κB inhibitory effect as previously shown for the heliangolides possessing a similar structure (Siedle et al., 2004). Interestingly, the low concentrations (0.1 μM) of SL **3** even induced a slight upregulation of NF-κB, whereas higher concentrations reversed this effect. This behaviour has not been reported up to now, because very low concentrations of SLs without TNF-α-treatment have not yet been studied.

NF-κB regulates the transcription of various cytokines, e.g. that of IL-8. This chemokine participates in recruitment of leukocytes from the blood to the injured tissue and in their activation, thus contributing to inflammatory processes (Wuyts et al., 1998). In order

Table 3
^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectroscopic data for diterpene **4** in pyridine and CD$_3$OD and for **5** in pyridine

Position	**4**				**5**		
	^{13}C δ/ppm	^{13}C δ/ppm	^{1}H δ/ppm (H; mult; J/Hz)	gHMQCR (^{13}C–^{1}H)	^{13}C δ/ppm	^{1}H δ/ppm (H; mult; J/Hz)	gHMQCR (^{13}C–^{1}H)
1a	41.8	41.3	0.82 (1H; *ddd*; 3.2, 12.8, 13.6)	20	41.2	0.81 (1H; *ddd*; 3.2, 13.0, 13.0)	20
1b			1.78 (1H; *m*)			1.74 (1H; *m*)	
2a	20.5	20.6	1.50 (1H; *m*)		20.5	1.47 (1H; *m*)	
2b			2.20 (1H; *m*)			2.18 (1H; *m*)	
3a	38.4	38.1	1.10 (1H; *m*)		38.2	1.10 (1H; *m*)	18
3b			2.71 (1H; *br d*; 13.3)			2.71 (1H; *m*)	
4	45.3	44.9		2a, 5, 18	44.9		2a, 5, 18
5	58.6	58.3	1.10 (1H; *)	18, 20	58.2	1.10 (1H; *m*)	18, 20
6a	22.9	22.9	2.01 (1H; *m*)		22.9	2.00 (1H; *m*)	
6b			2.22 (1H; *m*)			2.20 (1H; *m*)	
7a	43.3	43.2	1.44 (1H; *m*)	15b	43.3	1.43 (1H; *m*)	15b
7b			1.78 (1H; *m*)			1.76 (1H; *m*)	
8	46.4	46.0		9, 15a, 15b	45.4		9, 14a,b, 15a,b
9	57.4	56.7	1.01 (1H; *br d*; 6.9)	15a, 15b, 20	56.7	1.03 (1H; *br d*; 8.5)	15a,b, 20
10	40.9	40.5		5, 20	40.5		9, 20
11a	19.3	19.2	1.45–1.60 (2H; *m*)		19.5	1.48 (1H; *m*)	
11b						1.63 (1H; *m*)	
12a	27.8	27.7	1.40–1.60 (2H; *m*)	9	27.1	1.43 (1H; *m*)	9, 14a,b
12b						1.82 (1H; *m*)	
13	49.6	49.7	2.12 (1H; *m*)	17	46.4	2.43 (1H; *m*)	
14a,b	38.5	38.5	2.01 (2H; *m*)	9, 15a	38.1	2.05 (2H; *m*)	9, 15a
15a	58.9	59.2	1.67 (1H; *d*; 13.7)	17	54.5	1.73 (1H; *d*; 13.5)	14a,b
15b			1.95 (1H; *d*; 13.7)			1.83 (1H; *d*; 13.5)	
16	78.2	78.4		15b, 17	82.2		14a,b 15b,
17a	24.4	25.6	1.58 (3H; *s*)	15a	67.0	4.06 (1H; *d*; 10.5)	15a
17b						4.15 (1H; *d*; 10.5)	
18	29.7	29.7	1.46 (3H; *s*)		29.7	1.45 (3H; *s*)	
19	177.4	176.3		5, 18, 1′	176.3		5, 18, 1′
20	17.1	17.2	1.13 (3H; *s*)	5, 9	17.2	1.13 (3H; *s*)	5, 9
1′	93.9	93.8	6.35 (1H; *d*; 7.5)	2′	93.8	6.35 (1H; *d*; 7.6)	2′
2′	82.7	83.4	4.28 (1H; *m*)	3′, 1″	83.3	4.28 (1H; *dd*; 7.6, 6.5)	1″
3′	78.6	78.9	4.30 (1H; *m*)	2′, 4′	78.9	4.30 (1H; *dd*; 6.5, 6.5)	2′
4′	70.9	71.3	4.34 (1H; *m*)		71.3	4.35 (1H; *m*)	2′, 3′
5′	79.9	79.6	3.96 (1H; *m*)		79.6	3.96 (1H; *m*)	
6′ a,b	62.5	62.8	4.35–4.45 (2H; *m*)		62.8	4.35-4.45 (2H; *m*)	
1″	106.7	107.6	5.17 (1H; *d*; 7.3)	2′, 2″ , 5″a, 5″b	107.6	5.18 (1H; *d*; 7.3)	2′, 2″ , 5″a,b
2″	73.5	74.2	4.50 (1H; *dd*; 7.3, 9.0)	3″	74.2	4.50 (1H; *dd*; 7.3, 7.0)	
3″	74.3	75.1	4.16 (1H; *dd*; 3.5, 9.0)	1″, 2″ , 5″ b	75.1	4.18 (1H; *dd*; 3.5, 7.0)	2″, 5″b
4″	69.8	70.1	4.29 (1H; *m*)	5″ a, 5″ b	70.1	4.29 (1H; *m*)	5″ a,b
5″a	67.6	68.1	3.80 (1H; *dd*; 1.0, 12.0)	1″	68.1	3.80 (1H; *dd*; 1.0, 12.0)	
5″b			4.32 (1H; *br d*; 12.0)			4.32 (1H; *br d*; 12.0)	

