Articles

In Vitro and *in Vivo* Anti-Leishmanial Activity of Triterpenoid Saponins Isolated from *Maesa balansae* and Some Chemical Derivatives

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The methanolic extract from the leaves of the Vietnamese medicinal plant *Maesa balansae* showed potent *in vitro* and *in vivo* activity against the tropical protozoal parasite *Leishmania infantum*. Bioassay-guided purification of the extract led to the identification of six triterpenoid saponins, maesabalides I−VI (**1**−**6**), each having a strong and specific anti-leishmanial activity. Maesabalide III (**3**) and IV (**4**) were the most potent with IC_{50} values against intracellular amastigotes of about 7 and 14 ng/mL. In comparison, the IC_{50} value of sodium stibogluconate, the reference drug for treatment of leishmaniasis, is only 5.6 μg/mL. No cytotoxicity was present on a human fibroblast (MRC-5) cell line (CC_{50} > 32μg/mL). *In vivo* evaluation in the BALB/C mouse model demonstrated that >90% reduction of liver amastigote burdens was obtained 1 week after a single subcutaneous dose at 0.2−0.4 mg/kg was administered. Several chemical derivatives of maesabalides I−VI were prepared in order to study the structure−activity relationship.

Introduction

In the course of a drug-screening project on medicinal plants for biologically active components, the Vietnamese medicinal plant *Maesa balansae* was examined and found to have strong anti-leishmanial potential.[1] *Maesa balansae* Mez. (Myrsinaceae), a shrub growing in the northern part of Vietnam,[2] is used in traditional medicine for the treatment of allergies, sprains, worm infections, skin ulcers, drunkenness, and headache.[3] The polar extract of the leaves exhibited potent and selective *in vitro* and *in vivo* activity against intracellular *Leishmania infantum* amastigotes.[4] Bioassay-guided fractionation led to the isolation and characterization of six anti-leishmanial triterpene saponins designated as maesabalides I (**1**), II (**2**), III (**3**), IV (**4**), V (**5**), and VI (**6**)[5] (Chart 1).

Leishmaniasis is a growing public health problem in many parts of the world with about 350 million people living in endemic areas and an annual incidence of about 2 million cases.[6] As for most other tropical diseases, inadequate resources are available to tackle this disease and treatment options are limited to pentavalent antimonials as first-line chemotherapeutics and to amphotericin and pentamidine as second-line chemotherapeutics. The recent approval of miltefosine as the first oral treatment of visceral leishmaniasis was a major step forward, but new drugs are still urgently needed and drug-screening initiatives should continue.[7] Natural products may offer unexpected chemical diversity for that purpose.

Within the group of the saponin-containing plant species, anti-leishmanial activity has been reported only for the families Hedera,[8] Dracaena,[9] and Yucca.[10] Unfortunately, the *in vitro* screening methodologies mostly relied on promastigote models and/or the test concentrations were unrealistically high. In addition, conflicting results against intracellular amastigotes and no *in vivo* data in animal models were generated.

This study evaluated the *in vitro* and *in vivo* anti-leishmanial activity and structure−activity relationships of maesabalides I−VI (**1**−**6**) and some semisynthetic derivatives (**7**−**15**) in an attempt to (a) establish a structure−activity relationship and (b) simplify and thereby increase the yield of the basic plant extract by derivatization. The fact that anti-leishmanial activity had been claimed for ursolic acid[11] at least opened some theoretical possibilities.

