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Synthesis and anti-inflammatory activity of ent-kaurene derivatives

Idaira Hueso-Falcón ^{a, b, 1}, Irene Cuadrado ^{c, 1}, Florencia Cidre ^d, Juan M. Amaro-Luis ^e, Ángel G. Ravelo ^{a, b}, Ana Estevez-Braun ^{a, b, *}, Beatriz de las Heras ^{c, *}, Sonsoles Hortelano ^{d, *}

^a Instituto Universitario de Bio-Orgánica "Antonio González", Universidad de La Laguna, Avda. Astrofísico Fco. Sánchez 2, 38206 La Laguna, Tenerife, Spain

^b Instituto Canario de Investigaciones del Cáncer (ICIC)², Spain

^c Departamento de Farmacología, Facultad de Farmacia, Universidad Complutense, Plaza Ramón y Cajal s/n, 28040 Madrid, Spain

^d Unidad de Inflamación y Cáncer, Área de Biología Celular y del Desarrollo, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra Majadahonda-Pozuelo, Km 2,200, 28220 Majadahonda, Madrid, Spain

^e Departamento de Química, Facultad de Ciencias, Universidad de los Andes, Mérida, Venezuela

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ABSTRACT

A series of kaurene derivatives (1-63) were prepared and evaluated for anti-inflammatory activity. Thirteen of the tested compounds were able to inhibit NO production with an IC₅₀ between 2 and 10 μ M. Compounds **11**, **12**, **14** and **23** showed low percentage of cell viability, whereas compounds **9**, **10**, **17**, **28**, **37**, **48**, **55**, **61** and **62** were non-cytotoxic at the concentration up to 25 μ M. Some structure–activity relationships were outlined. Compounds **28**, **55** and **62**, were selected as representative compounds and they potently inhibited the protein expression of NOS-2. We also determined that inhibition of NF- κ B activation might be the mechanism involved in anti-inflammatory effects of these kaurene derivatives. As expected, cytokines IL-6, IL-1 α , TNF- α and IFN- γ were downregulated in the presence of compound **28**, **55** and **62** after stimulation with LPS. These results indicate that kaurene derivatives might be used for the design of new anti-inflammatory agents.

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1. Introduction

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, including infection and tissue injury. If the acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and acquires new characteristics.

Macrophages play a key role in the inflammatory response and serve as an essential interface between innate and adaptive immunity. On sensing the presence of pathogens through Toll-like (TLRs) and other receptors, macrophages are stimulated to secrete a battery of cytokines that recruit effector cells into the infected area. Thus, stimulation of TLR4 by lipopolysaccharide (LPS) triggers the recruitment of the cytoplasmic adapter protein MyD88 and subsequently culminates in the activation of downstream signaling pathways. These pathways induce the expression of various inflammatory mediators, including nitric oxide (NO), prostaglandins (PGs), chemokines and inflammatory cytokines that are wellknown to be involved in the pathogenesis of inflammatory response [1–4].

One of the most important regulators of the inflammatory response is the transcription factor NF- κ B. NF- κ B exists mainly as a heterodimer comprised of subunits of the Rel family p50 and p65, which is normally sequestered in the cytosol as an inactive complex by association with the inhibitory proteins I κ B [5]. The activation of NF- κ B involves the phosphorylation of I κ Bs at two critical serine residues (Ser32, Ser36) *via* the I κ B kinase (IKK) signalosome complex [6,7]. Once I κ Bs have been phosphorylated, they are ubiquitinated and degraded by 26S proteosome [8,9]. Degradation of I κ B allows the NF- κ B to translocate to the nucleus, where it regulates the transcription of all significant pro-inflammatory chemokines/cytokines, many angiogenic growth factors, TNF- α , inducible nitric oxide synthase (NOS-2), COX-2, ICAM-1, VCAM-1, E-selectin and pathways of cell proliferation and survival [10–12].

Abbreviations: NO, nitric oxide; NOS-2, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PGs, prostaglandins; TLCK, N_{α} -Tosyl-1-lysine chloromethyl ketone hydrochloride; TLRs, Toll-like receptors.

Corresponding authors. Tel.: +34 918223291; fax: +34 918223269.

E-mail addresses: aestebra@ull.es (A. Estevez-Braun), lasheras@farm.ucm.es (B. de las Heras), shortelano@isciii.es (S. Hortelano).

¹ Both authors contributed equally to this work.

² http://www.icic.es.

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Therefore, inhibition of NF-κB activation and the following release of inflammatory mediators provide a promising strategy for the development of potential anti-inflammatory agents. Indeed, the anti-inflammatory effect of steroids, retinoids and a variety of antirheumatic drugs act through a mechanism that involves inhibition of NF-kB activation [13,14]. Moreover, recent evidence suggests that anti-inflammatory drugs could also be beneficial in a number of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, which have a prominent neuroinflammatory component [15,16]. In this context, continuing our study in the development of potential anti-inflammatory agents, the present work describes the preparation and evaluation of anti-inflammatory activity of kaurene derivatives (1-63).

2. Chemistry

Compounds 1–34 were synthesized as described in Ref. [17] (Fig. 1). Compounds 35-40 were obtained by modifications at carbon C-19 of several kaurene derivatives (Scheme 1). Thus, the reaction of kaurenoic acid (1) with Me₃SiCHN₂ in Et₂O yielded the derivative 35 (68%) together the methyl ester 2 (26%). When compounds 9 and 25 were reacted with Ac₂O in the presence of pyridine the corresponding acetylated derivatives 36 and 37 were quantitatively obtained. The dimer compound 38 was formed in 50% yield under treatment of compound **9** with 1 equiv of thionyl chloride and 1 equiv of pyridine in DCM. Compound **39** with an amide moiety was prepared by reaction of compound **26** with 2 equiv of oxalvl chloride and 3 equiv of aniline in DCM under reflux. The reduction of compound **31** with LiAlH₄/Et₂O afforded the corresponding dihydroxylated compound 40 in 54% yield. Compounds (41-51) were obtained by transformations on functional groups present in C and D rings of several kaurene derivatives as it is shown in Scheme 2. The esterification of the hydroxyl group at C-15 in compound 10 with lauroyl chloride or pent-4-enoyl chloride in the presence of Et₃N, DMAP in DCM vielded the corresponding esters 41 and 42. Epoxidation of the double bond in 33 using MCPBA produced the epoxide derivative **43** in 89% yield. The reaction of compound **11** with NH₂OH· HCl provided the oxime derivative 44 in high yield (91%). From the two double bonds present in compound 17 only the exocyclic





Fig. 1. Structures of kaurene derivatives (1-34).

18

20



Scheme 1. Obtention of derivatives (35-40) by modifications at C-19.

double bond was reduced under hydrogenation conditions and compound **45** was consequently obtained. The spiro compounds **46** and **47** were obtained when the dihydroxyl derivative **14** was treated with 3 equiv of SOCl₂ and pyridine in DCM. Esterification of compound **18** with 2-furoyl chloride afforded a mixture 1.2:1 of the corresponding mono- (**48**) and di-furoate (**49**) compounds.

Epoxidation of the double bonds present in compound **17** produced the epoxide isomers (**50**) and (**51**). Compounds **52–63** were obtained by ozonolysis of the exocyclic double bond of kaurene derivatives **2**, **8**, **10**, **12**, **30** and **35** (Scheme 3). With compound **2** and **30** only the expected ketones (**52**) and (**59**) were obtained. The treatment of compound **8** in dry DCM under N₂



Scheme 2. Obtention of derivatives (41-51) by ring C and D modifications.

atmosphere at -78° with O_3 for 30 s and then, with 2.7 equiv of Me₂S yielded after purification three compounds **53** (54%), **54** (21%) and **55** (20%). The structures of **54** and **55** were ratified by the correlations detected in the HMBC spectrum. A plausible

explanation to the formation of the lactone moiety is based on a Baeyer–Villiger oxidation on the carbonyl compound formed in the ozonolysis. Other tentative formation is shown in Scheme 4 according to the result published by Schank et al. [18].



Scheme 3. Derivatives (52-63) obtained by ozonolysis.

Derivative **55** apparently is formed by opening of the corresponding lactone but **55** does not possess the corresponding hydroxyl group at C-16. In the ozonolysis of compound **10** furthermore of the carbonyl derivative **56** another unexpected derivative **57** was obtained in low yield (7%). Its structure was determined on the basis of the following HMBC correlations H-17/C-15, C-16 and H-7/C-15. The orientation of the hydrogen H-15

and the methylendioxy moiety was established by the NOE effects detected between H-15 and the hydrogens H-5 and H-9. When the α , β -unsaturated carbonyl compound (**12**) was ozonized only the anhydride **58** was obtained in 39% yield. Finally four compounds **60–62** were obtained from the reductive ozonolysis of **35**. The isolation of compound **63** having a dioxirane moiety supports the proposed mechanism illustrated in Scheme 4.



Scheme 4. Plausible formation of compound 54.

3. Results and discussion

3.1. Lipopolysaccahride (LPS)-induced NO production

To evaluate whether kaurene derivatives (1-63) regulate inflammatory response, we investigated the anti-inflammatory effects and underlying mechanisms of action of these derivatives using LPS-induced inflammatory responses. In murine RAW 264.7 macrophage cells treatment with LPS induces NOS-2 and NO release. NO production can be detected by measuring the accumulation of nitrite with the Griess method [19]. RAW 264.7 cells were pre-incubated with a range of concentrations $(1-25 \mu M)$ of compounds (Table 1) or the vehicle solution dimethyl sulfoxide (DMSO) for 15 min before their stimulation with LPS (250 ng/ml). Thirteen of the tested compounds were able to inhibit NO production with an IC₅₀ between 2 and 10 μ M (Table 1). To discard that the inhibitory effect on NO release was due to cytotoxicity, we analyzed the percentage of cell viability by MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) assays. Compounds 11, 12, 14 and 23 showed low percentage of cell viability, whereas compounds 9, 10, 17, 28, 37, 48, 55, 61 and 62 were non-cytotoxic at the concentration up to 25 µM.

From the obtained results some structure—activity relationships can be established. The higher inhibitory activities were exerted by compounds **10**, **12**, **14**, **17**, **28**, **55**, **61** and **62**. We observed two different behaviors depending on the existence or not of the double bond at C-9—C-11. In the series of kaurenoic acid (1), without the mentioned double bond, only good activities were achieved with the introduction of different groups at D ring. Thus, the introduction of a hydroxyl or a carbonyl group at C-15 led to an increased activity (compare 2 vs **10** and **12**). Compound (**12**) resulted be cytotoxic but similar NO inhibitory activity was obtained with compound **28** having a carboxylic acid instead of the methyl ester and a 95% of cell viability. The replacement of the exocyclic double bond at C-16—C-17 by epoxy (compounds **4** and **5**), aldehyde (compound **6**), or oxime (compound **7**) function did not produce an improved activity. The esterification of the hydroxyl group at C-15 of compound **10** with different acylation agents yielded inactive compounds (compare **10** vs **30** and **41**).

