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Cytotoxic versus Anti-Inflammatory Effects in HeLa, Jurkat T and Human Peripheral Blood Cells Caused by Guaianolide-Type Sesquiterpene Lactones

Fatima Hilmi,[a] Jürg Gertsch,[a] Paul Bremner,[b] Silvia Valovic,[a] Michael Heinrich,[b] Otto Sticher[a] and Jörg Heilmann[a,*]

[a]*Department of Chemistry and Applied BioSciences, Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH) Zurich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland*
[b]*Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, UK*

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Abstract—Four guaianolide type sesquiterpene lactones (SL), namely the new 1,2-dihydro-3-oxo-costic acid guaianyl ester 3β-*O*-(1,2-didehydro-3-oxo-costoyloxy)-4β,10β-dihydroxy-guaia-1(2)-en-6β,12-olide (**1**) and 3β-*O*-(1,2-didehydro-3-oxo-costoyloxy)-4β,10β-dihydroxy-guaia-1(2)-en-6α,12-olide (**2**), as well as the known moroccolide A [5αH-2β,4-epoxy-3β-hydroxy-guaia-1(10),11(13)-dien-6β,12-olide, **3**] and 3β-*O*-(2-methylbutyryl)-moroccolide A [5αH-2β,4-epoxy-3β-(2-methylbutyryloxy)-guaia-1(10),11(13)-dien-6β,12-olide, **4**] were examined for their cytotoxic and anti-inflammatory effects in HeLa, Jurkat T and human peripheral blood mononuclear cells. Compounds **1**, **2** and **4** were found to exert a strong cytotoxicity similar in potency in all investigated cell types, whereas **3** was significantly less active. Along with the cytotoxic effect compounds **1** and **4** showed a potent and comparable down-regulation of the mRNAs of the house-keeping genes β-actin and GAP-DH in PBMCs after 20 h. In contrast, the down-regulation of the PMA-induced mRNA levels of the NF-κB-driven pro-inflammatory genes IL-2, IL-6, GM-CSF, TNF-α, and IL-1β in PBMCs is significantly stronger with compound **4**. Compound **3** did not significantly modulate cytokine mRNAs levels at biochemically relevant concentrations. The electromobility shift assay (EMSA), revealed a stronger inhibition of NF-κB for **1** (IC_{50} 2.5 μM) than for **4** (IC_{50} 5 μM). Both compounds were also subjected to an IL-6 luciferase reporter gene assay and showed IC_{50} values of 1.0 (**1**) and 1.2 μM (**4**). Thus, the NF-κB inhibition measured by EMSA, as well as the IL-6 luciferase assay did not reflect the differential modulation of pro-inflammatory genes measured with RT-rt-PCR.
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Introduction

In the traditional medicine of Morocco the leaves of the endemic plant *Warionia saharae* Benth. & Coss. are used to treat inflammatory diseases, such as rheumatoid arthritis.[1] In the course of our search for anti-inflammatory and cytotoxic compounds from medicinal plants, we have recently reported on the isolation of 12 guaianolide-type SLs.[2,3] Further investigation of the methanol soluble part of the dichloromethane extract resulted in the isolation of the two new cytotoxic dimeric SLs (**1** and **2**), the skeleton of which has only been reported from *Podachaenium eminens*.[4,5] Due to the traditional use of *W. saharae*, we were particularly interested in the anti-inflammatory action of **1** and **2** and two further guaianolide-type SLs (**3** and **4**) previously isolated from the same plant.[2]

The cytotoxicity of SLs, and a broad variety of other conspicuous biological effects, for example their potent in vitro and in vivo anti-inflammatory activity, has been attributed to the reactivity of α,β-unsaturated carbonyl groups, most commonly α,β-en-one or α-methylene-γ-lactone substructures, that can undergo reactions with sulfhydryl groups of functional proteins via a Michael-type reaction.[6,7] However, the exact molecular mechanism of the different biological effects is often a point of discussion. Recent investigations on the biological activity of SLs focus mainly on the explanation of the