Results and Discussion

The dried leaves of *M. balansae* were extracted sequentially with dichloromethane and methanol. The residue obtained after evaporation of the methanol extract was partitioned between *n*-BuOH and water. The *n*-BuOH soluble fraction was evaporated to dryness. After the sample was stirred in acetone, the acetone-insoluble fraction (the "purified extract") was repeatedly subjected to semipreparative reversed-phase HPLC in order to obtain the pure saponins, maesabalides I (**1**)

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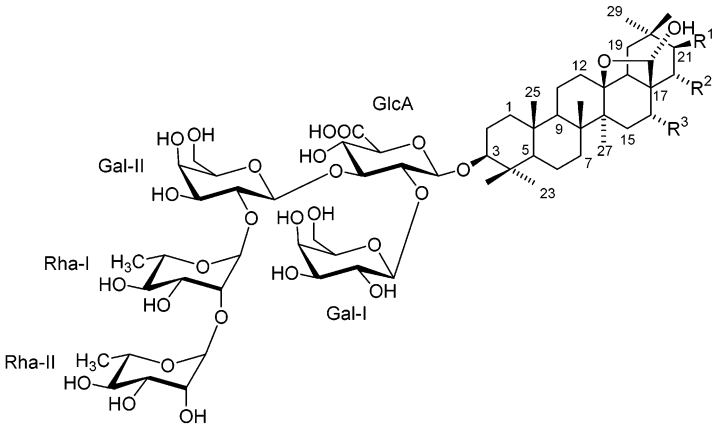
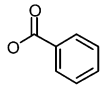
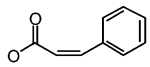
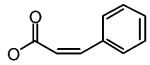
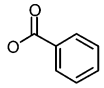
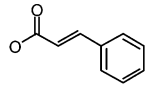
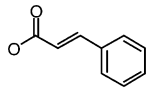
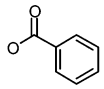
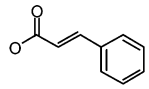
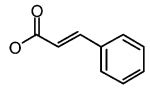
Chart 1. Saponins from the Leaves of *Maesa balansae*



	R^1	R^2	R^3
Maesabalide I (**1**)			OH
Maesabalide II (**2**)			OH
Maesabalide III (**3**)			OH
Maesabalide IV (**4**)			OH
Maesabalide V (**5**)			
Maesabalide VI (**6**)			

(8.2%), II (**2**) (4.0%), III (**3**) (35.8%), IV (**4**) (35.8%), V (**5**) (8.0%), and VI (**6**) (8.2%).[5]