In the series of grandiflorenic acid (16) with a double bond at C-9–C-11, the methyl ester 17 presented an IC_{50} of 5 μ M, and the importance of the mentioned double bond for the activity is evident when we compared the activity of 17 vs 2. The selective hydrogenation of compound **17** afforded the inactive compound **45**, which indicated that the exocyclic double bond plays an important role for the activity. The replacement of the two double bonds by epoxides yielded the inactive compounds 50 and 51. Respect to the nature of the substituents at C-19 the methyl ester (17) resulted to be more active than the carboxylic acid (16) and the hydroxyl derivative (25). The introduction of different groups at the ring D led to derivatives less active and more cytotoxic (compare 17 vs 19 and 23). The tricyclic acids 55 and 62 obtained by ozonolisis showed good inhibitory activity with an IC₅₀ of 7 μ M. With respect to the unexpected lactones 54 and 61 formed also by ozonolisis, only 61 showed inhibitory effect on NO release which indicated the importance of the nature of esters at C-4.

In summary, these results indicate that compounds **10**, **17**, **28**, **55**, **61** and **62** can be pointed as the most active derivatives, exerting their anti-inflammatory activity in a dose dependent manner (Fig. 2).

3.2. Compounds **28**, **55** and **62** inhibit NOS-2 protein and mRNA expression

Although the six compounds exert inhibitory effects on NO release with similar IC_{50} values, we selected compounds **28**, **55** and **62** for further evaluation as they showed a more potent inhibition at higher concentrations (Fig. 2). In order to discard cytotoxicity of these compounds, we measured cell viability with MTT assay. As we can observe in Fig. 3, none of them was toxic. To further analyze the signaling pathways modulated by compounds **28**, **55** and **62**, we studied the activation of the pro-inflammatory gene NOS-2.

Table 1 IC₅₀ values (μM) of compounds 1–63 on NO inhibition and cell viability.

Compound	NO inhibition	Cell viability (%)
1	>25 ± 1.2	70
2	25 ± 2.3	90
3	$>\!25\pm1.7$	72
4	$>\!25\pm4.6$	90
5	$>25\pm3.1$	66
6	$>25\pm1.8$	90
7	$>25\pm1.2$	25
8	$>25\pm3.5$	46
9	10 ± 1.1	80
10	5 ± 0.7	97
11	5 ± 1.1	/6
12	4 ± 0.4 20 ± 1.4	97
13	5 ± 12	69
15	>25 + 2.2	90
16	15 ± 1.9	90
17	5 ± 1.3	100
18	$>\!25\pm6.2$	35
19	$>\!25\pm4.2$	67
20	25 ± 1.7	18
21	$>\!25\pm1.9$	30
22	$>25\pm2.3$	50
23	10 ± 1.9	50
24	$>25 \pm 1.3$	23
25	$>25 \pm 6.9$	50
20	$>25 \pm 1.5$	28 100
27	25 ± 2.0	95
29	$>25 \pm 0.5$ $>25 \pm 4.6$	48
30	$>25 \pm 3.5$	40
31	Not tested	Not tested
32	25 ± 3.7	52
33	$>\!25\pm5.5$	100
34	$>25 \pm 1.2$	82
35	$>25 \pm 3.7$	95
36 27	12 ± 2.5	99
37	10 ± 2.0	90
39	$>25 \pm 1.5$ $>25 \pm 2.8$	85
40	$>25 \pm 2.5$ $>25 \pm 1.3$	95
41	$>25 \pm 4.7$	95
42	Not tested	Not tested
43	14 ± 3.5	95
44	$>\!25\pm3.2$	99
45	$>\!25\pm0.5$	95
46	17 ± 3.5	95
47	$>25 \pm 4.1$	60
48	10 ± 0.5	99 Not tootod
49 50	$>25 \pm 1.3$	
51	Not tested	Not tested
52	>25 + 1.2	98
53	12 ± 0.7	95
54	$>\!25\pm1.9$	50
55	7 ± 0.6	95
56	$>\!25\pm2.8$	95
57	Not tested	Not tested
58	$>25 \pm 2.5$	95
59	$>$ 25 \pm 3.8	99 Not toot - 1
0U 61		NUT TESTED
62	5 ± 1.1 7 ± 0.9	95
63	Not tested	Not tested

 IC_{50} values refer to the concentration needed to inhibit 50% of NO release after LPS stimulation in the presence of the compounds.

RAW 264.7 cells were treated with the compounds in a range of concentrations of 1–25 μM in the presence of LPS (250 ng/ml) for 24 h. Supernatants were analyzed for NO release by the Griess reaction and cell viability was determined by MTT assay. IC_{50} values are means \pm SD of three independent experiments carried out by triplicate.

Macrophages were pre-incubated with derivatives and stimulated with LPS for 24 h. All compounds potently inhibited the protein expression of NOS-2 (Fig. 4A). Inhibitory effects were exerted at the transcriptional level as revealed analysis of NOS-2 mRNA by quantitative PCR (Fig. 4B).

3.3. Anti-inflammatory effects of derivatives **28**, **55** and **62** are mediated by NF- κ B inhibition

An important transcription factor in the regulation of inflammation is NF- κ B. The binding of LPS to TLR4 on macrophages activates NF- κ B leading to the upregulation of pro-inflammatory enzymes and cytokines such as NOS-2, COX-2, IL-1 β , TNF- α , MCP-1 and IL-6. Thus, NF- κ B signaling and pathways that regulate its activity have become a focus for intense drug development and application screening [20,21]. To investigate the mechanism of action of kaurene derivatives on macrophage function, we examined the effect of compounds **28**, **55** and **62** on the levels of cytosolic IkB α protein in LPS-activated macrophages. As we can observe in Fig. 5, degradation of IkB α was impaired in activated cells pretreated with compounds **28**, **55** and **62**. These results suggest that inhibition of NF- κ B activation might be the mechanism involved in anti-inflammatory effects of these kaurene derivatives.

Inhibition of NF- κ B activation can occur by different mechanisms including (a) inhibiting the activation of IKK complex, (b) targeting the proteasomal degradation of I κ Bs or (c) interfering the translocation of NF- κ B to the nucleus, or the binding of NF- κ B to DNA. Nevertheless, the most effective and selective approach for the inhibition of NF- κ B activation is provided by inhibitors of the IKK activity [20,21]. Since, we have previously described that several kaurane diterpenes such as foliol and linearol specifically targeting IKK kinase activity [22], we cannot rule out this mechanism for the anti-inflammatory effects of compounds **28**, **55** and **62**. Nonetheless, further experiments must be accomplished to determine the degree of specificity on NF- κ B inhibition of our compounds.

3.4. Kaurene derivatives inhibit cytokine release

Macrophage activation is also associated with the release of several pro-inflammatory cytokines and chemokines. In order to explore whether other inflammatory mediators such as cytokines were affected by treatment with kaurene derivatives, we analyzed levels of IL-6, IL-1 α , TNF- α and IFN- γ . As expected, all cytokines were downregulated in the presence of compound 28, 55 and 62 after stimulation with LPS (Fig. 6). Interestingly, similar results have been described for several IKK inhibitors that inhibit TNF- α production upon LPS stimulation in murine models [20]. The most potent inhibition was observed on IL-6 production; a proinflammatory cytokine which is known to increase dramatically in response to inflammation and brain injury, and it has been described to play a key role in pathologies such as rheumatoid arthritis. There are several binding sites for a number of transcription factors in the 5' region of the IL-6 gene, including NF- κ B, CREB, NF-IL-6, and AP-1 box. Our results have shown that compounds 28, 55 and 62 antagonized the increase of IL-6 induced by LPS, indicating that NF-κB activation is the only transcription factor involved on IL-6 release. These data are according with recent studies on the IL-6 promoter that demonstrate that IL-6 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner. Thus, NF-KB has been shown to regulate the induced transcription of IL-6 in murine macrophages [23].



Fig. 2. Dose-dependent effects of compounds 10, 17, 28, 55, 61 and 62 on NO release. RAW 264.7 cells were pre-treated with diterpenoids (1, 5, 10, 25 and 50 μ M) for 15 min and then stimulated with 250 ng/ml LPS for 24 h. The accumulation of nitrite in the culture medium was measured with the Griess reagent. Experiments were carried out in triplicate and the results are the means \pm S.D. of three different experiments.

4. Conclusion

In an attempt to discover novel anti-inflammatory agents, a series of kaurene derivatives were synthesized and evaluated for their ability to suppress NO production in LPS-stimulated RAW 264.7 macrophages. Thirteen compounds were especially potent inhibitors of NO release, although the anti-inflammatory effects of compounds **11**, **12**, **14** and **23** were attributable to their cytotoxicity as assessed by MTT assay. Three of these analogs, compounds **28**, **55** and **62** showed the most potent anti-inflammatory effect. The existence of a carboxylic acid seems to play an important role for NO inhibitory activity and cell survival, since it is present in the three mentioned active compounds. The good activity of tricyclic derivatives **55** and **62** obtained by ozonolysis demonstrates that

the presence of the ring D can further enhance the activity. In the tetracyclic series the combination of methyl ester at C-19/hydroxyl group at C-15 or carboxylic acid at C-19/carbonyl group at C-15 led to an increase in the anti-inflammatory activity. Compounds **28**, **55** and **62** also demonstrated significant inhibitory effects on IkB α degradation. Therefore, the activity of these compounds may be at least in part due to its NF- κ B inhibitory activity. In addition to the inhibitory effects on NO production, compounds **28**, **55** and **62** were able to inhibit several cytokines involved in the inflammatory response after LPS stimulation such as IL-6, IL-1 β , TNF- α and IFN- γ .

In summary, we have identified three novel compounds with potential anti-inflammatory properties that might be use for the future development of a new class of anti-inflammatory agents.



Fig. 3. Cell viability of compounds **28**, **55** and **62**. RAW 264.7 cells were pre-treated with diterpenoids (1, 5, 10, 25 and 50 μM) for 15 min and then stimulated with 250 ng/ml LPS for 24 h. Cell viability was determined by the MTT assay. Experiments were carried out in triplicate and the results are the means ± S.D. of three different experiments.



Fig. 4. Compounds **28**, **55** and **62** inhibit NOS-2 expression. A) Macrophages were pre-incubated for 15 min with compounds **28**, **55** and **62** (5μ M) followed by stimulation with 250 ng/ml LPS for 24 h. NOS-2 protein was detected by Western blot. β -Actin content was used as a loading control. Results show a representative experiment of three. B) Macrophages were pre-incubated as in A and stimulated with 250 ng/ml LPS for 4 h. Relative expression of NOS-2 mRNA was determined by real-time quantitative PCR. Results show the means \pm S.D. of three independent experiments carried out in triplicate. **p < 0.01, ***p < 0.001 vs LPS.

5. Experimental

5.1. Chemistry

All solvents and reagents were purified by standard techniques reported in Perrin, D.D.; Amarego, W.L.F. Purification of Laboratory Chemicals, 3rd edition, Pergamon Press, Oxford, 1988 or used as supplied from commercial sources as appropriate. Reactions were monitored by TLC (on silica gel POLYGRAM SIL G/UV₂₅₄ foils). Purification by column flash-chromatography used Merk Kiesel 60-H (0.063–0.2 mm) as adsorbent and different mixtures of hexanes-ethylacetate as eluent. Pre-coated TLC plates SIL G-100 UV₂₅₄ (Machery-Nagel) were used for preparative-TLC purification. ¹H NMR spectra were recorded in CDCl₃ or C₆D₆ at 300 or 400 MHz, using a Bruker AMX300 or Bruker AMX400 instruments. For ¹H spectra, chemical shifts are given in parts per million (ppm) and are referenced to the residual solvent peak. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Proton assignments and stereochemistry are supported by ¹H-¹H COSY and ROESY where necessary. Data are reported in the following manner: chemical shift (multiplicity, coupling constant if appropriate, integration). Coupling constants (*I*) are given in Hertz (Hz) to the nearest 0.5 Hz. ¹³C NMR spectra were recorded at 75 and 100 MHz using a Bruker AMX300 or Bruker AMX400 instruments. Carbon spectra assignments are supported by DEPT-135 spectra, ¹³C-¹H (HMQC) and ¹³C-¹H (HMBC) correlations where necessary. Chemical shifts are quoted in ppm and are referenced to the appropriate residual solvent peak. MS and HRMS were recorded on a VG Micromass ZAB-2F. IR spectra were taken on a Bruker IFS28/55 spectrophotometer. Kaurenoic acid (1), grandiflorenic acid (16), 15*α*-acetoxy kaurenoic acid (26) and 16α -hydroxy kaurenoic acid (**31**) were used as starting material to synthesize the diterpenes. These diterpenes were isolated from *Espeletia chardonii* using the habitual previously described methodology [24–28].