* Multiplicity not determined (signal overlap).

to evaluate whether a correlation exists between the NF-κB inhibitory activity and the influence on IL-8 release, SLs **2** and **3** were studied in our IL-8 ELISA using HeLa 229 cells (for conditions, see Section 3). Parthenolide was chosen as a positive control as this SL was already proven to inhibit TNF-α-mediated IL-8 gene expression in cultured human respiratory epithelium (Mazor et al., 2000). 0.5 μM and higher concentrations of SLs **2** and **3** suppressed IL-8 production in a concentration dependent manner (see Fig. 4). In agreement with the results from the NF-κB EMSA SL **3** (IC$_{50}$ 1.62 μM) again proved to be nearly twice as active as SL **2** (4.55 μM). SLs are also known for their cytotoxic activity (Schmidt, 1999). To exclude that the results in the IL-8 ELISA are due to cytotoxic effects, the MTT assay was carried out under the same conditions. All concentrations used in the assays were not cytotoxic to the cells.

3. Experimental

3.1. General experimental procedures

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded in CDCl$_3$, CD$_3$OD, C$_6$D$_6$ and pyridine-d_5 using a Unity 300 (Fa. Varian) instrument. Chemical shift references were obtained by addition of TMS. MS data were taken with the following instru-

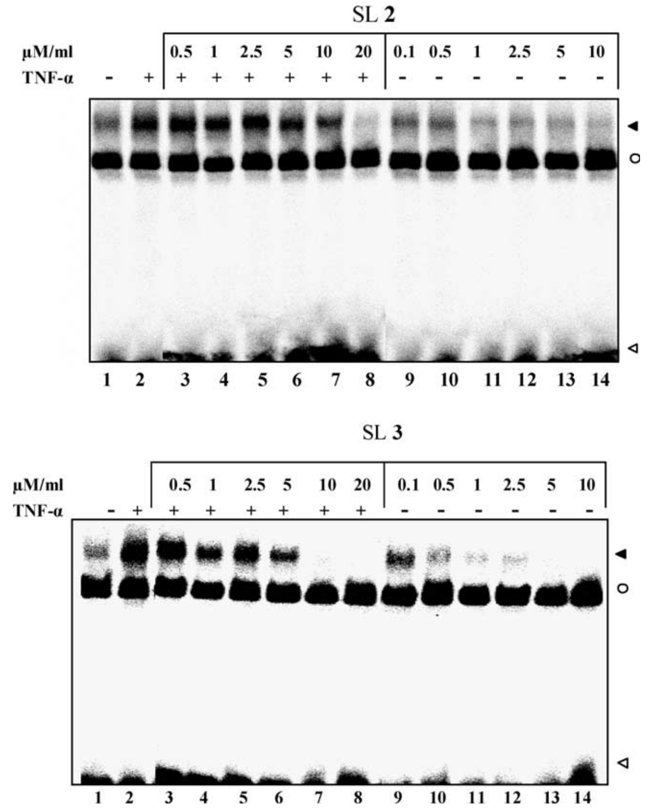


Fig. 3. The effect of SLs **2** and **3** on NF-κB DNA binding in HaCaT cells. Lane 1 shows unstimulated control cells, in lane 2 cells were treated with 1000 U/ml TNF-α alone. In lanes 3–8, cells were pretreated for one hour with various concentrations of SLs **2** or **3** and subsequently stimulated with TNF-α for one hour, in lanes 9–14 cells were only treated with the SLs for 2 h. A filled arrowhead indicates the position of NF-κB DNA complexes. The open circle denotes a non-specific activity binding to the probe. The open arrowhead shows unbound oligonucleotide. All experiments were carried out in duplicate.