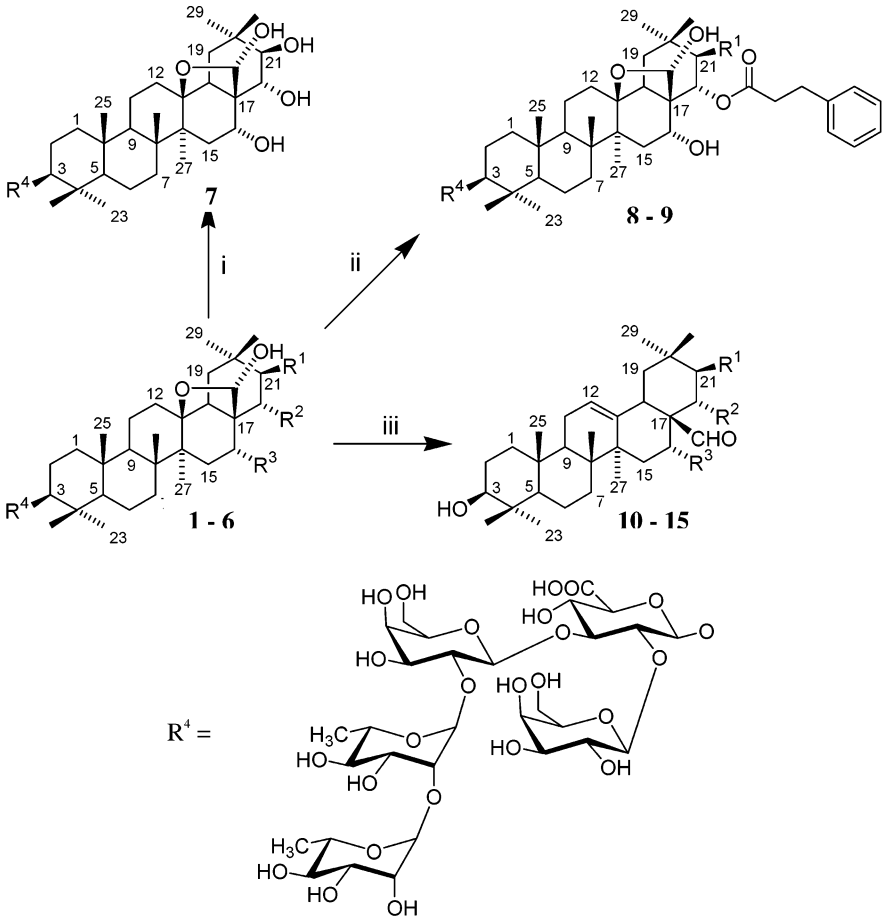
Several derivatives were prepared in order to have a better understanding of the structure−activity relationship (Scheme 1). 3-*β*-*O*-{[(α-L-Rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-16α,21*β*,22α,13*β*,28-oxidoolean-28α-tetrol (**7**) was obtained by basic hydrolysis of the crude extract followed by purification over column. 3-*β*-*O*-{[(α-L-Rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-16α-acetoxy-21*β*-benzoyloxy-22α-dihydrocinnamoyloxy-13*β*,28-oxidoolean-28α-ol (**8**) and 3-*β*-*O*-{[(α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-21*β*-angeloyloxy-22α-(*E*)-dihydrocinnamoyloxy-13*β*,28-oxidoolean-16α,28α-diol (**9**) were obtained after hydrogenation of the double bond of the cinnamoyl group attached at C-22. For this purpose the crude extract was hydrogenated over Pd/C, which after purification over the column afforded two hydrocinnamoyl derivatives. The other derivatives (**10**−**15**) were the semisynthetic aglycones obtained after acid hydrolysis of the corresponding maesabalides.

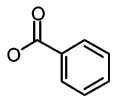
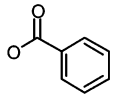
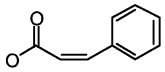
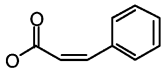
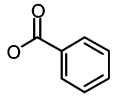
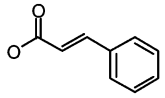
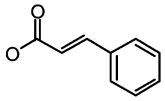
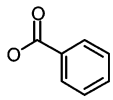
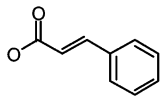
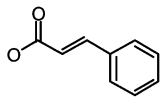
The characterization of compounds **1**−**6** and **11** has recently been described.[5]

Saponin **7** was obtained as a white amorphous powder. The negative-ion FABMS showed an $[M - H]^-$ anion at *m/z* 1297. The molecular formula was established as $C_{60}H_{98}O_{30}$ on the basis of ^{13}C distortionless enhancement by polarization transfer (DEPT) NMR and MS data. The signals corresponding to the ester groups in the ^1H and ^{13}C NMR spectra (D_2O) in comparison with the previous characterized compounds, **1**−**6**, disappeared. Compound **7** was therefore characterized as 3-*β*-*O*-{[(α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-16α,21*β*,22α,13*β*,28-oxidoolean-28α-tetrol.

Saponin **8** was obtained as a white amorphous powder. The negative-ion FABMS showed an $[M - H]^-$ anion at *m/z* 1533. The molecular formula was established as $C_{76}H_{110}O_{32}$ on the basis of ^{13}C DEPT NMR and MS data. The ^1H and ^{13}C NMR spectra (pyridine-d_5) indicated that saponin **8** had the same sugar chain and aglycone moiety as **1** but differed only in the cinnamoyl group linked to C-22 of the E ring. The two signals for the olefinic carbons of the double bond, C-32 and C-33 of the cinnamoyl group of saponin **1**, have disappeared, and two new methylene signals appeared in the ^{13}C NMR spectrum of saponin **8** at δ 36.36 and 31.33 ppm. These signals corresponded to two triplets at δ 2.43 and 2.71 ppm, respectively, in the ^1H NMR spectrum, each

Scheme 1[a]



	R^1	R^2	R^3
8			
9			
10			OH
11			OH
12			OH
13			OH
14			
15			

[a] (i) 1.1 equiv of K_2CO_3, MeOH, reflux, 10 h; (ii) H_2−Pd, MeOH, 12 h, room temp; (iii) 2 N HCl, MeOH−H_2O (1:1), reflux, 3 h.