5.1.1. Preparation of compound 35

To 489.5 mg of compound **1** (1.621 mmol) dissolved in Et₂O was added 1 ml of 2 M solution of Me₂SiCHN₂ in Et₂O. The reaction mixture was stirred at room temperature for 15 h. Then the solvent was removed and the residue was purified by chromatotron using hexanes/toluene (40%) to afford 30.1 mg of compound 2 (26%) as an amorphous white solid and 424.9 mg of compound 35 (68%) as vellow oil. ¹H NMR (300 MHz, CDCl₃) δ : 4.79 (1H, s, H-17a), 4.74 (1H, s, H-17b), 3.75 (1H, d, *J* = 14 Hz, H-21a), 3.57 (1H, d, *J* = 14 Hz, H-21b), 2.63 (1H, s, H-13), 2.03 (2H, s, H-15), 1.15 (3H, s, H-18), 0.80 (3H, s, H-20), 0.09 (9H, s, Si(CH₃)₃), 2.18-0.79 (18H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 178.1 (s, C-19), 155.4 (s, C-16), 102.8 (t, C-17), 56.8 (d, C-5), 56.6 (t, C-21), 54.8 (d, C-9), 48.7 (t, C-15), 43.9 (s, C-8), 43.7 (s, C-4), 43.5 (d, C-13), 41.1 (t, C-7), 40.5 (t, C-1), 39.3 (t, C-14), 39.2 (s, C-10), 38.0 (t, C-3), 32.8 (t, C-12), 28.7 (q, C-18), 21.8 (t, C-6), 18.9 (t, C-2), 18.1 (t, C-11), 15.2 (q, C-20), -3.13 (q, Si(CH₃)₃). EIMS m/z (%): 388 ([M]⁺, 92); 373 (95); 345 (29); 285 (28); 257 (100); 241 (34); 123 (52); 121 (26); 109 (33); 107 (25); 105 (27); 97 (23); 91 (30); 81 (38); 79 (23); 73 (42); 59 (35); 55 (29). HREIMS: 388.2794 (calcd for C₂₄H₄₀O₂Si [M]⁺ 388.2798). IR v_{max}: 2931, 2853, 1722, 1462, 1250, 1227, 1147, 860 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 202.0 (3.55) nm. $[\alpha]_D^{20}$: -68.42 (c 0.9, CHCl₃).

5.1.2. Preparation of compound 36

8.7 mg (0.030 mmol) of compound **9** dissolved in the minimum amount of pyridine were treated with an excess of Ac₂O (6.1 μ l, 3 equiv) in the presence of catalytic amount of DMAP. The reaction mixture was stirred for 24 h, after elimination of solvent, compound **36** (7.1 mg, 100%) was obtained as an amorphous white solid. ¹H NMR (300 MHz, CDCl₃) δ : 4.79 (1H, s, H-17a), 4.73 (1H, s,



Fig. 5. Kaurene derivatives inhibit NF- κ B activity. Macrophages were pre-treated for 15 min with kaurene derivatives (5 μ M) and then activated for the indicated times with 250 ng/ml LPS. The levels of I κ B α were determined by Western blot. β -Actin was used as a loading control. Results show a representative experiment of three.



Fig. 6. Cytokine release is inhibited by kaurene derivatives. Macrophages were treated or untreated with compound **28**, **55** and **62** (5μ M) for 15 min followed by stimulation for 24 h with LPS 250 ng/ml. Production of IL-6, IL-1 α , TNF- α and IFN- γ was determined in supernatant by ELISA. Results show the means \pm S.D. of three independent experiments carried out in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 vs LPS.

H-17b), 4.21 (1H, d, *J* = 11 Hz, H-19a), 3.87 (1H, d, *J* = 11 Hz, H-19b), 2.63 (1H, s, H-13), 2.04 (3H, s, OCOCH₃), 1.02 (3H, s, H-18), 0.93 (3H, s, H-20), 1.96–0.73 (20H, m). ¹³C NMR (75 MHz, CDCl₃): 171.2 (s, OCOCH₃), 155.6 (s, C-16), 102.7 (t, C-17), 66.9 (t, C-19), 56.5 (d, C-5), 55.9 (d, C-9), 48.8 (t, C-15), 43.9 (s, C-8), 43.7 (d, C-13), 41.3 (t, C-7), 40.1 (t, C-1), 39.4 (t, C-14), 38.9 (s, C-4), 36.8 (s, C-10), 36.1 (t, C-3), 32.9 (t, C-12), 27.3 (q, C-18), 20.8 (q, OCOCH₃), 20.2 (t, C-6), 18.0 (t, C-2), 18.0 (t, C-11), 17.8 (q, C-20). EIMS *m/z* (%): 330 ([M]⁺, 45); 315 (23); 270 (26); 257 (63); 255 (54); 227 (44); 167 (30); 149 (100); 147 (32); 145 (30); 135 (49); 133 (30); 123 (90); 121 (39); 109 (52); 107 (46); 105 (51); 95 (45); 93 (52); 91 (66); 81 (72); 79 (48); 67 (39); 57 (37); 55 (58). HREIMS: 330.2542 (calcd for C₂₂H₃₄O₂ [M]⁺ 330.2559). IR *ν*_{max}: 2927, 2855, 1740, 1448, 1370, 1239, 1032, 873 cm⁻¹. UV (EtOH) *λ*_{max} (log *ε*): 202.0 (3.58) nm. [*α*]₀²⁰: -40.13 (c 0.8, CHCl₃).

5.1.3. Preparation of compound 37

Following the same procedure described above, 8.7 mg (0.030 mmol) of compound **25** were treated with 8.63 μ l of Ac₂O (3 equiv) to yield quantitatively 10.0 mg of compound **37** as amorphous white solid. ¹H NMR (300 MHz, CDCl₃): 5.19 (1H, s, H-11), 4.91 (1H, s, H-17a), 4.78 (1H, s, H-17b), 4.30 (1H, d, *J* = 10.9 Hz, H-19a), 4.07 (1H, d, *J* = 10.9 Hz, H-19b), 2.73 (1H, s, H-13), 2.64 (1H, d, *J* = 15.6 Hz, H-15a), 2.17 (1H, d, *J* = 15.6 Hz, H-15b), 2.05 (3H, s, OCOCH₃), 1.07 (3H, s, H-18), 0.93 (3H, s, H-20), 2.41–0.90 (15H, m). ¹³C NMR (75 MHz, CDCl₃): 171.2 (s, OCOCH₃), 158.4 (s, C-9), 156.8 (s, C-16), 113.6 (d, C-11), 105.0 (t, C-17), 66.1 (t, C-19), 50.3 (t, C-15), 45.2 (d, C-5), 44.5 (t, C-14), 41.9 (s, C-4), 41.0 (d, C-13), 39.8 (t, C-1), 37.8 (s, C-8), 37.5 (t, C-3), 37.1 (s, C-10), 35.9 (t, C-12), 29.1 (t, C-7), 26.2 (q, C-18), 25.2 (q, C-20), 20.8 (q, OCOCH₃), 18.5 (t, C-2), 17.6 (t, C-6). EIMS *m*/*z* (%): 328 ([M]⁺, 78); 313 (96); 255 (44); 253 (100); 185 (25); 183 (27); 173 (29); 159 (29); 147 (43); 143 (27); 129 (27);

117 (29); 105 (47); 95 (22); 93 (28); 91 (54); 81 (33); 55 (44). HREIMS: 328.2405 (calcd for $C_{22}H_{32}O_2$ [M]⁺ 328.2402). IR ν_{max} : 2930, 2868, 1738, 1659, 1460, 1373, 1240, 1032, 983 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 202.0 (3.55) y 243.0 (3.11) nm. [α]²⁰_D: +22.59 (c 1.1, CHCl₃).

5.1.4. Preparation of compound 38

To 16.7 mg of compound 9 (0.058 mmol) dissolved in 5 ml of dry CH_2Cl_2 were added 4.69 µl of pyridine (1 equiv) and 4.22 µl of $SOCl_2$ (1 equiv) under N₂ atmosphere. The reaction mixture was stirred at room temperature for 4 h, until disappearance of the starting material (4 h). Then H₂O was poured and the mixture was extracted several times with CH₂Cl₂. The organic phases were collected and dried over anhydrous MgSO₄, then were filtered, concentrated and purified by preparative-TLC using hexanes/AcOEt (9.5:0.5). 9.1 mg of compound **38** (50%) were obtained. ¹H NMR (300 MHz, CDCl₃) δ : 4.80 (2H, s, H-17a, H-17'a), 4.74 (2H, s, H-17b, H-17'b), 4.15 (1H, d, *I* = 9.9 Hz, H-19a ó H-19'a), 4.01 (1H, d, *I* = 9.9 Hz, H-19a ó H-19'a), 3.77 (1H, d, *J* = 9.9 Hz, H-19b ó H-19'b), 3.63 (1H, d, *J* = 9.9 Hz, H-19b ó H-19'b), 2.64 (2H, s, H-13, H-13'), 1.03 (3H, s, H-18, H-18'), 0.98 (6H, s, H-20, H-20'), 2.31-0.73 (41H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 158.2 (s, C-9, C-9'), 156.6 (s, C-16, C-16'), 113.7 (d, C-11, C-11'), 105.1 (t, C-17, C-17'), 64.0 (t, C-19 ó C-19'), 63.6 (t, C-19 ó C-19'), 50.3 (t, C-15, C-15'), 45.3 (d, C-5 ó C-5'), 45.2 (d, C-5 ó C-5'), 44.5 (t, C-14, C-14'), 41.9 (s, C-8, C-8'), 41.0 (d, C-13, C-13'), 39.7 (t, C-1, C-1'), 37.8 (s, C-10, C-10'), 37.5 (t, C-12, C-12'), 37.4 (s, C-4 ó C-4'), 37.4 (s, C-4 ó C-4'), 35.5 (t, C-3 ó C-3'), 35.4 (t, C-3 ó C-3'), 29.1 (t, C-7, C-7'), 26.3 (q, C-18 ó C-18'), 26.2 (q, C-18 ó C-18'), 25.2 (q, C-20, C-20'), 18.4 (t, C-2 ó C-2'), 18.3 (t, C-2 ó C-2'), 17.6 (t, C-6, C-6'). EIMS m/z (%): 622 ([M]⁺, 5); 287 (79); 271 (54); 270 (50); 257 (79); 175 (23); 161 (63); 147 (47); 123 (47); 111 (100); 106 (37); 105 (64); 95 (49); 94 (59); 93 (43); 91 (38); 81 (61); 55 (35). HREIMS: 622.4421 (calcd for C₄₀H₆₂O₃S [M]⁺ 622.4420). IR ν_{max} : 2932, 2868, 1461, 1189, 972, 925, 874, 756 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.6 (3.29) nm. [α]_D²⁰: +46.75 (c 0.8, CHCl₃).