*Corresponding author. Tel.: +41-1-635-60-49; fax: +41-1-635-68-82; e-mail: joerg.heilmann@pharma.ethz.ch

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anti-inflammatory effects by inhibition of nuclear factor kappa B (NF-κB).[7–10] A specific inhibition of the transcription factor NF-κB has been postulated, and it has been demonstrated in vitro that the NF-κB unit p65 (relA) is alkylated by the bifunctional pseudoguaianolide-type SL helenalin and thus blocks binding to the promoter site.[8] In addition, the phosphorylation of I-κB was shown to be suppressed by SLs via inhibition of IKKs.[9] It was further postulated that SLs inhibit NF-κB by preventing the degradation of I-κBα and I-κBβ.[10] We have recently shown that mono- and bifunctional pseudoguaianolide SLs can also strongly down regulate mRNA levels of pro-inflammatory genes.[11] Although NF-κB has been most intensely studied for its involvement in immunity and inflammation processes, this transcription factor also regulates cell proliferation, apoptosis and cell migration. Because of the widespread importance of this factor, it is not entirely clear what role NF-κB inhibition might play with regard to the cytotoxic effects.[12]

To date, there is no study, which addresses in parallel, the cytotoxicity and anti-inflammatory action of SLs. In order to study the anti-inflammatory and the cytotoxic potential of the isolated guaianolides we carried out transcriptome analysis for several key inflammatory genes and the mRNA levels of IL-1β, IL-2, IL-6, GM-CSF and TNF-α were quantified by reverse transcription real-time PCR (RT-rt-PCR) as described previously.[11,13] The functional expression of these NF-κB controlled cytokines upon mitogen or antigen stimulation is a crucial step in the cellular immune response and inflammation.[14] In addition, the regulation of the housekeeping genes β-actin and GAP-DH was investigated to clarify whether the effects on the pro-inflammatory genes is specific or part of an overall down-regulation that is comparable in potency for all investigated genes. Furthermore, the cytotoxic activity of all compounds in HeLa, Jurkat and human peripheral blood mononuclear cells (PBMC) was investigated in a colorimetric cell viability assay.

It has been previously shown that SLs can inhibit the expression of NF-κB controlled genes in reporter gene assays,[9,15] but no study has addressed the question, whether this inhibition is stronger correlated with specific genuine gene expression or with results from cell viability assays. Therefore, compounds **1** and **4** were tested for their anti-inflammatory activity in an EMSA and an IL-6 luciferase reporter gene assay.

Results and Discussion

The ESILRMS of **1** gave a molecular ion at *m/z* 531 $[M+Na]^+$ and a pseudomolecular ion peak at *m/z* 531.231 in the MALDIHRMS consistent with the molecular formula $C_{30}H_{36}O_7$. The 1D and 2D NMR data indicated the presence of a heterodimeric sesquiterpene (Fig. 1). One of the monomers (B) corresponded to an esterified acid which was easily identified as the eudesmane-type sesquiterpene 1,2-didehydro-3-oxo-costic acid.[16] The ^{13}C NMR spectrum indicated the presence