ments: EIMS: MAT 8200 (Finnigan) or TSQ 7000 (Finnigan, 70 eV); CIMS: TSQ 7000 (Finnigan); ESIMS: TSQ 7000 (Finnigan, 70 eV); HREIMS: MAT 95 Sl (Finnigan); HRESIMS: LTQ-FT (Finnigan). Medium pressure liquid chromatography (MPLC) was carried out with Eurosil Bioselect® 100-30 C-18 (100 Å, 20–45 μm, irregular) (Fa. Knauer), or LiChrospher Si 60 (12 μm) (Fa. Merck), column fractions were monitored by TLC (Si gel 60 F_{254}) (Fa. Merck). Analytical TLC was carried out with an Automatic TLC Sampler (CAMAG). UV spectra were taken with a Uvikon 933 UV/Vis Spectrometer, Kontron Instruments in CH_3OH Uvasol® (Fa. Merck). Optical rotation was measured with a Perkin-Elmer 241 polarimeter at 27 °C.

3.2. Plant material

Aerial parts of *V. triflosculosa* were collected near Ciudad Quesada, Costa Rica, in February 2003 in an official collaboration between the university of Costa Rica and the university of Freiburg. Identification was done by L. Poveda, Professor of Botany, Universidad Nacional, Costa Rica. Voucher specimens, No. 85908, are deposited at the herbarium of the University of Costa Rica.

3.3. Extraction and isolation

Dried, powdered aerial parts of *V. triflosculosa* (810 g) were exhaustively extracted with methyl *tert*-butyl ether (MTBE)-methanol (8:2) at room temperature. The crude extract was treated with MeOH at −20 °C. After filtering and evaporation of the solvent, a viscous residue (25.3 g) was obtained. The MeOH soluble part was separated in two portions by CC on Sephadex LH-20 with MeOH as solvent. Twenty seven fractions were obtained and studied for their antibacterial activity in the agar plate diffusion test using *B. subtilis*. The most active fractions (7 (3.26 g), 8 (1.69 g) and 9 (2.16 g)) were combined and separated by CC on Sephadex LH 20 with cyclohexane–CH_2Cl_2–MeOH (7:4:1) yielding 13 subfractions which were again tested for their antibacterial activity against *B. subtilis*. Subfraction 2 (1318 mg) was submitted to MPLC on RP-18 with MeOH–H_2O

