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Activity of lupane triterpenoids from *Maytenus* species as inhibitors of nitric oxide and prostaglandin E_2

Carolina P. Reyes,[a] Marvin J. Núñez,[a] Ignacio A. Jiménez,[a] Jérôme Busserolles,[b] María J. Alcaraz[b] and Isabel L. Bazzocchi[a,*]

[a]*Instituto Universitario de Bio-Orgánica Antonio González, Universidad de La Laguna and Instituto Canario de Investigación del Cáncer, Avda. Astrofísico Francisco Sánchez 2, La Laguna, 38206 Tenerife, Canary Islands, Spain*
[b]*Departamento de Farmacología, Universidad de Valencia, Facultad de Farmacia, Av. Vicent Andrés Estellés s/n, Valencia, Spain*

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Abstract—In the present study, we report that three new lupane triterpenes (**1**–**3**), in addition to 16 known ones (**4**–**19**), were isolated from the root bark of *Maytenus cuzcoina* and the leaves of *Maytenus chiapensis*. Their structures were elucidated by spectral analysis, including homonuclear and heteronuclear correlation NMR experiments (COSY, ROESY, HSQC, and HMBC). The natural compounds and derivatives **6a**, **6b**, **9a**, and **9b** have been tested for potential anti-inflammatory activity, and several compounds including 3-*epi*calenduladiol (**2**), 11α-hydroxy-glochidone (**3**), rigidenol (**6**), acetoxy-rigidenol (**6a**), 11α-acetoxy-30-chloro-3-oxo-lup-20(29)-ene (**6b**), betulin (**9**), 28-acetoxy-betulin (**9a**), *epi*betulin (**12**), *epi*betulinic acid (**13**), and betulonic acid (**16**) exhibited potent inhibitory effects on NO and prostaglandin E_2 production in mouse macrophages (RAW 264.7) stimulated with bacterial endotoxin. The structure–activity relationship is discussed in detail.
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1. Introduction

Species of the family Celastraceae have a long history in the traditional medicine.[1] Over the last 30 years or so, a large number of secondary metabolites exhibiting a wide range of bioactivity have been extracted from species of the genus *Maytenus*, including a large number of dihydro-β-agarofuran sesquiterpenoids,[2] phenolic and quinone–methide triterpenes,[3], and a diverse array of triterpenoids.

Triterpenoids from the Celastraceae belonged to the lupane, oleanane, friedelane, taraxerane, glutinane, ursane, dammarane, and baccharane series.[4] Lupane triterpenoids are pentacyclic compounds with 30 carbon atoms, biosynthetically derived from the cyclization of squalene, and a vast class of natural products whose structural diversity includes a wide array of functional groups.[5] Compounds of this class are reported to be bioactive with cytotoxic,[6] antitumor-promoting,[7] antiviral,[8] and anti-inflammatory activities.[9]

Stimulation of macrophages with bacterial lipopolysaccharide (LPS) or cytokines causes the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) with the consequent generation of large quantities of NO and prostaglandins (PGs).[10] A variety of agents induce iNOS producing high concentrations of NO which contribute to the antimicrobial and antitumor action of macrophages.[11] Nevertheless, a high production of NO contributes to tissue injury as occurs in chronic inflammatory diseases.[12] Interaction of NO with superoxide anion produces the reactive species peroxynitrite which can lead to energy depletion and modification of proteins, lipids, carbohydrates, and nucleic acids. Thus, NO and related species have been implicated in atherosclerosis, arthritis, endotoxemia, and other pathologies.[13] COX activity catalyzes the rate-limiting step in prostanoid synthesis. The inducible COX-2 isozyme plays an important role in inflammation, pain, fever, and cancer,[14] whereas the pathogenesis of gastrointestinal damage by non-steroidal anti-inflammatory drugs has been mainly related to the inhibition of the constitutive isoform COX-1.[15] COX-2 is overexpressed in several human cancers and possesses proangiogenic as well as procarcinogenic effects.[16] Thus, inhibitors of NO and PG production in macrophages

* Corresponding author. Tel.: +34 922 318594; fax: +34 922 318571; e-mail: ilopez@ull.es

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are potential anti-inflammatory or cancer chemopreventive drugs.