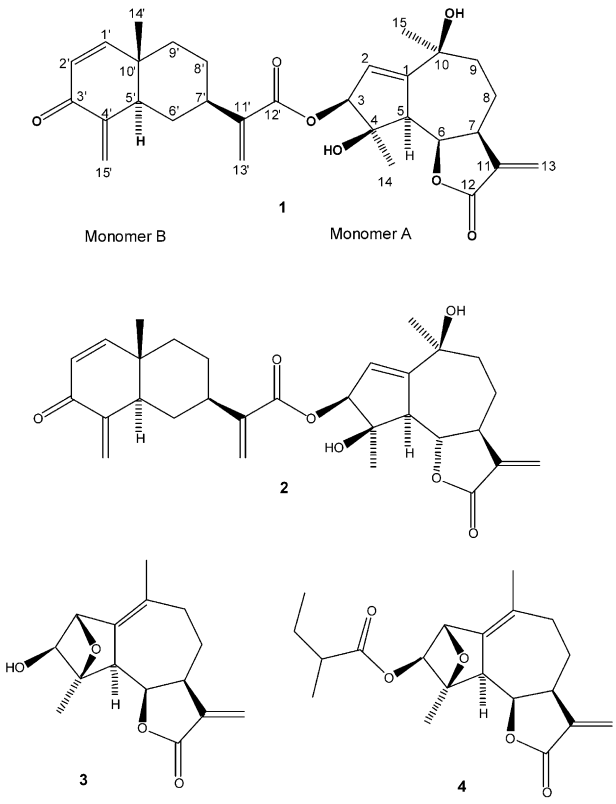

Figure 1. Structural formula of the investigated sesquiterpene lactones.

of an α-methylene-γ-lactone moiety in monomer A by the carbon signals at δ_C 172.2 (s, C-12), δ_C 141.7 (s, C-11), δ_C 81.6 (d, C-6; Table 2) and also by the characteristic ^1H NMR signals for H-13a and H-13b (δ_H 6.11 and 5.58, each d, $J=3.5$ and 3.2 Hz; Table 1). The ^1H, ^{13}C, and $^1H,^1H$ COSY spectra displayed the typical signals and correlations of an oxygen substituted guaianolide type sesquiterpene lactone.[2,3] $^1H,^{13}C$ HSQC and $^1H,^{13}C$ HMBC experiments were utilized to complete the assignments of all ^1H and ^{13}C signals. The connectivity of the monomers A and B in **1** was determined by a long-range correlation of C-12′ (δ_C 168.4) with H-3 in the HMBC spectrum. Due to the overlapping ^1H NMR resonances of H-5, H-7, H-5′ and H-7′ in CD_3OD, the relative stereochemistry of compound **1** was established by an $^1H,^1H$ ROESY experiment in C_6D_6. A NOE between H-6 and H-7, confirmed the *cis*-configuration of the lactone ring and a NOE between H-5′ and H-7′ indicated that these protons were on the same face of the molecule. Thus, the structure of **1** was established as 3β-*O*-(1,2-didehydro-3-oxo-costoyloxy)-4β,10β-dihydroxy-guaia-1(2),11(13)-dien-6β,12-olide. The structure of compound **2** as 3β-*O*-(1,2-didehydro-3-oxo-costoyloxy)-4β,10β-dihydroxy-guaia-1(2)-en-6α,12-olide was established applying the same strategy (MALDIHRMS, ^1H, ^{13}C, $^1H,^1H$ COSY, $^1H,^{13}C$ HSQC, $^1H,^{13}C$ HMBC, $^1H,^1H$ ROESY; Tables 1 and 2 for ^1H and ^{13}C NMR data). Besides the new dimeric sesquiterpene lactones also the known monomer 1,2-didehydro-3-oxo-costic acid was isolated.

Compounds **1**–**4** were subjected first to a WST-1 based cell viability assay in HeLa (KB) cells, where compounds

Table 1. ^{1}H NMR data of compounds **1** and **2** (500.1 MHz, 295 K, in CD_3OD, *J* in Hz, 295 K)