5.1.5. Preparation of compound 39

To a solution of 33.5 mg of **26** (0.093 mmol) in dry DCM (5 ml) at 0 °C were added 21.2 µl of oxalvl chloride (2 equiv) and one drop of DMF. The reaction mixture was stirred for 26 h. then 4.5 equiv of aniline were added and the reaction was left for 75 h at room temperature. The solvent was removed and the residue was purified by TC-preparative using hexanes/AcOEt (30%) to yield 18.8 mg of compound **39** (46%) as a yellow oil. ¹H NMR (300 MHz, CDCl₃) δ : 7.47 (2H, d, J = 7.5 Hz, H-2', H-6'), 7.32 (2H, t, J = 7.5 Hz, H-3', H-5'), 7.10 (1H, t, J = 7.5 Hz, H-4'), 5.26 (1H, s, H-15), 5.09 (2H, s, H-17), 2.77 (1H, s, H-13), 2.08 (3H, s, OCOCH₃), 1.27 (3H, s, H-18), 0.90 (3H, s, H-20), 2.19-0.85 (19H, m).¹³C NMR (75 MHz, CDCl₃) δ: 174.8 (s, C-19), 170.9 (s, OCOCH₃), 155.0 (s, C-16), 137.7 (s, C-1'), 128.8 (d, C-3', C-5'), 124.0 (d, C-4'), 120.0 (d, C-2', C-6'), 109.8 (t, C-17), 82.7 (d, C-15), 56.9 (d, C-5), 52.6 (d, C-9), 47.2 (s, C-8), 44.3 (s, C-4), 42.2 (d, C-13), 40.6 (t, C-1), 39.6 (s, C-10), 38.0 (t, C-3), 36.8 (t, C-7), 34.7 (t, C-14), 32.3 (t, C-12), 29.5 (q, C-18), 21.2 (t, C-6), 21.1 (q, OCOCH₃), 19.0 (t, C-2), 18.2 (t, C-11), 15.8 (q, C-20). EIMS *m*/*z*(%): 435 ([M]⁺, 25); 375 (12); 256 (21); 255 (100); 185 (11); 173 (28); 162 (17); 159 (15); 123 (15); 109 (12); 105 (11); 95 (11); 93 (87); 91 (12); 81 (17); 55 (12). HREIMS: 435.2754 (calcd for C₂₈H₃₇NO₃ [M]⁺ 435.2773). IR *v*_{max}: 3398, 2932, 2866, 1729, 1667, 1597, 1521, 1435, 1370, 1307, 1243, 1022, 752, 693 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 202.4 (4.03) y 241.6 (3.68) nm. $[\alpha]_{D}^{20}$: -96.80 (c 1.0, CHCl₃).

5.1.6. Preparation of compound 40

To 13.3 mg of compound 31 dissolved in dry THF were added 6.3 mg of LiAlH₄ (4 equiv) under N₂ atmosphere. The reaction mixture was stirred for six days. Then it was treated with a saturated solution of dipotassium salt of La L(+)-tartaric acid and extracted several times with AcOEt. The organic layers were separated, dried over anhydrous MgSO₄ and concentrated. The residue was purified by preparative-TLC using hexanes/AcOEt 40% to yield 6.9 mg of compound **40** (54%) as an amorphous white solid. ¹H NMR (300 MHz, CDCl₃) δ: 3.73 (1H, d, *J* = 10.9 Hz, H-19a), 3.44 (1H, d, J = 10.9 Hz, H-19b), 1.35 (3H, s, H-17), 1.01 (3H, s, H-18), 0.95 (3H, s, H-20), 1.90–0.73 (23H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 79.1 (s, C-16), 65.3 (t, C-19), 57.6 (t, C-15), 56.7 (d, C-5), 56.5 (d, C-9), 48.7 (d, C-13), 45.0 (s, C-8), 42.2 (t, C-7), 40.2 (t, C-1), 39.0 (s, C-4), 38.4 (s, C-10), 37.3 (t, C-3), 35.4 (t, C-14), 26.8 (q, C-17), 26.5 (t, C-12), 24.2 (q, C-18), 20.4 (t, C-6), 18.0 (q, C-20), 18.0 (t, C-2), 17.8 (t, C-11). EIMS m/ z (%): 306 ([M]⁺, 1); 302 (15); 288 (17); 275 (25); 258 (30); 257 (100); 161 (19); 147 (21); 135 (26); 123 (52); 121 (28); 109 (36); 107 (28); 105 (38); 95 (32); 94 (41); 91 (30); 81 (34); 79 (26); 55 (23). HREIMS: 306.2563 (calcd for $C_{20}H_{34}O_2$ [M]⁺ 306.2559). IR ν_{max} : 3365, 2926, 2864, 2850, 1697, 1447, 1122, 1030, 1010, 756, 462 cm⁻¹ $[\alpha]_D^{20}$: -25.94 (c 0.7, CHCl₃).

5.1.7. Preparation of compound 41

To a solution of 10.7 mg of compound **10** (0.032 mmol) in dry DCM were added 11.2 μ l of lauroyl chloride (1.5 equiv), 13.5 μ l of Et₃N (3 equiv) and catalytic amount of DMAP. The reaction mixture was stirred for 48 h under N₂ atmosphere. Then the solvent was removed and the crude was purified by preparative-TLC using hexanes/AcOEt 10% to yield 9.0 mg of compound **41** (54%) as a yellow oil. ¹H NMR (300 MHz, CDCl₃) δ : 5.25 (1H, s, H-17a), 5.07 (1H, s, H-17b), 5.07 (1H, s, H-15), 3.63 (3H, s, H-21), 2.77 (1H, s, H-13), 1.25 (20H, sa, H-2'-11'), 1.16 (3H, s, H-18), 0.89 (3H, m, H-12'), 0.82 (3H, s, H-20), 2.33–0.85 (18H, m). ¹³C NMR (75 MHz, CDCl₃) δ : 177.9 (s, C-19), 173.9 (s, C-1'), 155.3 (s, C-16), 109.6 (t, C-17), 82.4 (d, C-15), 56.3 (d, C-5), 52.6 (d, C-9), 50.9 (q, C-21), 47.2 (s, C-8), 43.5 (s,

C-4), 42.2 (d, C-13), 40.3 (t, C-1), 39.4 (s, C-10), 37.7 (t, C-3), 37.0 (t, C-7), 34.5 (t, C-14), 32.4 (t, C-12), 31.7 (t, C-2'), 29.4 (t, C-3'), 29.3 (t, C-4', C-5'), 29.1 (t, C-6'), 29.0 (t, C-7', C-8'), 28.9 (t, C-9'), 28.4 (t, C-10'), 24.9 (q, C-18), 22.4 (t, C-11'), 20.6 (t, C-6), 18.8 (t, C-2), 18.1 (t, C-11), 15.4 (q, C-20), 13.9 (q, C-12'). EIMS *m*/*z* (%): 514 ([M]⁺, 9); 332 (42); 315 (30); 314 (100); 299 (66); 273 (14); 255 (74); 254 (37); 239 (35); 183 (29); 147 (22); 121 (28); 107 (24); 71 (22); 57 (45); 55 (32). HREIMS: 514.4027 (calcd for $C_{33}H_{54}O_4$ [M]⁺ 514.4022). IR ν_{max} : 2927, 2855, 1729, 1463, 1233, 1189, 1154 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 202.0 (3.41) nm. [α]^D_D: -39.67 (c 0.9, CHCl₃).

5.1.8. Preparation of compound 42

To a solution of 9.4 mg (0.028 mmol) of compound 10 in 4 ml of dry DCM were added 10 μ l of Et₃N (2.5 equiv), 10 μ l of 4-pentenoyl chloride (3.2 equiv) and catalytic amount of DMAP. The reaction mixture was stirred for 42 h under N₂ atmosphere. Then the solvent was eliminated and the crude was purified by TLC using hexanes/ AcOEt 10% to yield 4.5 mg of compound 42 (38%) as an amorphous white solid. ¹H NMR (300 MHz, CDCl₃) δ: 5.84 (1H, m, H-4'), 5.27 (1H, s, H-17a), 5.08 (3H, sa, H-17b, H-15, H-5'a), 5.01 (1H, dd, J = 1.6 y 11.8 Hz, H-5'b), 3.64 (3H, s, H-21), 2.78 (1H, s, H-13), 2.41 (4H, m, H-2', H-3'), 1.16 (3H, s, H-18), 0.83 (3H, s, H-20), 2.19-0.78 (18H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 178.2 (s, C-19), 172.9 (s, C-1'), 155.3 (s, C-16), 136.5 (d, C-4'), 115.2 (t, C-5'), 109.7 (t, C-17), 82.7 (d, C-15), 56.4 (d, C-5), 52.6 (d, C-9), 51.0 (q, C-21), 47.2 (s, C-8), 43.8 (s, C-4), 42.2 (d, C-13), 40.6 (t, C-1), 39.4 (s, C-10), 38.0 (t, C-3), 37.0 (t, C-2'), 34.9 (t, C-3'), 33.6 (t, C-7), 32.4 (t, C-14), 28.8 (t, C-12), 28.4 (q, C-18), 20.7 (t, C-6), 19.1 (t, C-2), 18.1 (t, C-11), 15.2 (q, C-20). EIMS m/z (%): 414 ([M]⁺, 14); 332 (34); 315 (28); 314 (94); 299 (68); 255 (91); 254 (41); 239 (58); 211 (20); 147 (24); 121 (39); 109 (23); 107 (33); 105 (30); 93 (24); 91 (37); 83 (49); 81 (25); 79 (23); 55 (100). HREIMS: 414.2773 (calcd for $C_{26}H_{38}O_4$ [M]⁺ 414.2770). IR ν_{max} : 2929, 2855, 1727, 1458, 1232, 1155, 992, 908, 773 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.6 (3.51) nm. $[\alpha]_D^{20}$: -41.11 (c 0.4, CHCl₃).

5.1.9. Preparation of compound 43

To 26.7 mg of compound **33** (0.225 mmol) dissolved in dry DCM were added 42.3 mg (2 equiv) of MCPBA and 31.8 mg of NaHCO₃ (4.5 equiv). The reaction mixture was left to room temperature for 4 h, then the organic phase was separated and treated with a saturated solution of sodium thiosulfate. The organic phase was separated again and dried over anhydrous magnesium sulfate, the mixture was filtered, the solvent was removed under reduced pressure and the crude was purified by preparative-TLC using hexanes/AcOEt 20% to yield 25.1 mg of compound **43** (89%) as an amorphous white solid.