As part of an intensive investigation on the biological activities of natural products from plants of the family Celastraceae, we have isolated 19 lupane triterpenes (**1**–**19**) (Fig. 1) from the root bark of *Maytenus cuzcoina* and the leaves of *Maytenus chiapensis*. The natural compounds and derivatives **6a**, **6b**, **9a**, and **9b** were investigated for their pharmacological activity as inhibitors of NO and PGE_2 production in macrophages. The structures of the new compounds (**1**–**3**) and the derivative of rigidenol, 11α-acetoxy-30-chloro-3-oxo-lup-20(29)-ene (**6b**), were determined on the basis of spectroscopic data, including ^{1}H–^{13}C heteronuclear correlation (HSQC), long-range correlation with inverse detection (HMBC), and ROESY experiments. The known compounds (**4**–**19**, **6a**, **9a**, and **9b**) were identified as 28-hydroxy-3,20-dioxo-29-norlupane (**4**),[17] nepeticin (**5**),[18] rigidenol (**6**),[18] glochidone (**7**),[19] lupeol (**8**),[19] betulin (**9**),[17] betulinic aldehyde (**10**),[20] betulin 3-caffeate (**11**),[21] *epi*betulin (**12**),[17] *epi*betulinic acid (**13**),[20] lupenone (**14**),[19] betulone (**15**),[17] betulonic acid (**16**),[17] 3-*epi*glochidiol (**17**),[19] glochidiol (**18**),[19] glochidonol (**19**),[19] acetoxy-rigidenol (**6a**),[18] 28-acetoxy-betulin (**9a**),[22] and 3, 28-diacetoxy-betulin (**9b**)[22] by comparison of their spectral data with values reported in the literature.

2. Results and discussion

Repeated chromatography of the *n*-hexane/Et_2O (1:1) extract of the root bark of *M. cuzcoina* and the CH_2Cl_2 extract of the leaves of *M. chiapensis* on silica gel and Sephadex LH-20 yielded three new lupane triterpenoids (**1**–**3**).

Compound **1** was assigned the molecular formula $C_{30}H_{50}O_2$ by HREIMS. The IR spectrum suggested that it contained hydroxyl groups (3413 cm^{-1}) and a terminal double bond (2926, 1731, and 883 cm^{-1}). The ^{1}H NMR spectrum (Table 1) showed six methyl groups [δ_H 0.77, 0.85, 0.94, 0.97, 1.03, 1.05 (each 3H, s)], an isopropenyl group [δ_H 1.68 (3H, s), 4.58 (1H, br s), and 4.71 (1H, br s)], and two oxymethine protons geminal to hydroxyl groups [δ_H 3.36 (1H, t, J = 7.3 Hz) and 3.92 (1H, dt, J = 5.3, 10.7 Hz)], and a typical lupene H_β-19 proton signal δ_H 2.35 (1H, dt, J = 4.9, 10.8 Hz). All these data indicate that compound **1** is a two-substituted lupane triterpene.

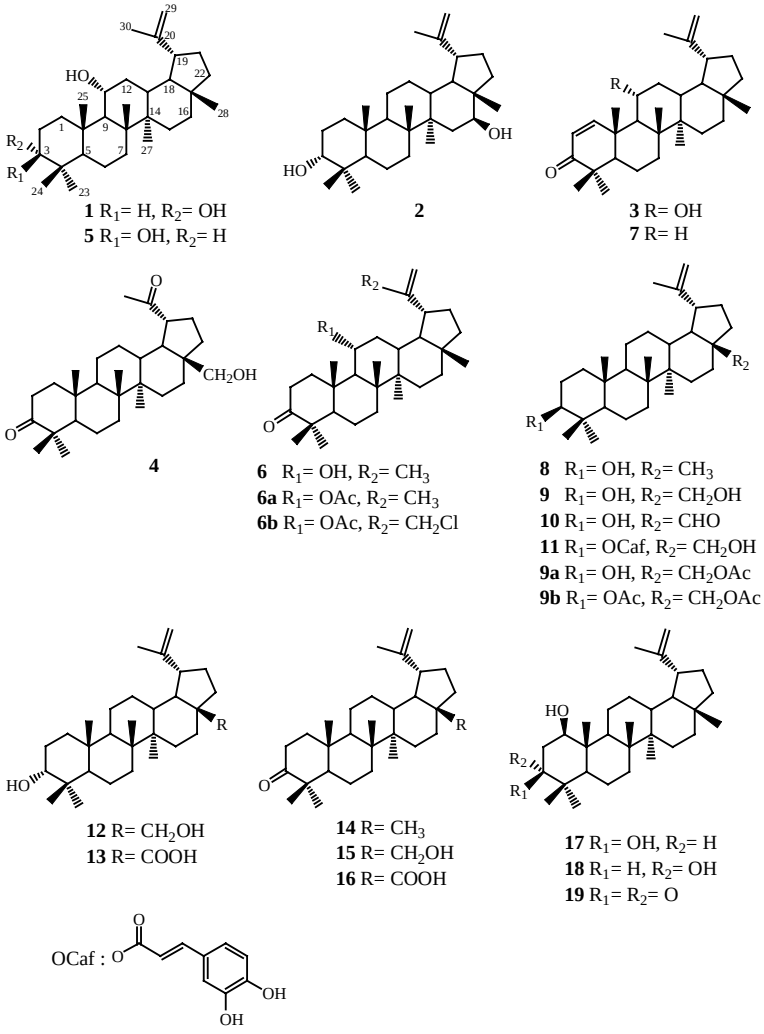

Figure 1. Chemical structures of compounds **1**–**19** isolated from *Maytenus chiapensis*, *Maytenus cuzcoina*, and the derivatives **6a**, **6b**, **9a**, and **9b**.