H	1	2
2	6.05 t (2.9)	5.97 m[a]
3	5.17 dd (0.6, 3.0)	5.26 m[a]
5	3.22 dd (2.5, 11.3)	3.28 m[a]
6	4.43 dd (9.8, 11.3)	4.26 dd (9.7, 11.3)
7	2.85 m[a]	3.25 m[a]
8a	2.11 m	2.27 m
8b	1.81 m[a]	1.53 m[a]
9a	2.00 m[a]	1.97 m[a]
9b	1.82 m[a]	1.77 m[a]
13a	6.11 d (3.5)	6.11 d (3.5)
13b	5.58 d (3.2)	5.56 d (3.2)
14	1.46 s	1.42 s
15	1.46 s	1.46 s
1′	7.00 d (9.9)	7.01 d (9.9)
2′	5.97 d (9.9)	5.99 m[a]
5′	2.69 dd (2.4, 11.8)	2.70 dd (2.5, 11.9)
6′a	1.93 m[a]	1.94 m[a]
6′b	1.59 m[a]	1.56 d (12.7)
7′	2.65 m[a]	2.65 m[a]
8′a	1.77 m[a]	1.76 m[a]
8′b	1.66 m[a]	1.67 m[a]
9′a	1.79 m[a]	1.79 m[a]
9′b	1.66 m[a]	1.66 m[a]
13′a	6.25 s	6.29 s
13′b	5.70 s	5.72 s
14′	0.98 s	0.99 s
15′a	6.01 dd (1.4, 2.1)	6.01 dd (1.4, 2.1)
15′b	5.26 s	5.26 m[a]

[a]Multiplicity not determined due to overlapping signals.

Table 2. ^{13}C NMR data of **1** and **2** (75.5 MHz, 295 K, in CD_3OD, multiplicities determined by DEPT spectra)

C	1	2
1	156.7, s	156.7, s
2	124.9, d	125.3, d
3	83.3, d	83.5, d
4	80.5, s	80.2, s
5	59.6, d	59.6, d
6	81.6, d	82.3, d
7	49.9, d	47.5, d
8	23.2, t	24.6, t
9	38.8, t	38.7, t
10	71.8, s	73.0, s
11	141.7, s	141.7, s
12	172.2, s	172.2, s
13	119.5, t	119.4, t
14	29.5, q	31.0, q
15	21.8, q	22.5, q
1′	164.2, d	166.2, d
2′	127.4, d	127.4, d
3′	191.4, s	191.4, s
4′	147.6, s	147.6, s
5′	49.5, d	49.4, d
6′	30.0, t	30.1, t
7′	40.7, d	40.5, d
8′	28.0, t	28.0, t
9′	37.9, t	37.9, t
10′	38.9, s	38.9, s
11′	146.6, s	146.5, s
12′	168.4, s	168.3, s
13′	124.2, t	124.3, t
14′	18.1, q	18.1, q
15′	118.9, t	118.9, t

1 (IC_{50} 2.0 μM), **2** (IC_{50} 2.0 μM) and **4** (IC_{50} 2.9 μM) showed very similar cytotoxicity. **3** (IC_{50} 17.1 μM) was significantly less active (Table 3). In PBMCs, the compounds showed a similar cytotoxicity pattern, **1** and **2** (IC_{50} 1.0 μM) being most active, followed by **4** (IC_{50} 2.9 μM) and **3** (IC_{50} 12.2 μM). In human Jurkat T leukemia cells **3** (IC_{50} 19.1 μM) and **4** (IC_{50} 7.3 μM) were less cytotoxic, whereas **1** and **2** showed almost identical effects as in PBMCs (Table 3). We have chosen compounds **1**, **3**, and **4** to look at their effects on mitogen (phorbol-12-myristate-13-acetate, PMA)-induced cytokine gene expression in PBMCs.[11,13] This was achieved by relatively quantifying mRNA levels of IL-1β, IL-2, IL-6, GM-CSF, TNF-α, as well as the house-keeping genes β-actin and GAP-DH under the influence of 10 μM of the respective SL. Along with the strong cytotoxic effect observed in the cell viability assay, compounds **1** and **4** showed a potent and comparable down-regulation of the mRNA levels of the house-keeping genes β-actin and GAP-DH after 20 h. It is of interest that the PMA-induced mRNA up-regulation of the NF-κB-driven pro-inflammatory genes IL-2, IL-6, GM-CSF, TNF-α, and IL-1β in PBMCs is inhibited to a greater degree by compound **4**, and it is not strongly correlated to the down-regulation of the house-keeping genes and the cytotoxic effect (Fig. 2). These results suggest that despite of an unspecific down-regulation of the investigated mRNA levels (connected with a reduced cell viability), compound **4** is significantly more specific than **1** as an inhibitor of PMA-induced mRNA expression of the investigated pro-inflammatory genes. Compound **3** only weakly modulated the mRNA levels and its activity is