Table 1. Pharmacological and Cytotoxic Activity of Purified Saponins and Some Derivatives against *L. infantum* and MRC-5 Cells

	activity against *L. infantum*[a]			
compound	IC_{50} (μg/mL)	IC_{90} (μg/mL)	CC_{50} (μg/mL)	vivo[b] (mg/kg)
purified extract	0.023	0.1	>32	0.4
amphotericin B	0.285	0.373	>32	>10
stibogluconate	5.6	>32	>32	>40
1	0.018	0.031	0,5	0.8
2	0.023	0.094	0,5	>0.8
3	0.007	0.012	0.5	0.2
4	0.014	0.021	0.1	0.4
5	0.046	0.125	8	>0.8
6	0.041	0.125	8	>0.8
7	>32	>32	>32	>40
8	0.009	0.015	0.3	nd
9	0.018	0.03	0.6	nd
10	>32	>32	>32	nd
11	22	>32	>32	nd
12	8	>16	32	>40
13	>32	>32	>32	nd
14	>32	>32	>32	nd
15	>32	>32	>32	nd

[a] nd: not determined. IC_{50}/IC_{90}: 50%/90% inhibitory concentration against intracellular amastigotes of *L. infantum* (the mean of three replicate values). CC_{50}: 50% cytotoxic concentration for MRC-5 cells (mean of three replicate values). [b] vivo: lowest dose (single subcutaneous administration) with >90% reduction of liver amastigote burdens in BALB/C mouse model (three animals/group).

integrating for two protons, which were strongly correlated in the $^1H-^1H$ COSY spectrum. A long-range HMBC coupling was also observed between C-22 and δ_H 2.43 ppm and C-34 and δ_H 2.71 ppm. From the above evidence the structure of saponin **8** was elucidated as 3-*β*-*O*-{[(α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-21*β*-benzoyloxy-22α-hydrocinnamoyloxy-13*β*,28-oxidoolean-16α,28α-diol.

Saponin **9**, also a white amorphous powder, showed an $[M - H]^-$ anion at *m/z* 1511 in the negative-ion FABMS. This result corresponded to the hydrogenation of only one of the double bonds in saponin **2** and **4** and not both (angeloyl and cinnamoyl groups). The molecular formula was determined as $C_{74}H_{112}O_{32}$ on the basis of the MS and ^{13}C DEPT data. As for saponin **8**, the two signals for the olefinic carbons of the double bond of the cinnamoyl group, C-32 and C-33, disappeared and two new methylene signals appeared in the ^{13}C NMR spectrum (pyridine-d_5) of saponin **9** at δ 36.39 and 31.51 ppm. The C−H correlation showed the corresponding triplets in the ^1H NMR at δ 2.56 and 2.91 ppm, each integrating for two protons. A long-range HMBC coupling was also observed between C-22 and H-32 and between C-34 and H-33. The characteristic signals for the angeloyl group were present in the ^1H NMR (δ 6.51 ppm (H-42), 2.13 ppm (H-43), 2.04 ppm (H-44)) and in the ^{13}C NMR spectrum (δ 128.98 ppm (C-41), 138.02 ppm (C-42), 16.16 ppm (C-43), and 21.24 ppm (C-44)). The structure of saponin **9** was characterized as 3-*β*-*O*-{[(α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-21*β*-angeloyloxy-22α-hydrocinnamoyloxy-13*β*,28-oxidoolean-16α,28α-diol.