Table 1. ^{1}H NMR (400 MHz) data (δ, $CDCl_3$, J are given hertz in parentheses) of **1**–**4**

H	1	2	3	4[a]
1	2.30, 1.47	1.22 dd (12.4, 17.2) 1.38 dd (6.4, 12.4)	8.30 d (10.4)	1.00, 1.52
2	1.97, 1.48	1.53 dd (6.0, 12.4) 1.93 dt (6.0, 18.8)	5.71 d (10.4)	1.35, 2.36
3	3.36 t (7.3)	3.38 t (2.6)		
5	1.20	1.20	1.55	1.10[b]
6	1.28	1.41[b]	1.35,1.90	1.25
7	1.32	1.41[b]	1.44	1.45
9	1.47	1.40[b]	1.75	1.16
11	3.92 dt (5.3, 10.7)	1.20, 1.43	4.85 dt (4.8, 10.7)	1.17
12	1.18, 1.99	1.05, 1.69	1.98	1.37
13	1.77 t (10.9)	1.65	1.90 t (10.9)	1.40
15	1.02, 1.56	1.31 d (5.0) 1.56 dd (8.0, 13.6)	1.24, 1.40[b]	0.96
16	1.45, 2.28	3.60 dd (4.7, 11.2)	1.45[b], 1.56	1.10[b], 1.98
18	1.42	1.40m[b]	1.45[b]	2.25 t (11.7)
19	2.35 dt (4.9, 10.8)	2.50 dt (5.6,10.4)	2.40 dt (4.9, 10.8)	2.31 m
21	1.34, 1.92	1.40[b], 1.98	1.40[b], 2.01	1.90
22	1.25, 1.40	1.28, 1.65 t (10.4)	1.31, 1.40[b]	1.23, 2.10
23	0.94 s	0.93 s	1.12 s[b]	1.07 s
24	0.85 s	0.82 s	1.12 s[b]	0.99 s
25	1.05 s	0.84 s	1.26 s	0.73 s
26	1.03 s	1.04 s	1.11 s	0.89 s
27	0.97 s	1.00 s	0.98 s	0.93 s
28	0.77 s	0.79 s	0.80 s	2.98, 3.55 d_{AB} (10.6)
29	4.58 br s 4.71 br s	4.59 br s 4.68 br s	4.61 br s 4.73 br s	
30	1.68 s	1.68 s	1.70 s	1.84 s

[a] C_6D_6.
[b] Overlapping signals.

Its ^{13}C NMR spectrum (Table 2) revealed 30 carbon signals, which were assigned by DEPT experiments as seven methyls, nine methylenes, five methines, five quaternary carbons, two alcoholic methines, and two olefinic carbons. The two sp^2 carbons observed at δ_C 150.2 (s) and 109.8 (t) in the ^{13}C NMR spectrum confirmed the $\Delta^{20,29}$-functionality of a lupane skeleton. The positions of the two hydroxyl groups were determined through spectral data. Thus, the multiplicity and ^{1}H NMR shift of the oximethine group at δ_H 3.36 agree with those of the biogenetic hydroxyl group at the C-3 position and were confirmed by an HMBC experiment in which the protons of the geminal methyl groups (δ_H 0.94, 0.85) at C-4 showed ^{2}J correlations with C-4 (δ_C 37.8) and ^{3}J interactions with C-3 (δ_C 75.9) and C-5 (δ_C 48.9). An HMBC experiment established the regiosubstitution of the second hydroxyl group at C-11 since ^{2}J correlations of the oxymethine proton signal (δ_H 3.92) to C-9 (δ_C 55.6) and C-12 (δ_C 37.6), and ^{3}J correlations to C-13 (δ_C 37.1) and C-10 (δ_C 39.1), were observed.

The relative stereochemistry of the oxymethine proton at the C-3 position was established as β, on the basis of the coupling constants and confirmed by ROESY experiment, showing NOE effects with the Me-23 and Me-24 resonating at δ_H 0.94 and 0.85, respectively, which indicated an equatorial position for H-3 on the β-face and an axial orientation for the hydroxyl group on the α-face. Similarly, the relative stereochemistry of the hydroxyl group at C-11 was assigned as α on the

Table 2. ^{13}C NMR (100 MHz) data (δ, $CDCl_3$) of **1**–**4**[a]