Table 3. Cytotoxicity of the investigated sesquiterpene lactones **1**–**4** against HeLa, Jurkat T and PBM cells (IC_{50} in μM, means ± SD, $n = 4$, 72 h)

Compd	HeLa (KB) cells	Jurkat T cells	PBMCs
1	2.0 ± 0.28	2.2 ± 0.33	1.0 ± 0.14
2	2.0 ± 0.24	2.1 ± 0.30	1.0 ± 0.14
3	17.1 ± 1.30	19.1 ± 1.41	12.2 ± 0.38
4	2.9 ± 1.50	7.3 ± 0.29	2.9 ± 0.29
Helenalin	0.64 ± 0.1	0.15 ± 0.05	0.3 ± 0.07

comparable in quality to the effects of **4**. It should be noticed here that there is also a difference in the cytotoxicity of compounds **1** and **4** when the kinetic profile (and not only the cytotoxicity after 72 h) is taken into account. During the first 20 h, the effect of compound **1** is significantly stronger in all cell lines and the differences vanish the longer the assay is performed (data not shown).

Since the esterified SLs **1** and **4** were the most active compounds, we performed NF-κB EMSA analysis and a IL-6 reporter gene assay (luciferase assay) in HeLa cells to assess a possible correlation with the modulation of gene transcription. Interestingly, **1** (IC_{50} 2.5 μM) more strongly inhibited NF-κB binding than **4** (IC_{50} 5 μM). Hence, the stronger NF-κB inhibition by **1** did not correlate with a stronger down-regulation of NF-κB controlled mRNA expression, and the results resembled the pattern obtained for the cytotoxicity tests. The NF-

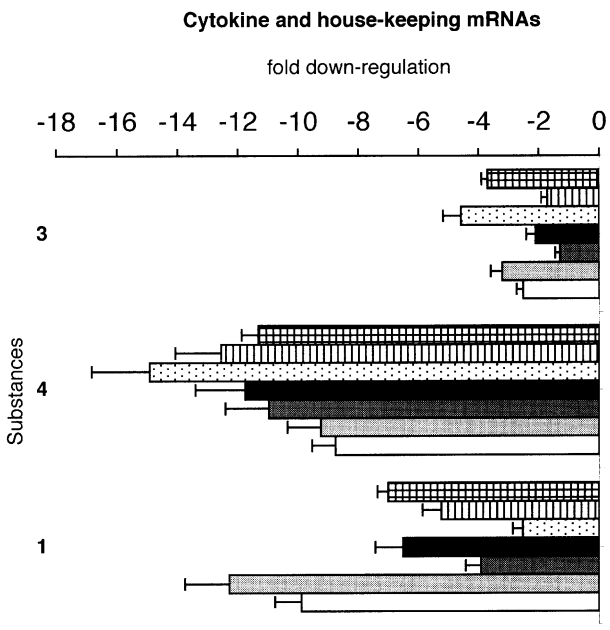

Figure 2. Down-regulation of the PMA-induced cytokine, β-actin and GAP-DH mRNA transcription after 20 h incubation with the investigated sesquiterpene lactones (10 μM, **1**, **3**, **4**), shown as means (+ SE). White bars: β-actin, grey bars: GAP-DH, dark grey bars: IL-2, black bars: IL-6, dotted bars: GM-CSF, striped bar: IL-1β, checkered bar: TNF-α.

κB driven IL-6 reporter gene assay (luciferase assay) in HeLa cells showed for compounds **1** and **4** a strong and nearly identical inhibition of the luciferase activity, with IC_{50} values of 1.0 and 1.2 μM, respectively. Thus, the obtained results did not reflect the differential modulation of gene transcription.