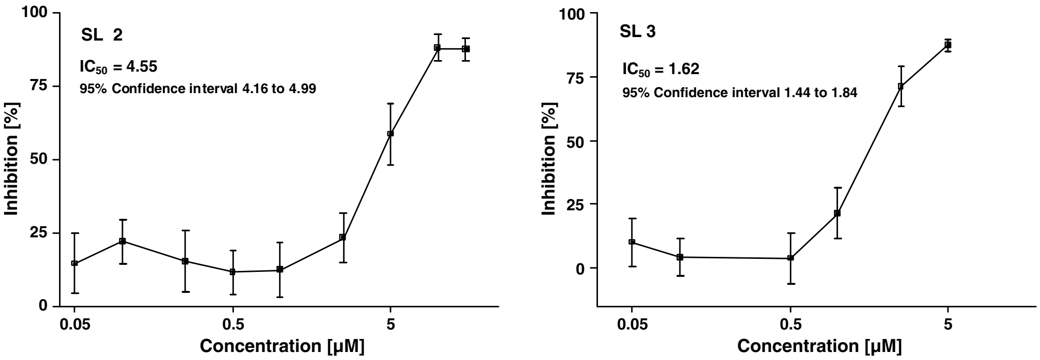


Fig. 4. ELISA results as dose–response curves demonstrating the effect of SLs **2** and **3** on TNF-α-mediated production of IL-8 in HeLa 229 cells. HeLa 229 cells were pretreated with indicated concentrations of the SL for 1 h before being stimulated with TNF-α (6 ng/ml) for 6 h. IL-8 production was analyzed as described in Section 3. Each point shows the mean ± SD of at least three independent experiments carried out in triplicate.

mixtures of increasing polarities (50–100%, $\Delta = 10\%$). Subsequently subfraction 2.3 (160 mg) was chromatographed by MPLC on silica gel with *n*-hexane–CH_2Cl_2–MeOH (6:3.5:0.5) yielding **1** (15.7 mg). Compound **3** (29.7 mg) was isolated from subfraction 2.4 (92 mg) after MPLC on RP-8 with MeOH–H_2O (50–100%). Subfraction 3 (547 mg) was used for the isolation of **2** (29.1 mg) by MPLC on RP-18 with MeOH–H_2O (40–80%). Subfractions 5, 6 and 7 were combined (total 369 mg) and chromatographed by MPLC on RP-18 with MeOH–H_2O (50–100%) affording **4** (10.6 mg) and **5** (3.9 mg).

16β-Hydroxy-*ent*-kauran-19-oic acid (**6**, 1.8 mg) was obtained from subfraction 8 (263 mg) after MPLC separation on RP-18 with MeOH–H_2O (30–100%). The flavonols **7** (quercetin-3,3′-dimethylether) (10.4 mg) and **8** (quercetin-3-methylether) (16.4 mg) were isolated from fractions 20 (55 mg) and 25 (44 mg) by CC on Sephadex LH 20 with MeOH.

3.3.1. 8α-(4-Hydroxymethacryloyloxy)-10α-hydroxy-1,13-dimethoxy-hirsutinolide (1)

Colorless crystals; m.p. 69–72 °C; $[\alpha]_D^{20}$ +37° (*c*. 0.36; MeOH); for ^1H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* (rel. int.): 849 $[(2 \times M) + H]^+$ (7), 463 $[M + K]^+$ (7), 447 $[M + Na]^+$ (100), 291 (20); HREIMS *m*/*z* 424.1747 $[M]^+$ (Calc. for $C_{21}H_{28}O_9$, 424.1733).

3.3.2. 8α-(4-Hydroxymethacryloyloxy)-10α-hydroxy-13-methoxy-hirsutinolide (2)

Colorless crystals; m.p. 84–87 °C; for ^1H and ^{13}C NMR, see Tables 1 and 2; EIMS *m*/*z* (rel. int.): 410 $[M]^+$ (4), 378 $[M - MeOH]^+$ (2), 308 $[M - C_4H_6O_3]^+$ (4), 276 $[M - C_4H_6O_3 - MeOH]^+$ (24), 234 (53), 216 (41), 205 (11), 191 (21), 188 (29), 174 (20), 163 (25), 148 (55), 99 (18), 85 $[C_4H_5O_2]^+$ (48), 69 $[C_4H_5O]^+$ (16), 57 (17), 43 (100); CIMS (isobutane) *m*/*z* (rel. int.): 411 $[M + H]^+$ (1), 393 $[M - H_2O]^+$ (3), 327 (17), 309 $[M - C_4H_6O_3]^+$ (100), 277 (14), 103 $[C_4H_7O_3]^+$ (21), 85 $[C_4H_5O_2]^+$ (9), 69 $[C_4H_5O]^+$ (3).