C	1	2	3	4
1	35.4 t	33.3 t	123.6 d	39.3 t
2	25.6 t	25.2 t	165.1 d	33.5 t
3	75.9 d	76.2 d	205.3 s	214.9 s
4	37.8 s	37.5 s	45.0 s	46.9 s
5	48.9 d	49.0 d	52.9 d	54.6 d
6	18.3 t	18.2 t	18.9 t	19.6 t
7	35.1 t	34.1 t	34.5 t	33.8 t
8	42.7 s	41.1 s	42.9 s	40.7 s
9	55.6 d	49.7 d	48.9 d	49.5 d
10	39.1 s	37.2 s	40.6 s	36.6 s
11	70.6 d	20.7 t	70.6 d	21.2 t
12	37.6 t	24.7 t	37.3 t	27.6 t
13	37.1 d	37.2 d	37.1 d	36.3 d
14	42.7 s	44.2 s	42.8 s	42.5 s
15	27.3 t	36.8 t	27.3 t	27.3 t
16	35.4 t	77.0 d	35.3 t	34.1 t
17	43.0 s	48.6 s	43.0 s	47.8 s
18	47.7 d	47.7 d	47.5 d	49.0 d
19	47.7 d	47.6 d	47.7 d	51.7 d
20	150.2 s	150.0 s	150.2 s	209.4 s
21	29.8 t	29.9 t	29.7 t	27.1 t
22	39.8 t	37.7 t	39,8 t	29.0 t
23	22.3 q	28.2 q	21.4 q	26.6 q
24	28.7 q	22.1 q	28.2 q	21.0 q
25	16.2 q	15.9 q	19.9 q	14.5 q
26	17.2 q	16.0 q	17.4 q	15.6 q
27	14.6 q	16.3 q	14.4 q	15.7 q
28	18.0 q	11.7 q	18.0 q	60.1 t
29	109.8 t	109.8 t	113.5 t	
30	19.3 q	19.3 q	19.3 q	28.8 q

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

basis of the coupling constants and confirmed by a ROESY experiment, showing a correlation between the oxymethine proton at δ 3.92 and the protons of the methyl group at C-26 (δ_H 1.03). All of these data and comparison with those found in the literature for nepeticin (**5**)[18] established the structure of **1** as 3-*epi*nepeticin.

The structure of compound **2** with the same molecular weight as **1** was elucidated by spectral methods (Tables 1 and 2), showing it was related to **1**, the difference being the presence of a secondary hydroxyl group at C-16 (δ_H 3.60, dd, $J = 4.7$, 11.2 Hz) instead of C-11 (δ_H 3. 92) as in **1**. This difference was well established by an HMBC experiment showing ^2J correlations of the oxymethine proton signal at δ_H 3.60 to C-17 (δ_C 48.6) and ^3J interactions of the carbons C-14 (δ_C 44.2), C-18 (δ_C 47.7), and C-28 (δ_C 11.7). The relative stereochemistry of the oxymethine proton at C-16 was assigned as α since NOESY correlations with the signals corresponding to Me-27 and H-18, resonating at δ 1.00 and 1.40, respectively, were observed. These data and those given in the literature for calenduladiol[23] revealed that **2** was 3-*epi*calenduladiol.

Compound **3** with the molecular formula $C_{30}H_{46}O_2$ was shown in the study of its IR, UV, ^{1}H, and ^{13}C NMR data (Tables 1 and 2) and 2D ^{1}H–^{1}H (COSY and ROESY) and ^{1}H–^{13}C (HSQC and HMBC) experiments to be a lupane triterpene with an unsaturated α,β-ketone and one secondary hydroxyl group, whose positions were established by an HMBC experiment. These data and comparison with those found in the literature for glochidone (**7**)[19] established the structure of **3** as 11α-hydroxyglochidone.

The detailed ^{1}H and ^{13}C NMR (Tables 1 and 2) assignments of the one known lupane triterpene 28-hydroxy-3,20-dioxo-29-norlupane (**4**),[17] which have not been previously reported, were achieved by 1D and 2D techniques including DEPT, HMBC, HSQC, COSY, and ROESY.

The betulin (**9**) analogues **9a** and **9b** were prepared by acetylation reaction,[22] where the reactivity of the hydroxyl group on C-28 is much higher than that on C-3, the C-28 acetoxy could be obtained in a higher yield (77.5%) keeping the temperature reaction at 0 °C.

We prepared by standard acetylation reaction the acetoxy-rigidenol (**6a**). We want to introduce a double bond at C-1 by the treatment of **6a** with phenylselenyl chloride in ethyl acetate and the sequential addition of pyridine and *m*-chloroperbenzoic acid.[24] However, instead of the unsaturated α,β-ketone desired we obtained a 11α-acetoxy-30-chloro-3-oxo-lup-20(29)-ene (**6b**) by the chlorination of the isopropenyl group at C-19. This was verified with the studies of the spectroscopic data of **6b**, including ^{1}H–^{13}C heteronuclear correlation (HSQC), long-range correlation with inverse detection (HMBC), and ROESY experiments.