¹H NMR (300 MHz, CDCl₃) δ: 3.62 (3H, s, H-21), 2.64 (1H, s, H-15), 1.41 (3H, s, H-17), 1.16 (3H, s, H-18), 0.80 (3H, s, H-20), 2.18–0.83 (19H, m). ¹³C NMR (75 MHz, CDCl₃): 177.7 (s, C-19), 67.8 (d, C-15), 61.1 (s, C-16), 56.5 (d, C-5), 50.9 (q, C-21), 49.3 (d, C-9), 43.6 (s, C-8), 43.5 (s, C-4), 40.6 (t, C-1), 39.0 (s, C-10), 38.8 (d, C-13), 37.8 (t, C-3), 35.5 (t, C-7), 31.9 (t, C-14), 28.5 (q, C-18), 26.7 (t, C-12), 20.4 (t, C-6), 18.8 (t, C-2), 18.0 (t, C-11), 14.9 (q, C-20), 14.4 (q, C-17). EIMS *m/z* (%): 332 ([M]⁺, 63); 317 (25); 314 (11); 289 (28); 273 (76); 257 (45); 255 (23); 173 (18); 159 (23); 149 (23); 147 (26); 135 (64); 133 (33); 123 (53); 121 (100); 109 (49); 107 (76); 105 (39); 95 (43); 93 (45); 91 (48); 81 (50); 79 (45); 67 (33); 55 (47). HREIMS: 332.2343 (calcd for C₂₁H₃₂O₃ [M]⁺ 332.2351). IR *ν*_{max}: 2947, 2848, 1724, 1446, 1232, 1194, 1153, 986, 845, 771 cm⁻¹. UV (EtOH) *λ*_{max} (log ε): 201.8 (2.91) nm. [α]_D²⁰: -45.88 (c 1.0, CHCl₃).

5.1.10. Preparation of compound 44

To a solution of 19.8 mg of hydroxylamine hydrochloride (7.2 mg, 3 equiv) and 19.8 mg sodium acetate (3.2 equiv) in 0.4 ml of H_2O , 29.8 mg of aldehyde **11** in 3 ml of EtOH was added. The

reaction mixture was stirred under reflux for 23 h. Then the EtOH was removed, and the residue was extracted with DCM (3×10 ml). The organic phases were collected and dried over anhydrous MgSO₄, filtered and concentrated. The residue was purified by preparative-TLC using hexanes/AcOEt (4:1) and 23.5 mg of compound 44 (91%) were obtained as an amorphous yellow solid. ¹H NMR (300 MHz, CDCl₃) δ : 7.85 (1H, s, H-17), 5.82 (1H, s, H-15), 3.64 (3H, s, H-21), 2.94 (1H, d, J = 3 Hz, H-13), 1.17 (3H, s, H-18), 0.86 (3H, s, H-20), 2.18–0.76 (19H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 177.8 (s, C-19), 147.3 (d, C-17), 147.0 (d, C-15), 139.4 (s, C-16), 56.4 (d, C-5), 51.0 (q, C-21), 49.7 (s, C-8), 46.4 (d, C-9), 43.6 (s, C-4), 43.0 (t, C-7), 40.5 (t, C-1), 39.6 (d, C-13), 39.5 (s, C-10), 38.5 (t, C-14), 37.8 (t, C-3), 28.5 (q, C-18), 24.8 (t, C-12), 20.4 (t, C-6), 18.8 (t, C-2), 18.6 (t, C-11), 15.0 (q, C-20). EIMS m/z (%): 345 ([M]⁺, 18); 330 (11); 329 (51); 328 (100); 312 (32); 268 (79); 267 (54); 252 (35); 162 (21); 123 (66); 121 (29); 117 (24); 109 (47); 108 (24); 107 (36); 91 (35); 81 (38); 79 (29); 67 (32); 55 (40). HREIMS: 345.2301 (calcd for C₂₁H₃₁NO₃ [M]⁺ 345.2304). IR v_{max}: 3421, 3286, 2939, 2851, 1723, 1464, 1446, 1233, 1191, 1159, 970, 950, 738 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 248.4 (3.83) nm. $[\alpha]_D^{20}$: -107.94 (c 1.0, CHCl₃).

5.1.11. Preparation of compound 45

72.6 mg (0.231 mmol) of compound 17 dissolved in 4 ml of dry THF were hydrogenated in the presence of catalytic amount of Pd/C 10%. The reaction mixture was stirred for 1 h until disappearance of the starting material. After elimination of solvent the resulting residue was purified by preparative-TLC using hexanes/AcOEt (20%) to yield 27.1 mg of compound 45 (37%) as a white amorphous solid. ¹H NMR (300 MHz, CDCl₃) δ : 5.18 (1H, t, J = 3 Hz, H-11), 3.64 (3H, s, H-21), 1.16 (3H, s, H-18), 1.00 (3H, d, J = 8 Hz, H-17), 0.89 (3H, s, H-20), 2.41–1.04 (19H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 177.9 (s, C-19), 158.1 (s, C-9), 114.8 (d, C-11), 51.0 (q, C-21), 49.7 (t, C-15), 46.5 (d, C-5), 45.8 (t, C-14), 44.6 (s, C-4), 42.2 (s, C-8), 41.0 (t, C-1), 38.3 (s, C-10), 38.2 (t, C-3), 37.5 (d, C-13), 36.8 (d, C-16), 30.1 (t, C-12), 29.6 (t, C-7), 27.9 (q, C-18), 23.1 (q, C-20), 20.1 (t, C-2), 18.6 (q, C-17), 18.4 (t, C-6). EIMS *m*/*z* (%): 316 ([M]⁺, 38); 301 (77); 271 (41); 257 (49); 241 (100); 197 (63); 159 (36); 134 (40); 121 (53); 109 (94); 107 (62); 95 (53); 93 (46); 91 (98); 81 (54); 79 (37); 57 (47); 55 (55). HREIMS: 316.2390 (calcd for C₂₁H₃₂O₂ [M]⁺ 316.2402). IR *v*_{max}: 2933, 2867, 1723, 1460, 1376, 1220, 1149, 978, 757 cm⁻¹. $[\alpha]_D^{20}$: +37.33 (c 0.9, CHCl₃).

5.1.12. Preparation of compounds 46 and 47

To 33.8 mg of compound 14 (0.097 mmol) dissolved in 5 ml of dry CH_2Cl_2 were added 23.5 µl of pyridine (3 equiv) and 21.1 µl of SOCl₂ (3 equiv) under N₂ atmosphere. The reaction mixture was stirred at room temperature for 1 h, until disappearance of the starting material (4 h). Then H₂O was poured and the mixture was extracted several times with CH₂Cl₂. The separated organic phases were dried over anhydrous MgSO₄, filtered and concentrated. The residue was purified by preparative-TLC using toluene to yield 11.8 mg of compound **46** (31%) and 12.2 mg of compound **47** (32%) as amorphous white solids. Compound 46: ¹H NMR (300 MHz, CDCl₃) δ : 4.58 (1H, d, J = 8.7 Hz, H-17a), 4.42 (1H, d, J = 8.7 Hz, H-17b), 3.63 (3H, s, H-21), 2.28 (1H, d, J = 15.2 Hz, H-15a), 2.16 (1H, d, J = 15 Hz, H-14a), 2.14 (1H, s, H-13), 1.16 (3H, s, H-18), 0.81 (3H, s, H-20), 2.05–0.74 (18H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 177.7 (s, C-19), 98.9 (s, C-16), 69.7 (t, C-17), 56.5 (d, C-5), 54.7 (d, C-9), 53.1 (t, C-15), 51.0 (q, C-21), 44.9 (d, C-13), 44.6 (s, C-8), 43.5 (s, C-4), 40.7 (t, C-7), 40.3 (t, C-1), 39.1 (s, C-10), 37.8 (t, C-3), 37.7 (t, C-14), 28.4 (q, C-18), 26.8 (t, C-12), 21.7 (t, C-6), 18.8 (t, C-2), 18.1 (t, C-11), 15.2 (q, C-20). EIMS *m*/*z* (%): 396 ([M]⁺, 9); 364 (14); 337 (100); 336 (35); 315 (67); 314 (37); 273 (60); 255 (59); 147 (34); 135 (23); 123 (67); 121 (90); 109 (59); 107 (50); 105 (30); 95 (35); 93 (37); 91 (42); 81 (41); 79 (39); 67 (33); 55 (39). HREIMS: 396.1966 (calcd for C₂₁H₃₂O₅S [M]⁺

396.1970). IR ν_{max} : 2946, 2875, 1727, 1467, 1212, 1161, 962, 819, 762, 681 cm⁻¹. [α]_D²⁰: -64.49 (c 1.2, CHCl₃). Compound **47**: ¹H RMN (300 MHz, CDCl₃) δ : 4.71 (1H, d, *J* = 8.6 Hz, H-17a), 4.33 (1H, d, *J* = 8.6 Hz, H-17b), 3.64 (3H, s, H-21), 2.60 (1H, s, H-13), 1.16 (3H, s, H-18), 0.81 (3H, s, H-20), 2.29–0.72 (20H, m). ¹³C NMR (75 MHz, CDCl₃) δ : 177.7 (s, C-19), 98.6 (s, C-16), 71.1 (t, C-17), 56.5 (d, C-5), 55.3 (t, C-15), 54.8 (d, C-9), 51.0 (q, C-21), 46.1 (d, C-13), 44.5 (s, C-8), 43.5 (s, C-4), 40.9 (t, C-7), 40.4 (t, C-1), 39.1 (s, C-10), 38.0 (t, C-3), 37.7 (t, C-14), 28.4 (q, C-18), 26.6 (t, C-12), 21.7 (t, C-6), 18.8 (t, C-2), 18.8 (t, C-11), 15.3 (q, C-20). EIMS *m*/*z* (%): 396 ([M]⁺, 4); 337 (100); 315 (37); 273 (49); 255 (20); 123 (43); 121 (45); 109 (37); 107 (29); 95 (23); 91 (24); 81 (27); 79 (23); 55 (24). HREIMS: 396.1988 (calcd for C₂₁H₃₂O₅S [M]⁺ 396.1970). IR ν_{max} : 2948, 2875, 1726, 1467, 1209, 1161, 963, 822, 685 cm⁻¹. [α]_D²⁰: -44.84 (c 1.2, CHCl₃).