Conclusion

Summing up, our results revealed that guaianolide type SLs can differentially modulate gene transcription of pro-inflammatory and house-keeping genes despite of similar activity in NF-κB EMSA, IL-6 reporter gene, and cytotoxicity assays. The less cytotoxic compound **4** showed a significantly more pronounced down-regulation of pro-inflammatory gene transcription than the more cytotoxic SL **1**. This is a hint that the observed anti-inflammatory effect on the transcriptome level and the cytotoxicity of guaianolide type SLs can have different structural requirements. This will open up new possibilities to optimize SL based lead structures showing strong anti-inflammatory, but reduced cytotoxic effects. Due to the fact that NF-κB EMSA analysis and IL-6 reporter gene assay did not allow a differentiation between the anti-inflammatory activity of compounds **1** and **4** we stress the usefulness of a functional transcriptomics assay to characterize the anti-inflammatory potential of SLS. It should be mentioned that differential effects on the modulation of pro-inflammatory and regulatory genes have also been found for the pseudoguainolide type sesquiterpene lactones helenalin, 11α,13-dihydrohelenalin acetate and chamissonolide.[11] Because compound **4** is the major bioactive SL (approximately 0.5%) in *W. saharae*, its use in Moroccan traditional medicine could be validated pharmacologically. Further studies on the toxicity of this compound would be needed to promote its external use in the treatment of inflammatory conditions.

Experimental

General

^{13}C NMR spectra of compounds **1** and **2** were measured on a Bruker AMX-300 spectrometer (operating at 300.13 MHz for ^{1}H and 75.47 for ^{13}C) at 295 K. All other NMR spectra were recorded on a Bruker DRX-500 spectrometer (operating at 500.13 MHz for ^{1}H). Spectra were measured in CD_3OD, and C_6D_6 and referenced against residual CH_3OH (δ_H 3.35) and CD_3OD (δ_C 49.0), or residual C_6H_6 (δ_H 7.16) and C_6D_6 (δ_C 128.0). ESILRMS spectra were measured on a VG-ZAB-2SEQ spectrometer. MALDIHR spectra were obtained on an IonSpec Ultima FTMS spectrometer. HPLC separations were performed with a Merck-Hitachi L-4250 UV/VIS detector, a Merck D-2500 Chromato-Integrator and Knauer HPLC column (Spherisorb, S 10 ODSII, 5 μm column, 250×16 mm) detecting at 254 nm. Column chromatography was performed with silica gel (Merck, 63–200 μm). TLC was performed on precoated aluminum sheets (Merck, 0.2 mm, 60 F_{254}) and compounds detected by UV illumination and spraying with vanillin/sulfuric acid reagent.

Plant material

The leaves of *W. saharae* Benth. & Coss. were collected north of Agadir, Morocco, in May 1998. The plant was identified by Dr. A. Benchâabane, University Smlallia, Marrakech, Morocco, and Dr. F. Jacquemoud, Conservatoire et Jardin botaniques de Genève, Geneva, Switzerland. A voucher specimen is deposited at the Conservatoire et Jardin botaniques de Genève, Geneva, Switzerland with the identification number 3A/98.

Extraction and isolation

Air-dried and powdered leaves of *W. saharae* (1 kg) were percolated with dichloromethane at room temperature. The extract (157.2 g) was partitioned between n-hexane and methanol. The alcoholic phase (30.45 g) was subjected to VLC (silica gel) using a step gradient of hexane/ethyl acetate (9:1 to 1:1) and final washing with MeOH to give seven fractions (F1–F7). Fraction F3 was further separated by VLC (silica gel) eluting with a gradient of hexane/ethyl acetate/MeOH (9:1:0 to 0:1:1) to yield eight subfractions (F3.1–F3.8). The subfraction F3.6 was subjected to an open column chromatography with silica gel, eluting with a stepwise gradient mixture of chloroform/2-butanone/hexane (1:1:3 to 1:1:0) to give eight subfractions (F3.6.1–F3.6.8). Subfraction F2.6.5 (chloroform/2-butanone/hexane, 1:1:1, 50 mL) was purified by HPLC (Spherisorb RP 18, 5 μm) using ACN/H_2O (2:3, flow 5 mL/min) as solvent to give compounds **1** (t_R = 20 min, 2.1 mg) and **2** (t_R = 25 min, 1.8 mg), respectively. The known substance 1,2-didehydro-