The structure−activity relationship of the individual saponins is presented in Table 1. Saponin **3** was the most active compound with an *in vitro* IC_{50} of 7 ng/mL. This was also reflected in a potent *in vivo* activity, producing more than 90% reduction in liver amastigote burdens after a single subcutaneous dose at 0.2 mg/kg. Further activity ranking yielded saponin **8** > saponin **4** > saponin **9**. Since **8** and **9** are the dihydrocinnamoyl derivatives of **3** and **4**, the effect of the double bond in the cinnamoyl group on the pharmacological activity is negligible. The corresponding *Z*-isomers, saponins **1** and **2**, were only marginally less active. A more significant drop in activity was noted with the saponins carrying an acetyl group at C-16 (saponins **5** and **6**); the *in vitro* potency dropped by a factor of about 40×, and no *in vivo* activity was obtained at 1 × 0.8 mg/kg subcutaneously.

These results indicate that the saponin core structure can be subject to minor structural changes without losing pharmacological activity. However, replacement of the OH group by an acetoxy group at C-16 has a more pronounced effect on the activity (30−60 times less active *in vitro*). The fact that maesabalides III and IV contribute to more than 70% in the total purified saponin mixture explains why the *in vitro* and *in vivo* potency of the purified extract is little different from that of the individual maesabalides I−IV. The least potent maesabalides V and VI contribute to about 20% in the mixture.[4]

Compound **7** and the aglycones **10**−**15** possess no activity at all. This fact strongly suggests that both parts of the molecule, i.e., the ester part and the sugar part, are necessary for activity. The role of the oxygen bridge (between C-13 and C-17) is still unclear and remains to be investigated. However, indirect evidence suggests that it may be critical in the activity because related saponins from other Maesa plant species were also active[4] while many other plant species known to contain saponins were found to be inactive.[12]

Experimental Section

General Experimental Procedures. Preparative HPLC was performed on C18 BDS (Hypersil BDS, 8 μm, 200 g) using a column with axial compression (50 mm i.d., packed at 60 bar).

^1H, ^{13}C, and 2D NMR spectra were recorded using a Bruker AVANCE-400 spectrometer. The NMR data of the saponins were measured in pyridine-d_5 (**1**−**6**, **8**, **9**) and in D_2O (**7**), and the NMR data for the semisynthetic aglycones were recorded in $CDCl_3$. Chemical shifts were expressed in δ (ppm) with TMS as the reference. The negative-ion mode FAB-MS spectra (for the saponins) were recorded on a Micromass VG70SEQ instrument with glycerol as the liquid matrix. The positive-ion ES-MS spectra (for the aglycones) were recorded on a Micromass ZMD spectrometer coupled to an Alliance (Waters) HPLC system. Optical rotations were determined on an AA-10 automatic polarimeter (Optical Active Ltd.).

Plant Material. Leaves of *Maesa balansae* were collected from Deo Khe, Dai Tu district, Thai Nguyen province in Vietnam (November 2000), and were identified by Dr. Tran Ngoc Ninh (Institute of Ecology and Biological Resources, NCST, Hanoi, Vietnam). Voucher specimens are deposited at the herbarium of that institute.

Preparation of a Purified Extract. The powdered leaves (3 kg) were subsequently extracted in a percolator with chloroform (10 × 5 L) and methanol−water (9:1) (10 × 5 L). The extracts were filtered and evaporated to dryness under reduced pressure with a rotavapor at 40 °C. The dried methanol extract was partitioned between *n*-BuOH (saturated with water) (1 L) and water (1 L). The aqueous fraction was extracted six times with *n*-BuOH (1 L) (saturated with water).

The combined *n*-BuOH soluble fractions were evaporated to dryness, and the residue was suspended in acetone (1 L) and stirred for 4 h. After filtration, the residue gave the purified extract (100 g, yield 3.33%).