5.1.13. Preparation of compounds 48 and 49

To 9.3 mg of compound 18 (0.027 mmol) dissolved in 3 ml of toluene were added 8.5 µl of 2-furoyl (3 equiv), Et₃N (15 µl, 4 equiv) and catalytic amount of DMAP. The reaction mixture was stirred under reflux for 18 h. The solvent was removed and the residue was purified by preparative-TLC using hexanes/AcOEt 40%, to yield 10.0 mg of compound **48** (30%) and 3.6 mg of compound **49** (25%) as amorphous white solids. 49 resulted be unstable and decomposes with the time. Compound **48**: ¹H NMR (300 MHz, CDCl₃) δ : 7.59 (1H, d, J = 1.0 Hz, H-5'), 7.21 (1H, d, J = 3.5 Hz, H-3'), 6.53 (1H, dd, J = 1.0, 3.5 Hz, H-4'), 5.16 (1H, s, H-11), 4.33 (1H, d, J = 11.3 Hz, H-17a), 4.27 (1H, d, J = 11.3 Hz, H-17b), 3.65 (3H, s, H-21), 1.17 (3H, s, H-18), 0.90 (3H, s, H-20), 2.48–0.83 (16H, m). ¹³C NMR (75 MHz, CDCl₃) δ : 177.7 (s, C-19), 158.5 (s, C-9), 157.4 (s, C-1'), 146.3 (d, C-5'), 144.1 (s, C-2'), 118.1 (d, C-11), 113.3 (d, C-3'), 111.7 (d, C-4'). 82.6 (s. C-16), 70.3 (t, C-17), 54.7 (t, C-15), 51.1 (q, C-21), 46.4 (d, C-5), 44.6 (d, C-13), 44.6 (s, C-4), 42.7 (s, C-8), 42.6 (t, C-14), 40.7 (t, C-1), 38.4 (s, C-10), 38.1 (t, C-3), 30.2 (t, C-12), 29.6 (t, C-7), 27.9 (q, C-18), 23.1 (q, C-20), 19.9 (t, C-2), 18.2 (t, C-6). EIMS m/z (%): 424 ([M⁺ - 18] 12); 312 (67); 297 (36); 273 (25); 271 (17); 255 (23); 253 (37); 237 (43); 213 (26); 197 (34); 173 (20); 172 (25); 157 (23); 145 (27); 144 (26); 143 (27); 131 (40); 129 (25); 117 (26); 112 (33); 109 (26); 107 (19); 105 (39); 95 (100); 91 (50); 81 (18); 79 (19); 55 (29). HREIMS: 424.2264 (calcd for C₂₆H₃₂O₅ ([M]⁺ – 18) 424.2250). IR ν_{max} : 3501, 2931, 2858, 1720, 1469, 1397, 1297, 1226, 1179, 1151, 1122, 971, 762 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 202.0 (3.51) y 251.0 (3.60) nm. $[\alpha]_D^{20}$: +7.60 (c 0.5, CHCl₃). Compound **49**: ¹H NMR (300 MHz, $CDCl_3$) δ : 7.55 (2H, s, H-5', H-5"), 7.12 (1H, d, J = 3.4 Hz, H-3' \circ H-3"), 7.08 (1H, d, J = 3.4 Hz, H-3' ó H-3"), 6.47 (2H, m, H-4', H-4"), 5.28 (1H, J = 12.3 Hz, H-17a), 5.23 (1H, s, H-11), 4.37 (1H, d, J = 12.3 Hz, H-17b), 3.64 (3H, s, H-21), 2.77 (1H, s, H-13), 1.17 (3H, s, H-18), 0.91 (3H, s, H-20), 2.48-0.83 (17H, m). ¹³C NMR (75 MHz, CDCl₃): 177.7 (s, C-19), 158.0 (s, C-9), 157.0 (s, C-1', C-1"), 146.2 (d, C-5' ó C-5"), 145.9 (d, C-5' ó C-5"), 145.1 (s, C-2' ó C-2"), 144.1 (s, C-2' ó C-2"), 117.8 (d, C-11), 117.8, 117.4 y 113.8 (4C, d, C-3', C-3", C-4', C-4"), 93.2 (s, C-16), 65.6 (t, C-17), 53.6 (t, C-15), 51.1 (q, C-21), 46.2 (d, C-5), 44.6 (s, C-4), 42.7 (t, C-14), 42.4 (d, C-13), 42.3 (s, C-8), 40.7 (t, C-1), 38.4 (s, C-10), 38.0 (t, C-3), 29.9 (t, C-12), 29.5 (t, C-7), 27.8 (q, C-18), 23.2 (q, C-20), 19.9 (t, C-2), 18.1 (t, C-6). EIMS m/z (%): 424 $([M]^+ - 112(C_5H_4O_3), 7); 312(44); 297(30); 253(16); 237(24); 197$ (16); 145 (15); 111 (23); 105 (18); 97 (11); 95 (100); 91 (21); 57 (21); 55 (24). HREIMS: 424.2262 (calcd for $C_{26}H_{32}O_5$ ([M]⁺ – 112 $(C_5H_4O_3))$ 424.2250).

5.1.14. Epoxidation of compound 17 to obtain compounds 50 and 51

To 70.7 mg of compound **17** (0.225 mmol) dissolved in 15 ml of dry DCM were added 222.6 mg (4 equiv) of MCPBA and 153.3 mg of NaHCO₃ (8.1 equiv). The reaction mixture was left to room temperature for 4 h, then the organic phase was separated and treated with a saturated solution of sodium thiosulfate. The organic

phase was separated again and dried over anhydrous magnesium sulfate, the mixture was filtered, the solvent was removed under reduced pressure and the crude was purified by preparative-TLC using hexanes/AcOEt 20% to yield 9.4 mg of compound 50 (12%) and 11.6 mg of compound 51 (15%) as amorphous white solids. Compound **50**: ¹H NMR (300 MHz, CDCl₃) δ: 3.63 (3H, s, H-21), 3.08 (1H, dd, J = 3.7, 9.8 Hz, H-11), 2.83 (1H, d, J = 5.4 Hz, H-17a), 2.79 (1H, d, *I* = 5.2 Hz, H-17b), 1.19 (3H, s, H-18), 0.65 (3H, s, H-20), 2.27–0.85 (18H, m). ¹³C NMR (100 MHz, CDCl₃) δ: 177.7 (s, C-19), 69.1 (s, C-9), 66.0 (s, C-16), 54.7 (t, C-17), 53.9 (d, C-11), 51.1 (q, C-21), 48.5 (d, C-5), 46.0 (t, C-15), 44.0 (s, C-4), 42.1 (s, C-8), 39.3 (t, C-1), 39.0 (t, C-3), 37.9 (s, C-10), 37.6 (t, C-14), 36.0 (d, C-13), 31.9 (t, C-12), 28.4 (q, C-18), 26.6 (t, C-7), 19.6 (t, C-2), 18.9 (t, C-6), 14.6 (q, C-20). EIMS *m*/*z* (%): 346 ([M]⁺, 10); 330 (8); 287 (19); 286 (13); 258 (19); 257 (12); 173 (23); 150 (26); 149 (100); 147 (27); 136 (70); 135 (57); 133 (27); 123 (71); 122 (74); 121 (60); 109 (49); 107 (65); 105 (53); 95 (39); 93 (46); 91 (55); 81 (41); 79 (42); 67 (32); 55 (56). HREIMS: 346.2137 (calcd for $C_{21}H_{30}O_4$ [M]⁺ 346.2144). IR v_{max} : 2929, 2855, 1722, 1458, 1226, 1148, 1036, 983, 893, 757 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.2 (3.10) nm. [α]_D²⁰: -0.64 (c 0.47, CHCl₃). Compound **51**: ¹H NMR (300 MHz, CDCl₃) δ: 3.64 (3H, s, H-21), 3.07 (1H, d, J = 3.4 Hz, H-11), 2.85 (1H, d, J = 4.5 Hz, H-17a), 2.80 (1H, d, J = 4.5 Hz, H-17b), 2.23 (1H, d, J = 13.5 Hz, H-15a), 1.21 (3H, s, H-18), 0.67 (3H, s, H-20), 2.10–0.98 (17H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 177.7 (s, C-19), 69.6 (s, C-9), 68.8 (s, C-16), 53.5 (d, C-11), 51.1 (q, C-21), 50.5 (t, C-17), 48.5 (d, C-5), 45.4 (t, C-14), 44.1 (s, C-4), 43.2 (s, C-8), 39.3 (t, C-1), 38.7 (t, C-3), 38.7 (d, C-13), 37.9 (s, C-10), 37.7 (t, C-12), 29.5 (t, C-7), 28.9 (t, C-15), 28.4 (q, C-18), 19.6 (t, C-2), 18.8 (t, C-6), 14.8 (q, C-20). EIMS m/z (%): 346 ([M]⁺, 49); 328 (35); 317 (37); 288 (72); 269 (32); 257 (35); 253 (53); 225 (40); 211 (57); 173 (71); 159 (45); 149 (79); 147 (52); 145 (51); 135 (57); 133 (54); 129 (43); 121 (86); 118 (78); 109 (61); 107 (84); 105 (96); 95 (53); 93 (60); 91 (100); 81 (60); 79 (67); 67 (48); 55 (81). HREIMS: 346.2144 (calcd for C₂₁H₃₀O₄ [M]⁺ 346.2144). IR *v*_{max}: 2926, 2855, 1722, 1459, 1383, 1226, 1147, 1038, 981, 775 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.0 (2.95) nm. $[\alpha]_D^{20}$: -6.92 (c 0.5, CHCl₃).

5.1.15. Preparation of compound 52

A solution of 24.4 mg of compound 2 (0.077 mmol) in 30 ml of dry CH₂Cl₂ at -78 °C was ozonized until the color of the solution changed to dark blue-gray (30 s). The reaction mixture was then quenched with 10 µl of dry Me₂S (1.76 equiv) and concentrated under vacuum. The residue was purified by preparative-TLC using hexanes/AcOEt (30%) to afford 4.3 mg of compound 52 (18%) as an amorphous white solid. ¹H NMR (300 MHz, CDCl₃) δ : 3.66 (3H, s, H-20), 2.39 (1H, s, H-13), 1.16 (3H, s, H-17), 0.88 (3H, s, H-19), 2.30-0.82 (20H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 222.5 (s, C-16), 177.7 (s, C-18), 56.5 (d, C-5), 54.7 (t, C-15), 53.7 (d, C-9), 51.0 (q, C-20), 47.5 (d, C-13), 43.5 (s, C-4), 42.2 (s, C-8), 40.8 (t, C-7), 40.4 (t, C-1), 39.3 (s, C-10), 37.7 (t, C-3), 37.0 (t, C-14), 29.3 (t, C-12), 28.5 (q, C-17), 20.5 (t, C-6), 18.8 (t, C-2), 18.5 (t, C-11), 15.7 (q, C-19). EIMS *m*/*z* (%): 318 ([M]⁺, 26); 300 (4); 286 (16); 259 (100); 149 (12); 133 (10); 123 (17); 121 (23); 109 (23); 107 (19); 95 (15); 93 (12); 79 (15); 67 (12); 55 (15). HREIMS: 318.2185 (calcd for C₂₀H₃₀O₃ [M]⁺ 318.2195). IR ν_{max} : 2945, 2870, 1726, 1463, 1235, 1192, 1150 cm⁻¹. $[\alpha]_D^{20}$: -67.07 (c 0.4, CHCl₃).