3.3.3. 8α-Methacryloyloxy-10α-hydroxy-13-methoxy-hirsutinolide (3)

Colorless crystals; m.p. 74–77 °C; ^{13}C NMR, see Table 2; ESIMS *m*/*z* (rel. int.): 827 $[2 \times M + K]^+$ (15), 789 $[2 \times M + H]^+$ (10), 445 $[M + MeOH + H_2O + H]^+$ (100), 431 $[M + K]^+$ (18), 417 $[M + Na]^+$ (50), 394 $[M]^+$ (8).

3.3.4. 16β-Hydroxy-ent-kauran-19-oic acid-19-[a-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranosyl]-ester (4)

Colorless crystals; m.p. 154–155 °C; $[\alpha]_D$ −28.9 (MeOH; *c*. 0.42); ^1H, ^{13}C NMR see Table 3; ESIMS *m*/*z* (neg. ions) 1227 $[(2 \times M) - H]^+$ (16), 649 (63), 613 $[M - H]^+$ (100), 534 (9), 492 (12), 450 (20), 361 (24); ESIMS *m*/*z* (pos. ions): 1251 $[(2 \times M) + Na]^+$ (25), 773 (15), 637 $[M + Na]^+$ (100); HRESIMS *m*/*z* 637.31945 $[M + Na]^+$ (Calc. for $C_{31}H_{50}O_{12} + Na$ 637.3199).

3.3.5. 16β,17-Dihydroxy-ent-kauran-19-oic acid 19-[a-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranosyl] ester (5)

Colorless crystals; 161–163 °C; $[\alpha]_D$ −23.0 (MeOH; *c*. 0.15); ^1H, ^{13}C NMR see Table 3; ESIMS (neg. ions) *m*/*z* 1259 $[(2 \times M) - H]^+$ (85), 823 (8), 743 (7), 629 $[M - H]^+$ (100), 509 (13), 467 (15), 377 (23); ESIMS *m*/*z* (pos. ions): 1283 $[(2 \times M) + Na]^+$ (25), 789 (5), 653 $[M + Na]^+$ (100); HRESIMS *m*/*z* 653.31436 $[M + Na]^+$ (Calc. for $C_{31}H_{50}O_{13} + Na$ 653.3146).

3.4. Cell culture

HeLa 229 cells were maintained in RPMI (Rochester Polytechnical Medicinal Institute) 1640 medium; HaCat cells in Dulbecco's modified Eagle's medium (Gibco-BRL). Both were supplemented with 10% fetal calf serum (Sigma) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Roche).

For EMSA, cells were plated in small Petri dishes in a density of about 700,000–100,0000 cells, the day before using 5 ml of starved medium. The starved medium consists of DMEM without FCS, but with 100 IU/ml penicillin and 100 μg/ml streptomycin. A lower base stimulation is obtained under these starving conditions.

3.5. Electrophoretic mobility shift assays

The assays were carried out as previously described (Garcia-Pineres et al., 2001). However, [^{32}P]-labelled oligonucleotide was replaced by [^{33}P]-labelled one.

3.6. IL-8 ELISA

The IL-ELISA was performed as previously reported (Kos et al., 2005).

3.7. Vitality test

The cell viability was studied by the MTT test. HeLa 229 cells were plated, incubated with test compounds and stimulated for 6 h. Next steps followed as described previously (Kos et al., 2005).

3.8. Statistics

Concerning the IL-8 data statistical analysis was performed using the GraphPad Prism 4.0 software. Data are reported as means ± SD and analyzed using the independent *t* test (two groups); $p < 0.05$ is considered statistically significant. EMSAs was carried out twice.

Acknowledgements

The authors thank the Volkswagenstiftung for financial support. O.K. is grateful to the Friedrich Naumann Stiftung for a grant. The authors acknowledge the help of B. Schuler, Department of Pharmaceutical Biology and Biotechnology for experimental assistance, of Dr. J. Wörth and C. Warth, Institute of Organic Chemistry, for taking the mass spectra, of Dr. Schröder, Thermo Finnigan MAT GmbH, Bremen, for recording the HRMS spectra, of V. Brecht, Department of Pharmaceutical and Medical Chemistry, for measuring the NMR spectra and of M. Wagner, Department of Pharmaceutical and Medical Chemistry for measuring the spectra for the specific rotation.

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