The isolated lupane triterpenes **1**–**19** and the derivatives (**6a**, **6b**, **9a**, and **9b**) were tested for their inhibitory activity on the production of NO (measured as nitrite) and PGE_2 in mouse macrophages (RAW 264.7), as a preliminary screening assay system to find new anti-inflammatory compounds. Stimulation of RAW 264.7 macrophages with LPS (1 μg/mL) for 18 h induced iNOS and COX-2. Non-stimulated cells generated 175.3 ± 18.5 ng/mL nitrite and 4.4 ± 0.9 ng/mL PGE_2. Control cells were incubated with vehicle and LPS for 18 h, and generated 1628.3 ± 86.8 ng/mL nitrite and 31.1 ± 3.1 ng/mL PGE_2. The compounds were tested in this system at concentrations not significantly affecting cell viability (Table 3). As expected, the reference drugs 1400 W (iNOS inhibitor) and NS 398 (COX-2 inhibitor) potently decreased the production of nitrite and PGE_2, respectively. Several lupane triterpenes inhibited both NO and PGE_2 generation, with some differences in potency and IC_{50} values in the micromolar range (Table 3). The most effective compounds were **2**, **3**, **6**, **9**, **12**, **13**, and **16.** In addition, **8**, **11**, and **18** preferentially inhibited the generation of PGE_2.

The preliminary structure–activity relationship of these natural lupane triterpenes revealed the following trends. The stereochemistry of the hydroxyl group at C-3 can modify the activity (**1** and **17** vs **5** and **18**, respectively). Interestingly, the esterification by caffeic acid of the C-3 hydroxyl group in compound **9** suppresses the activity on NO (**11**). It can also be observed that the presence of oxygenated groups at C-28 has important consequences on the activity. In this respect, the substitution of a C-28 methyl (**8**, **14**) by a hydroxymethyl group (**9**, **15**) increases the potency mainly on NO, whereas the presence of a C-28 carboxyl group (**13**, **16**) increases the potency on NO and PGE_2 production and also the cytotoxicity. In contrast, the introduction of either a C-28 (**10**) or C-20 (**4**) carbonyl group is detrimental. On the other hand, the presence of the α-hydroxyl group at C-11 (**3** vs **7**) results in a higher inhibitory activity, especially for NO. The acetylation of **9** at C-28 (**9a**) increases the potency and reduces the cytotoxicity of this compound, although the double acetylation at C-28 and C-3 strongly reduces the activity (**9b**). Also, the acetylation of **6** at C-11 (**6a**) or the chlorination at C-30 (**6b**) increases the potency of the compound.

In summary, our data indicate that several lupane triterpenes are effective in vitro inhibitors of NO and PGE_2 production by macrophages. Compounds able to reduce the excessive production of these mediators have a potential for the prevention and treatment of different inflammatory pathologies.

3. Experimental

3.1. General

Optical rotations were measured on a Perkin Elmer 241 automatic polarimeter and the $[\alpha_D]$ are given in 10^{-1} deg cm^2 g^{-1}. IR (film) spectra were recorded on a Bruker IFS 55 spectrophotometer, and UV spectra were

Table 3. Effect of compounds **1**–**19** on NO and PGE_2 production

Compound	Viability[a] (10 μM)	NO		PGE_2	
		% inhibition[a]	IC_{50}[b] (μM)	% inhibition[a]	IC_{50}[b] (μM)
1	100.0 ± 0.0	22.0 ± 3.6[c]	N.D.	20.2 ± 0.2[c]	N.D.
2	91.4 ± 1.5	38.7 ± 3.8[c]	15.6	52.1 ± 2.9[d]	6.9
3	88.4 ± 1.9	53.1 ± 4.2[d]	8.2	53.6 ± 5.9[d]	5.4
4	94.9 ± 3.3	19.4 ± 2.8[c]	N.D.	0.0 ± 0.0	N.D.
5	96.8 ± 3.1	4.0 ± 4.0	N.D.	27.1 ± 4.2[c]	N.D.
6	100.0 ± 0.0	42.7 ± 1.7[d]	12.9	44.6 ± 4.7[d]	20.7
6a	90.9 ± 2.3	67.7 ± 4.8[d]	5.3	59.6 ± 3.9[d]	3.9
6b	98.4 ± 1.2	69.4 ± 4.3[d]	4.7	78.8 ± 5.3[d]	0.3
7	100.0 ± 0.0	0.0 ± 0.0	N.D.	33.2 ± 0.6[c]	N.D.
8	98.8 ± 0.7	8.0 ± 2.5	N.D.	42.1 ± 2.1[d]	18.0
9	69.9 ± 1.2[c]	50.1 ± 3.0[d]	5.0	47.7 ± 4.3[d]	12.9
9a	84.4 ± 1.2	55.9 ± 5.0[d]	4.7	69.5 ± 5.1[d]	1.7
9b	90.1 ± 1.6	27.4 ± 4.4[c]	N.D.	32.5 ± 6.6[c]	N.D.
10	100.0 ± 0.0	19.5 ± 4.0	N.D.	0.0 ± 0.0	N.D.
11	98.2 ± 1.0	9.3 ± 3.2	N.D.	52.1 ± 2.6[d]	10.8
12	91.0 ± 5.3	44.9 ± 5.7[d]	12.8	49.3 ± 2.7[d]	9.6
13	67.1 ± 4.1[c]	89.1 ± 4.4[d]	0.7	68.7 ± 5.7[d]	0.6
14	92.7 ± 3.1	7.5 ± 3.3	N.D.	35.2 ± 4.9[c]	N.D.
15	100.0 ± 0.0	27.3 ± 2.3[c]	N.D.	34.5 ± 12.7[c]	N.D.
16	72.1 ± 2.9[c]	69.2 ± 5.1[d]	0.3	58.4 ± 3.9[d]	2.7
17	96.1 ± 2.2	26.5 ± 2.8[c]	N.D.	0.0 ± 0.0	N.D.
18	100.0 ± 0.0	25.8 ± 0.5[c]	N.D.	47.2 ± 5.1[d]	15.5
19	99.0 ± 1.0	14.3 ± 1.7	N.D.	0.0 ± 0.0	N.D.
1400 W	99.0 ± 0.2	71.8 ± 6.4[d]	0.4	N.D.	N.D.
NS 398	98.2 ± 0.4	N.D.	N.D.	100.0 ± 0.9[d]	2.5 nM