5.1.16. Preparation of compounds 53, 54 and 55

A solution of 19.7 mg of compound **8** (0.050 mmol) in 30 ml of dry CH₂Cl₂ at -78° C was ozonized until the color of the solution changed to dark blue-gray (30 s). The reaction mixture was then quenched with 10 µl of dry Me₂S (2.7 equiv) and concentrated under vacuum. The residue was purified by preparative-TLC using hexanes/AcOEt (20%) to afford 10.6 mg of compound **53** (54%), 4.4 mg of compound **54** (21%) and 4.2 mg (20%) of compound **55** as

amorphous white solids. Compound **53**: ¹H NMR (300 MHz. CDCl₃) δ: 7.35 (5H, m, H-22–H-26), 5.14 (1H, d, *J* = 12.4 Hz, H-20a), 5.07 (1H, d, J = 12.4 Hz, H-20b), 2.37 (1H, s, H-13), 1.21 (3H, s, H-17), 0.82 (3H, s, H-19), 2.37–0.76 (21H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 222.6 (s, C-16), 176.9 (s, C-18), 135.9 (s, C-21), 128.3 (d, C-23, C-25), 128.0 (d, C-22, C-26), 127.9 (d, C-24), 68.8 (t, C-20), 56.7 (d, C-5), 54.7 (t, C-15), 53.7 (d, C-9), 47.5 (d, C-13), 43.7 (s, C-8), 42.2 (s, C-4), 40.8 (t, C-7), 40.4 (t, C-1), 39.4 (s, C-10), 37.7 (t, C-3), 36.9 (t, C-14), 29.2 (t, C-12), 28.6 (q, C-17), 20.6 (t, C-6), 18.8 (t, C-2), 18.5 (t, C-11), 15.8 (q, C-19). IEMS *m*/*z* (%): 394 ([M]⁺, 14); 303 (30); 285 (11); 259 (15); 257 (22); 149 (15); 121 (11); 109 (12); 107 (13); 95 (12); 93 (11); 91 (100); 81 (14); 79 (12); 55 (13). HREIMS: 394.2522 (calcd for $C_{26}H_{34}O_3$ [M]⁺ 394.2508). IR ν_{max} : 2947, 2872, 1723, 1455, 1232, 1146, 752, 699 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 202.0 (3.53) nm. [α]_D²⁰: -40.19 (c 1.1, CHCl₃). Compound **54**: ¹H NMR (300 MHz, CDCl₃) δ: 7.36 (5H, m, H-22–H-26), 5.14 (1H, d, J = 12.4 Hz, H-20a), 5.06 (1H, d, J = 12.4 Hz, H-20b), 4.76 (1H, sa, H-13), 2.25 (2H, m, H-15), 1.21 (3H, s, H-17), 0.76 (3H, s, H-19), 2.27-0.80 (18H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 176.8 (s, C-18), 172.1 (s, C-16), 135.7 (s, C-21), 128.3 (d, C-23, C-25), 128.0 (d, C-22, C-26), 127.9 (d, C-24), 75.6 (d, C-13), 65.9 (t, C-20), 56.6 (d, C-5), 52.8 (d, C-9), 47.9 (t, C-15), 43.6 (s, C-4), 42.8 (t, C-7), 40.9 (t, C-1), 39.2 (s, C-10), 37.5 (t, C-3), 33.6 (s, C-8), 32.8 (t, C-14), 28.6 (q, C-17), 28.5 (t, C-12), 19.8 (t, C-6), 18.9 (t, C-2), 16.1 (t, C-11), 16.0 (q, C-19). IEMS *m*/*z* (%): 410 ([M]⁺, 4); 319 (16); 273 (17); 259 (11); 121 (8); 107 (10); 91 (100); 81 (11); 55 (10). HREIMS: 410.2458 (calcd for $C_{26}H_{34}O_4$ [M]⁺ 410.2457). IR ν_{max} : 2951, 2930, 1725, 1458, 1227, 1143, 996, 697 cm⁻¹. UV (EtOH) λ_{max} $(\log \epsilon)$: 207.6 (3.43) nm. $[\alpha]_D^{20}$: -61.00 (c 0.4, CHCl₃). Compound **55**: ¹H NMR (300 MHz, CDCl₃) δ: 7.34 (5H, m, H-22–H-26), 5.15 (1H, d, I = 12.4 Hz, H-20a), 5.04 (1H, d, I = 12.4 Hz, H-20b), 2.72 (1H, d, J = 13.0 Hz, H-15a), 2.18 (1H, d, J = 13.0 Hz, H-15b), 1.20 (3H, s, H-17), 0.83 (3H, s, H-19), 2.54–0.65 (21H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 177.2 (s, C-18), 176.9 (s, C-16), 135.9 (s, C-21), 128.2 (d, C-23, C-25), 127.9 (d, C-22, C-26), 127.8 (d, C-24), 65.8 (t, C-20), 56.9 (d, C-5), 50.9 (d, C-9), 44.9 (t, C-15), 43.6 (s, C-4), 41.8 (t, C-7), 40.7 (t, C-1), 39.5 (s, C-10), 37.6 (t, C-3), 36.7 (s, C-8), 30.3 (t, C-12, C-14), 28.7 (q, C-17), 21.8 (t, C-6 ó C-13), 20.3 (t, C-6 ó C-13), 19.7 (t, C-2), 19.1 (t, C-11), 18.1 (q, C-19). EIMS *m*/*z* (%): 412 ([M]⁺, 1); 394 (2); 352 (21); 303 (10); 277 (16); 275 (21); 261 (14); 135 (10); 121 (10); 109 (14); 107 (15); 95 (13); 91 (100); 81 (14); 55 (8). HREIMS: 412.2628 (calcd for C₂₆H₃₆O₄ [M]⁺ 412.2614). IR *v*_{max}: 2934, 2870, 1721, 1461, 1225, 1140, 698 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 205.4 (3.52) nm. [α]_D²^U: -40.19 (c 1.1, CHCl₃).

5.1.17. Preparation of compounds 56 and 57

A solution of 44.0 mg of compound 10 (0.133 mmol) in 30 µl of dry CH_2Cl_2 at -78° C was ozonized until the color of the solution changed to dark blue-gray (2 min). The reaction mixture was then quenched with 20 µl of dry Me₂S (2.0 equiv) and concentrated under vacuum. The residue was purified by preparative-TLC using hexanes/AcOEt (15%) to afford 16.8 mg of compound 56 (38%) and 3.2 mg of compound **57** (7%) as amorphous white solids. Compound **56**: ¹H NMR (300 MHz, CDCl₃) δ: 3.65 (3H, s, H-20), 3.63 (1H, s, H-15), 3.35 (1H, d, J = 1.92 Hz, H-13), 1.20 (3H, s, H-17), 0.90 (3H, s, H-19), 2.52–0.82 (19H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 221.8 (s, C-16), 177.7 (s, C-18), 80.8 (d, C-15), 56.5 (d, C-5), 53.5 (d, C-9), 51.0 (q, C-20), 46.4 (d, C-13), 44.7 (s, C-8), 43.5 (s, C-4), 40.4 (t, C-1), 39.4 (s, C-10), 37.7 (t, C-3), 33.9 (t, C-7), 33.6 (t, C-14), 29.4 (t, C-12), 28.5 (q, C-17), 19.7 (t, C-6), 18.8 (t, C-2), 18.8 (t, C-11), 16.0 (q, C-19). EIMS m/z (%): 334 ([M]⁺, 96); 319 (10); 302 (18); 291 (15); 274 (100); 259 (23); 247 (17); 215 (16); 175 (11); 147 (17); 135 (22); 123 (47); 121 (82); 109 (55); 107 (53); 95 (37); 93 (35); 81 (44); 79 (35); 55 (44); 53 (12). HREIMS: 334.2153 (calcd for $C_{20}H_{30}O_4$ [M]⁺ 334.2144). IR v_{max} : 3467, 2934, 2854, 1725, 1464, 1234, 1155, 1002, 736 cm⁻¹. $[\alpha]_D^{20}$: -23.91 (c 0.2, CHCl₃). Compound **57**: ¹H NMR (300 MHz, CDCl₃) δ : 5.08 (1H, s, H-17a), 5.02 (1H, s, H-17b), 3.66 (1H, s, OH), 3.64 (3H, s, H-21), 3.64 (1H, s, H-15), 1.18 (3H, s, H-18), 0.84 (3H, s, H-20), 2.20–0.87 (19H, m). 13 C NMR (75 MHz, CDCl₃) δ : 177.9 (s, C-19), 111.5 (s, C-16), 93.9 (t, C-17), 93.7 (d, C-15), 56.6 (d, C-5), 52.6 (d, C-9), 51.0 (q, C-21), 45.7 (s, C-8), 43.5 (s, C-4), 42.1 (d, C-13), 40.3 (t, C-1), 39.2 (s, C-10), 37.8 (t, C-3), 34.8 (t, C-7), 34.5 (t, C-12), 28.5 (q, C-18), 25.7 (t, C-14), 20.4 (t, C-6), 18.8 (t, C-2), 17.9 (t, C-11), 15.2 (q, C-20). EIMS *m*/*z* (%): 364 ([M]⁺, 1); 334 (84); 274 (97); 259 (29); 247 (19); 149 (22); 133 (29); 123 (57); 121 (100); 109 (66); 107 (66); 105 (34); 95 (48); 93 (45); 91 (48); 81 (56); 79 (46); 67 (43); 55 (62). HREIMS: 364.2252 (calcd for C₂₁H₃₂O₅ [M]⁺ 364.2250). IR ν_{max} : 3439, 2943, 2854, 1724, 1464, 1234, 1159, 1097, 1064, 1002, 977 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 202.0 (3.23) nm. [α]₀²⁰: –24.48 (c 0.3, CHCl₃).

5.1.18. Preparation of compound 58

A solution of 40.0 mg of compound 12 (0.121 mmol) in dry CH_2Cl_2 at -78° C was ozonized until the color of the solution changed to dark blue-gray (2 h). The reaction mixture was then quenched with 20 µl of dry Me₂S (2.2 equiv) and concentrated under vacuum. The residue was purified by preparative-TLC using hexanes/AcOEt (40%) to afford 16.3 mg of compound 58 (39%) as an amorphous white solid. ¹H NMR (300 MHz, CDCl₃) δ : 3.65 (3H, s, H-20), 2.75 (1H, s, H-13), 1.16 (3H, s, H-17), 0.86 (3H, s, H-19), 2.43-0.77 (18H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 184.7 (s, C-15), 182.3 (s, C-16), 177.8 (s, C-18), 55.6 (d, C-5), 51.1 (q, C-20), 44.9 (d, C-9), 44.1 (s, C-8), 43.4 (s, C-4), 41.4 (t, C-7), 39.1 (t, C-1), 38.6 (s, C-10), 37.6 (t, C-3), 36.9 (d, C-13), 31.3 (t, C-14), 28.5 (q, C-17), 21.8 (t, C-12), 19.6 (t, C-6), 19.0 (t, C-2), 18.4 (t, C-11), 17.5 (q, C-19). EIMS m/z (%): 348 ([M]⁺, 15); 330 (6); 276 (36); 261 (38); 217 (60); 180 (34); 135 (33); 121 (100); 109 (60); 107 (50); 93 (43); 91 (41); 81 (42); 79 (59); 67 (39); 55 (52). HREIMS: 348.1925 (calcd for C₂₀H₂₈O₅ [M]⁺ 348.1937). IR v_{max}: 2948, 2875, 1712, 1457, 1239, 1151, 755 cm⁻¹. $[\alpha]_D^{20}$: -21.43 (c 0.6, CHCl₃).

5.1.19. Preparation of compound 59

A solution of 32.5 mg of compound **30** (0.087 mmol) in dry CH_2Cl_2 at -78° C was ozonized until the colour of the solution changed to dark blue-gray (2 h). The reaction mixture was then quenched with $15 \,\mu$ l of dry Me₂S (2.3 equiv) and concentrated under vacuum. The residue was purified by preparative-TLC using Hexanes/AcOEt (20%) to afford 11.1 mg of compound 59 (34%) as an amorphous white solid. ¹H NMR (300 MHz, CDCl₃) δ : 4.80 (1H, d, J = 1.89 Hz, H-15), 3.65 (3H, s, H-20), 2.56 (1H, s, H-13), 2.10 (3H, s, OCOCH₃), 1.17 (3H, s, H-17), 0.89 (3H, s, H-19), 2.27-0.82 (18H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 217.5 (s, C-16), 177.6 (s, C-18), 169.9 (s, OCOCH₃), 80.3 (d, C-15), 56.2 (d, C-5), 53.1 (d, C-9), 51.1 (q, C-20), 46.8 (d, C-13), 44.5 (s, C-8), 43.5 (s, C-4), 40.2 (t, C-1), 39.4 (s, C-10), 37.6 (t, C-3), 34.6 (t, C-7), 33.8 (t, C-14), 29.4 (t, C-12), 28.4 (q, C-17), 20.4 (q, OCOCH₃), 19.6 (t, C-6), 18.7 (t, C-2, C-11), 15.9 (q, C-19). EIMS m/z (%): 376 ([M]⁺, 51); 361 (29); 335 (21); 334 (100); 317 (29); 316 (43); 275 (42); 274 (65); 257 (36); 256 (27); 123 (26); 121 (67); 109 (34); 107 (34); 105 (22); 95 (29); 93 (25); 91 (26); 81 (34); 79 (28); 67 (24); 55 (32). HREIMS 376.2235 (calcd for C₂₂H₃₂O₅ [M]⁺ 376.2250). IR v_{max}: 3487, 2947, 2870, 1754, 1725, 1464, 1371, 1232, 1155, 1023, 756 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 200.6 (2.55) nm. [α]_D²⁰: -11.39 (c 1.1, CHCl₃).