3-oxo-costic acid was obtained from the subfraction F3.6.2 after purification with HPLC (Spherisorb RP 18, 5 μm) using CH_3CN/H_2O (3:2, flow 5 mL/min).

3β-*O*-(1,2-didehydro-3-oxo-costoyloxy)-4β,10β-dihydroxy-guaia-1(2),11(13)-dien-6β,12-olide (1). Colourless gum (2.1 mg); $[\alpha]_D^{22}$ −132° (*c* 0.1, EtOH); ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; ESILRMS (pos.): *m/z* 531 $[M+Na]^+$. MALDIHRMS *m/z* 531.2310 $[M+Na]^+$, calculated for $[C_{30}H_{36}O_7+Na]^+$, requires 531.2359.

3β-*O*-(1,2-didehydro-3-oxo-costoyloxy)-4β,10β-dihydroxy-guaia-1(2),11(13)-dien-6α,12-olide (2). Colourless gum (1.8 mg); $[\alpha]_D^{22}$ −74° (*c* 0.1, EtOH); ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; ESILRMS (neg.): *m/z* 507 $[M-H]^-$. MALDIHRMS *m/z* 531.2310 $[M+Na]^+$, calculated for $[C_{30}H_{36}O_7+Na]^+$, requires 531.2359.

Cytotoxicity assay

The cytotoxicity of the pure compounds against HeLa (KB cells, ATCC CCL17), Jurkat T CD4$^+$ cells (ATCC TIB-152), and peripheral blood mononuclear cells (PBMCs) was determined in a WST-1 based cell viability assay.[11,13] The PBMCs were isolated from healthy volunteers with Polymorphrep™ (Axon Shield) following the manufacturers instructions. All compounds were tested in a concentration range between 0.1 and 20 μg/mL.

Electromobility shift assay (EMSA)

Total HeLa cell lysate was prepared in nonylphenoxypolyethoxyethanol (Np-40) lysis buffer. Aliquots (10 μg protein) were mixed with Totex buffer (20 mM Hepes/KOH, pH 7.9, 0.35 M NaCl, 20% (v/v) glycerol, 1% (v/v) NP-40, 1 mM $MgCl_2$, 0.5 mM ethylenediamintetraacetic acid (EDTA), 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT)) and incubated on ice for 20 min followed by incubation with ^{32}P-radiolabeled oligonucleotides for additional 10 min. NF-κB oligonucleotides (Promega) specific to the NF-κB promotor region were labeled using γ-^{32}P-ATP (4500 Ci/mol) and a T4 polynucleotide kinase (Promega). DNA binding activity was analyzed by 4% acrylamide gel and autoradiography.[17]

IL-6 luciferase assay

HeLa cells were stably transfected with a luciferase reporter gene controlled by IL-6 promoter.[10] Cells were incubated for a period of 1 h with varying concentrations of **1** and **4**, respectively, followed by a 10 h stimulation with PMA (50 ng/mL). The luciferase assay was performed on an Anthos Lucy 1 luminometer (96-well format, with Stingray 1.5 software, Dazdaq Ltd) using the Promega dual-luciferase reporter system according to the manufacturer's instructions and to literature.[18] Experiments were repeated three times for each concentration.

Relative quantification of mRNA levels by reverse transcription-real time-PCR (RT-rt-PCR)

The test was performed as described in previous papers, but using PBMCs instead of Jurkat T CD4$^+$ cells (ATCC TIB-152).[11,13]

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