N.D., not determined.

[a] Data show means ± SEM ($n = 6$). Compounds were assayed at 10 μM. Percentages of inhibition for NO and PGE_2 production were obtained at 5 μM for **9**, **13**, and **16**.

[b] Values represent the concentration required to produce 50% inhibition of the response, along with the 95% confidence limits.

[c] $P < 0.05$.

[d] $P < 0.01$ with respect to the control group.

collected in absolute EtOH on a JASCO V-560 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-500, a Bruker Avance 400, or a Bruker Avance 300 spectrometer. EIMS and HREIMS were recorded on a Micromass Autospec spectrometer. Purification was performed using silica gel (particle size 40–63 μM, Merck and HPTLC-Platten-Sil 20 UV_{254}, Panreac) and Sephadex LH-20 (Pharmacia).

3.2. Plant material

Maytenus cuzcoina Loesener was collected at Huayllabamba-Urquillos, Province of Urubamba, Cusco (Peru), in December 1998, and was identified by Prof. Alfredo Tupayachi; a voucher specimen ('cuz' 02765 A.T. 1004 MO) is deposited in the herbarium of Vargas, Department of Botany, in the National University of San Antonio Abad de Cusco. *M. chiapensis* Lundell was collected at the Parque Nacional El Imposible, El Salvador, in August 1999 and was identified by Prof. Edi Montalvo; a voucher specimen (ISB-88) is on file in the Jardin Botánico La Laguna, El Salvador.

3.3. Extraction and isolation

The root bark of *M. cuzcoina* (900.0 g) was extracted with *n*-hexane–Et_2O in a Soxhlet apparatus. The extract (25.5 g) was chromatographed on Sephadex LH-20 (*n*-hexane/$CHCl_3$/MeOH, 2:1:1), to afford 64 fractions. In this way, after several chromatographies on Sephadex LH-20 (*n*-hexane/$CHCl_3$/MeOH, 2:1:1), silica gel (CH_2Cl_2–Et_2O of increasing polarity), and preparative HPTLC developed with *n*-hexane/Et_2O (4:6) gave rise to the new compounds **1** (20.5 mg) and **3** (12.3 mg), in addition to the known compounds nepeticin (**5**, 220.0 mg), rigidenol (**6**, 200.0 mg), glochidone (**7**, 75.0 mg), lupeol (**8**, 10.0 mg), betulin (**9**, 16.0 mg), betulinic aldehyde (**10**, 7.0 mg), *epi*betulin (**12**, 30.0 mg), *epi*betulinic acid (**13**, 28.0 mg), lupenone (**14**, 7.0 mg), betulone (**15**, 10.0 mg), betulonic acid (**16**, 3.0 mg), 3-*epi*glochidiol (**17**, 350.0 mg), glochidiol (**18**, 40.0 mg), and glochidonol (**19**, 125.0 mg).