5.1.20. Preparation of compounds 60–63

A solution of 77.3 mg of compound **35** (0.199 mmol) in 25 ml of dry CH₂Cl₂ at -78° C was ozonized until the colour of the solution changed to dark blue-gray (30 s). The reaction mixture was then quenched with 30 μ l of dry Me₂S (2 equiv) and concentrated under vacuum. The residue was purified by preparative-TLC using hexanes/AcOEt (20%) to afford 22.3 mg of compound **60** (29%),

9.8 mg of compound 61 (12%), 9.5 mg (20%) of compound 62 and 9.3 mg of compound **63** as yellow oils. Compound **60**: ¹H NMR $(300 \text{ MHz}, \text{CDCl}_3) \delta$: 3.74 (1H, dd, J = 4.0, 14.2 Hz, H-20a), 3.59 (1H, dd, J = 4.0, 14.2 Hz, H-20b), 2.39 (1H, s, H-13), 1.17 (3H, s, H-17), 0.86 (3H, s, H-19), 0.10 (9H, s, Si(CH₃)₃), 2.28-0.80 (20H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 222.5 (s, C-16), 178.1 (s, C-18), 56.8 (t, C-20), 56.7 (d, C-5), 54.7 (t, C-15), 53.6 (d, C-9), 47.5 (d, C-13), 43.8 (s, C-8), 42.2 (s, C-4), 40.8 (t, C-7), 40.5 (t, C-1), 39.3 (s, C-10), 37.8 (t, C-3), 37.0 (t, C-14), 29.2 (t, C-12), 28.7 (q, C-17), 20.7 (t, C-6), 18.8 (t, C-2), 18.5 (t, C-11), 15.7 (q, C-19), -3.1 (q, Si(CH₃)₃). EIMS *m*/*z* (%): 390 ([M]⁺, 47); 375 (100); 287 (15); 259 (82); 199 (19); 163 (20); 123 (33); 121 (24); 109 (34); 107 (32); 105 (21); 97 (34); 95 (41); 93 (33); 91 (32); 81 (52); 79 (38); 73 (61); 67 (35); 59 (49); 55 (59). HREIMS: 390.2586 (calcd for $C_{23}H_{38}O_3Si [M]^+$ 390.2590). IR ν_{max} : 2951, 2871, 1741, 1720, 1249, 1147, 858 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.2 (2.75) nm. $[\alpha]_{D}^{20}$: -44.06 (c 1.0, CHCl₃).

Compound **61**: ¹H NMR (300 MHz, CDCl₃) δ: 4.79 (1H, s, H-13), 3.76 (1H, d, J = 14 Hz, H-20a), 3.59 (1H, d, J = 14 Hz, H-20b), 1.18 (3H, s, H-17), 0.84 (3H, s, H-19), 0.10 (9H, s, Si(CH₃)₃), 2.24-0.84 (20H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 178.0 (s, C-18), 172.1 (s, C-16), 75.6 (d, C-13), 56.9 (t, C-20), 56.6 (d, C-5), 52.9 (d, C-9), 47.9 (t, C-15), 43.6 (s, C-4), 42.8 (t, C-7), 40.9 (t, C-1), 39.2 (s, C-10), 37.6 (t, C-3), 33.7 (s, C-8), 32.8 (t, C-14), 28.8 (q, C-17), 28.8 (t, C-12), 19.9 (t, C-6), 18.9 (t, C-2), 16.2 (t, C-11), 16.0 (q, C-19), -3.1 (q, Si(CH₃)₃). EIMS m/z (%): 406 ([M]⁺, 27); 391 (100); 303 (36); 275 (45); 215 (27); 193 (22); 121 (20); 107 (22); 95 (31); 81 (24); 73 (33); 59 (23); 55 (26). HREIMS: 406.2556 (calcd for C₂₃H₃₈O₄Si [M]⁺ 406.2539). IR *v*_{max}: 2954, 2874, 1724, 1223, 1146, 858 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.4 (2.79) nm. $[\alpha]_D^{20}$: -58.37 (c 0.9, CHCl₃). Compound **62**: ¹H NMR (300 MHz, CDCl₃) δ : 3.76 (1H, d, I = 14 Hz, H-20a), 3.76 (1H, d, *I* = 14 Hz, H-20b), 2.75 (1H, d, *I* = 12.8 Hz, H-15a), 2.17 (1H, d, *I* = 12.8 Hz, H-15b), 1.17 (3H, s, H-17), 0.87 (3H, s, H-19), 0.10 (9H, s, Si(CH₃)₃), 2.22–0.69 (19H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 178.4 (s, C-16), 177.6 (s, C-18), 56.8 (d, C-5), 56.8 (t, C-20), 51.0 (d, C-9), 45.0 (t, C-15), 43.7 (s, C-4), 41.9 (t, C-7), 40.7 (t, C-1), 39.5 (s, C-10), 37.8 (t, C-3), 36.8 (s, C-8), 30.3 (t, C-14, C-12), 28.8 (q, C-17), 21.9 (t, C-6 or C-13), 20.3 (t, C-6 or C-13), 19.8 (t, C-2), 19.1 (t, C-11), 18.1 (q, C-19), -3.1 (q, Si(CH₃)₃). EIMS m/z (%): 408 ([M]⁺, 9); 393 (51); 349 (41); 305 (19); 277 (100); 245 (21); 217 (79); 173 (14); 147 (21); 135 (40); 123 (27); 121 (33); 109 (52); 107 (28); 95 (38); 93 (24); 81 (37); 79 (20); 73 (36); 67 (22); 59 (32); 55 (28). HREIMS: 408.2679 (calcd for $C_{23}H_{40}O_4Si \ [M]^+ 408.2696$). IR ν_{max} : 2952, 2872, 1703, 1250, 1141, 858, 757 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.8 (2.89) nm. [α]_D²⁰: -34.11 (c 0.9, CHCl₃). Compound **63**: ¹H NMR (300 MHz, CDCl₃) δ : 3.75 (1H, d, J = 14.2 Hz, H-20a), 3.57 (1H, d, J = 14.2 Hz, H-20b), 3.10 (1H, s, H-13), 1.15 (3H, s, H-17), 0.83 (3H, s, H-19), 0.10 (9H, s, Si (CH₃)₃), 2.32–0.79 (20H, m). ¹³C NMR (75 MHz, CDCl₃) δ: 178.1 (s, C-18), 117.5 (s, C-16), 56.7 (t, C-20), 56.5 (d, C-13), 54.9 (d, C-5), 50.4 (t, C-15), 43.8 (s, C-4), 42.8 (s, C-8), 41.0 (t, C-7), 40.4 (t, C-1), 39.5 (s, C-10), 39.3 (d, C-9), 37.9 (t, C-3), 36.9 (t, C-14), 28.7 (q, C-17), 25.6 (t, C-12), 21.5 (t, C-6), 18.8 (t, C-2), 17.8 (t, C-11), 15.1 (q, C-19), -3.1 (q, Si (CH₃)₃). EIMS *m*/*z* (%): 406 ([M]⁺, 8); 391 (76); 375 (35); 347 (40); 303 (20); 275 (44); 259 (33); 231 (21); 215 (60); 199 (30); 157 (30); 149 (29); 135 (54); 133 (41); 123 (46); 121 (74); 109 (62); 107 (73); 105 (39); 95 (67); 93 (55); 91 (50); 81 (87); 79 (51); 73 (100); 67 (45); 59 (67); 55 (63). HREIMS: 406.2545 (calcd for C₂₃H₃₈O₄Si [M]⁺ 406.2539). IR *v*_{max}: 2950, 2850, 1721, 1250, 1154, 857, 757 cm⁻¹. UV (EtOH) λ_{max} (log ϵ): 201.4 (3.04) nm. [α]_D²⁰: -63.23 (c 0.9, CHCl₃).

5.2. Cell culture

The murine macrophage cell line RAW 264.7 was maintained in RPMI 1640 medium supplemented with 10% FCS, L-glutamine and antibiotics, as previously described [29].

5.3. Determination of NO synthesis

NO release was determined with Griess reagent as previously described [30]. Briefly, NO release was determined spectrophotometrically by the accumulation of nitrite in the medium. The absorbance at 548 nm was compared with a standard of NaNO₂.

5.4. MTT assay for cell viability

RAW 264.7 cells were plated at a density of 10⁵ cells/well in 96well plates. To determine the appropriate concentration not toxic to cells, cells were incubated in the presence of different concentrations of derivatives for 24 h, before they were then reacted with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) at 37 °C for 4 h. The reaction product, formazan, was extracted with dimethyl sulfoxide (DMSO) and the absorbance was read at 540 nm. Assays were performed in triplicate, and results are expressed as the percent reduction in cell viability compared to untreated control cultures for at least three independent experiments.

5.5. Preparation of cytosolic and nuclear extracts

Cells were washed twice with ice-cold buffer A (10 mM Hepes, pH 7.9; 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 2 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 μ g/ml TLCK (N_{α} -Tosyl-L-lysine chloromethyl ketone hydrochloride), 5 mM NaF, 1 mM NaV0₄, 10 mM Na₂MoO₄) containing 120 mM NaCl and scraped off the plate. Cells were lysed at 4 °C with 0.2 ml of buffer A supplemented with 0.5% Nonidet P-40 and under continuous shaking. After centrifugation of the cell lysate the supernatant was stored at -80 °C (cytosolic extract) and the pellets were resuspended in 50 μ l of buffer A supplemented with 20% glycerol-0.4 M KCl and gently shaken for 30 min at 4 °C. Protein content was assayed using the Bio-Rad protein reagent. All cell fractionation steps were carried out at 4 °C.

5.6. Western blot analysis

Western blot of cell extracts were prepared and incubated with anti-NOS-2, anti-I κ B α and anti- β -actin (Santa Cruz Biotechnology). Blots were developed by ECL according to the manufacturer's instructions (GE Healthcare). β -Actin was used as a loading control.

5.7. RNA analysis and quantitative PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Quantitative PCR (SYBRgreen) analysis was performed with an ABI 7700 sequence detector as described [30]. Each sample was run in duplicate, and all samples were analyzed in parallel for the expression of the housekeeping gene 36B4 (acidic ribosomal phosphoprotein P0), used as an endogenous control to normalize the expression level of target genes. Fold induction was determined from average replicate values. Primer sequences are available on request.

5.8. Assay of cytokines

Cytokine production in the cell culture was measured with ELISA kits from R&D systems according to the manufacturer's instructions.

5.9. Statistical analysis

The data shown are the mean \pm S.D. Statistical significance was estimated with Student's *t* test for unpaired observations using the InStart program (GraphPad Software). Values of **p* < 0.05 were considered significant.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.01.052.

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