The leaves of *M. chiapensis* (2.1 kg) were extracted with EtOH in a Soxhlet apparatus. Evaporation of the solvent under reduced pressure provided 400.2 g crude extract, which was partitioned into a CH_2Cl_2/H_2O (1:1, v/v) solution. Removal of the CH_2Cl_2 from the organic-soluble extract under reduced pressure yielded 77.0 g residue, which was chromatographed on a silica gel column using increasing polarity mixtures of *n*-hexane–EtOAc as an eluant to afford 54 fractions. Fractions 15–35 (8.0 g) were subjected to column chromatography over Sephadex LH-20 (*n*-hexane/$CHCl_3$/MeOH, 2:1:1) and silica gel (CH_2Cl_2–acetone of increasing polarity). Preparative HPTLC developed with CH_2Cl_2/EtO_2 (9:1) was used to

purify the new compound **2** (30.0 mg), in addition to the known compounds 28-hydroxy-3,20-dioxo-29-norlupane (**4**, 32.0 mg), glochidone (**7**, 24.0 mg), lupeol (**8**, 10.0 mg), betulin (**9**, 45.0 mg), betulin 3-caffeate (**11**, 45.0 mg), lupenone (**14**, 50.0 mg), betulone (**15**, 60.0 mg), 3-*epi*glochidiol (**17**, 60.0 mg), and glochidonol (**19**, 15.0 mg).

3.3.1. Compound 1. Lacquer; $[\alpha]_D^{20}$ 13.6 (*c* 0.5, $CHCl_3$); IR ν_{max} cm^{-1}: 3413, 2926, 2855, 1731, 1458, 1381, 1070, 990, 883, 757; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI/MS *m*/*z* %: 442 (M^+, 1), 424 (18), 406 (100), 391 (64), 255 (38), 216 (17), 201 (38), 189 (26), 175 (19), 159 (21), 121 (35), 95 (43), 81 (34); HR-EI/MS: *m*/*z* 442.3790 (calcd for $C_{30}H_{50}O_2$ 442.3811).

3.3.2. Compound 2. Lacquer; $[\alpha]_D^{20}$ +4.1 (*c* 0.87, $CHCl_3$); IR ν_{max} cm^{-1}: 3430, 3010, 2946, 2844, 1645, 1460, 1385, 1035, 900; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI/MS *m*/*z* %: 442 (M^+, 9), 424 (10), 406 (100), 363 (17), 201 (37), 189 (67), 147 (42), 134 (79), 107 (54), 95 (45), 69 (29), 55 (34); HR-EI/MS: *m*/*z* 442.3760 (calcd for $C_{30}H_{50}O_2$ 442.3811).

3.3.3. Compound 3. Lacquer; $[\alpha]_D^{20}$ +23.8 (*c* 0.6, $CHCl_3$); UV λ_{max} nm: 229; IR ν_{max} cm^{-1}: 3447, 2925, 2854, 1732, 1659, 1459, 1382, 1261, 1075, 937, 883, 803, 697; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI/MS *m*/*z* %:438 (M^+, 16), 420 (100), 405 (21), 339 (7), 329 (38), 283 (13), 255 (9), 229 (15), 216 (11), 201 (15), 189 (25), 175 (20), 150 (31), 121 (41), 95 (47), 69 (57); HR-EI/MS: *m*/*z* 438.3494 (calcd for $C_{30}H_{46}O_2$ 438.3498).

3.4. Acetylation of rigidenol

To solution of rigidenol (**6**) (160.3 mg, 0.4 mM) and catalytic amount of 4-dimethylaminopyridine (DMAP) in dichloromethane (8 mL) were added triethylamine (0.2 mL, 1.4 mM) and acetic anhydride (0.1 mL, 1.0 mM). After being stirred for 30 min at room temperature, until TLC showed complete conversion, the mixture was quenched with EtOH and stirred for 30 min. The mixture was evaporated to dryness and the residue was purified by flash chromatography on silica gel (eluting 20–40% ethyl acetate in *n*-hexane) to yield acetoxyrigidenol (**6a**) (169.4 mg, 87.9%) as an amorphous solid; $[\alpha]_D^{20}$ +31.2 (*c* 0.25, $CHCl_3$); IR ν_{max} cm^{-1}: 2959, 2926, 2854, 1728, 1712, 1643, 1456, 1379, 1243, 1018, 801; ^1H NMR δ: 0.74 (3H, s), 0.85 (3H, s), 0.99 (3H, s), 1.00 (3H, s), 1.04 (3H, s), 1.06 (3H, s), 1.48 (3H, s), 1.91 (3H, s), 2.25 (1H, m), 2.34 (1H, m), 2.48 (1H, m), 4.56 (1H, br s); 4.70 (1H, br s), 5.08 (1H, dt, J = 5.2, 11.1 Hz); ^{13}C NMR δ: 14.2 (q), 16.6 (q), 17.5 (q), 18.0 (q), 19.4 (q), 19.5 (t), 20.3 (q), 21.9 (q), 27.3 (t), 27.7 (q), 29.7 (t), 32.3 (t), 33.8 (t), 34.1 (t), 35.3 (t), 36.7 (d), 37.7 (s), 39.8 (t), 42.1 (t), 42.5 (s), 42.6 (s), 43.0 (s); 47.3 (d), 47.4 (s), 47.7 (d), 52.2 (d), 54.0 (d), 72.7 (d), 109.9 (t), 149.8 (s), 169.8 (s), 218.2 (s); EI/MS *m*/*z* %: 482 (M^+, 0.2), 440 (0.2), 422 (100.0), 407 (30.4), 379 (5.6), 339 (6.9), 311 (10.7), 216 (16.7), 203 (22.0), 147 (9.1), 121 (8.3), 81 (5.5); HR-EI/MS: *m*/*z* 482.3762 (calcd for $C_{32}H_{50}O_3$ 482.3760).

3.5. Chlorination of acetoxyrigidenol

A solution of **6a** (150.0 mg, 0.3 mM) and phenylselenyl chloride (98%) (76.0 mg, 0.4 mM) in EtOAc (65.0 mL) was stirred at room temperature for 3 h. To the stirred mixture, saturated aqueous $NaHCO_3$ solution was added. After most of the aqueous layer was removed, pyridine (0.3 mL, 0.8 mM) and *m*-chloroperbenzoic acid (50–60%) (284.0 mg, 0.8 mM) were added to the organic layer. The mixture was washed with 5% aqueous NaOH solution (three times), saturated aqueous NH_4Cl solution (three times), and saturated NaCl solution (three times); dried over anhydrous $MgSO_4$; and filtered. The mixture was evaporated to dryness and the residue was purified by flash chromatography on silica gel (eluting 40–70% ethyl acetate in *n*-hexane) to yield 11α-acetoxy-30-chloro-3-oxo-lup-20(29)-ene (**6b**) (55.3 mg, 32%) as an amorphous solid; $[\alpha]_D^{20}$ +5.5 (*c* 0.12, $CHCl_3$); IR ν_{max} cm^{-1}: 2957, 2870, 1728, 1712, 1643, 1456, 1381, 1246, 1019, 971, 910, 756, 580; ^1H NMR δ: 0.80 (3H, s), 0.90 (3H, s), 1.01 (3H, s), 1.04 (3H, s), 1.09 (3H, s), 1.09 (3H, s), 1.11 (3H, s), 1.85 (1H, m), 1.78 (1H, m), 1.96 (3H, s), 2.38 (1H, m), 4.05 (2H, s), 5.02 (1H, s), 5.08 (1H, s), 5.09 (1H, m); ^{13}C NMR δ: 14.3 (q), 16.7 (q), 17.5 (q), 17.9 (q), 19.5 (t), 20.3 (q), 21.9 (q), 27.3 (t), 27.7 (q), 32.2 (t), 33.7 (t), 34.0 (t), 34.1 (t), 35.2 (t), 36.6 (d), 37.8 (s), 39.6 (t), 42.1 (t), 42.5 (s), 42.7 (s), 43.1 (s), 43.3 (d), 47.5 (s), 48. 1 (t), 49.3 (d), 52.3 (d), 54.1 (d), 72.8 (d), 112.7 (t), 150.5 (s), 169.9 (s), 218.3 (s); EI/MS *m*/*z* %: 456 (M^+−60, 100.0), 441 (24.1), 422 (11.5), 407 (3.4), 311 (16.7), 250 (13.9), 237 (17.4), 107 (12.4), 81.1 (8.5); HR-EI/MS: *m*/*z* 456.3179 (calcd for $C_{30}H_{45}OCl$ 456.3159).

3.6. Acetylation of betulin

To a solution of betulin (**9**) (442.0 mg, 1.0 mM) in pyridine (4.0 mL) was added acetic anhydride (0.1 mL, 1.0 mM) at 0 °C. The reaction mixture was stirred for 4 h at 0 °C, until TLC showed complete conversion. The mixture was evaporated to dryness and the residue was purified by flash chromatography on silica gel (eluting 5–50% ethyl acetate in *n*-hexane) to give 28-acetoxybetulin (**9a**) (375.2 mg, 77.5%) and 3,28-diacetoxy-betulin (**9b**) (40.3 mg, 7.6%).

3.7. Pharmacological activity

3.7.1. Nitrite and PGE_2 production by stimulated macrophages. The mouse macrophage RAW 264.7 cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan. After incubation with test compounds for 18 h, cells were incubated with MTT (200 μg/mL) for 60 min. The medium was then removed and the cells were solubilized in DMSO (100 μL) to quantitate formazan at 550 nm.[25] Macrophages (4×10^5/well) were incubated with *Escherichia coli* [serotype 0111:B4] LPS (1 μg/mL) at 37 °C for 18 h in the presence of test compounds or vehicle

(methanol, 1%, v/v). Nitrite (as an index of NO production) and PGE_2 levels were determined in culture supernatants by a fluorimetric method[26] and by radioimmunoassay,[27] respectively.

3.7.2. Statistical analysis. The results are presented as means ± SEM. Inhibitory concentration 50% (IC_{50}) values were calculated by linear regression analysis. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

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