Basic Hydrolysis of the Crude Saponin Mixture. An amount of 1 g of the purified extract was dissolved in 50 mL of MeOH, and the mixture was stirred at room temperature for 30 min until all starting material was dissolved. To this solution K_2CO_3 (50 mg, 0.36 mmol) was added, and the reaction mixture was refluxed for 10 h. The solvent was evaporated *in vacuo*, and the residue was dissolved in water (100 mL) and extracted with ethyl acetate (3 × 50 mL). The water-soluble fraction was evaporated *in vacuo* and subjected to column chromatography. Column chromatography conditions were the following: column Hypersil C18-BDS, 5 μm, 21.2 mm × 250 mm; 30 mL/min; buffer (0.5% w/v ammonium acetate in H_2O) to buffer−MeOH (80−20) in 30 min; ELSD detection. After desalting over the same column, compound **7** was obtained (100 mg, 23%) as a white amorphous powder.

3-*β*-*O*-{[(α-L-Rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-16α,21*β*,22α,13*β*,28-oxidoolean-28α-tetrol (7). White amorphous powder. FABMS (negative ion mode) *m/z*: 1297 $[M - H]^-$. IR (KBr): ν_{max} 3400, 2921, 1611, 1079 cm^{-1}. $[\alpha]^{18}_D$ −43.3° (*c* 0.53, pyridine). ^1H NMR and ^{13}C NMR data are listed in Tables 2 and 3 in Supporting Information. Anal. ($C_{60}H_{98}O_{30}$): C, 55.89%; H, 7.48% (calculated C, 55.46%; H, 7.60%).

Hydrogenation of the Crude Saponin Mixture. An amount of 10 g of the purified extract was dissolved in 100 mL of MeOH, and the mixture was hydrogenated for 12 h with 2 g of Pd/C as catalyst. The catalyst was removed by filtration over dicalite under nitrogen. The filtrate was evaporated in vacuo and subjected to column chromatography. For each injection, 250 mg of sample was dissolved in 50 mL of water−methanol (1:1), injected onto the column, and eluted with a water (5% NH_4OAc w/v (A))−methanol (B)−acetonitrile (C) gradient: A−B−C 36:23:41 to 33:23:44 in 61 min. The column (50 mm i.d.) was packed with 200 g of BDS-C18, 8 μm at 60 bar: flow rate 80 mL/min; UV detection at 275 nm. The fractions (20 mL) were collected by level.

On the basis of the UV chromatogram, four fractions were obtained. These fractions were evaporated under reduced pressure to dryness and desalted over a column of 50 mm i.d. packed with 200 g of BDS-C18 (8 μm) at 60 bar with a flow rate of 80 mL/min. The two main compounds, **8** (545 mg, 5% w/w) and **9** (560 mg, 6% w/w), were characterized by NMR and FAB-MS.

3-*β*-*O*-{[(α-L-Rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-16α-acetoxy-21*β*-benzoyloxy-22α-dihydrocinnamoyloxy-13*β*,28-oxidoolean-28α-ol (8). A white amorphous powder. FABMS (negative ion mode) *m/z*: 1533 $[M - H]^-$. IR (KBr): ν_{max} 3411, 2933, 1720, 1603, 1278, 1072 cm^{-1}. $[\alpha]^{18}_D$ −48.5° (*c* 0.68, pyridine). λ_{max} = 230.9 nm. ^1H NMR and ^{13}C NMR data are listed in Tables 2 and 3 in Supporting Information. Anal. ($C_{76}H_{110}O_{32}$): C, 59.08%; H, 7.31%; (calculated C, 59.44%; H, 7.22%).

3-*β*-*O*-{[(α-L-Rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→3)]-[*β*-D-galactopyranosyl-(1→2)]-*β*-D-glucuronopyranosyl}-21*β*-angeloyloxy-22α-(*E*)-dihydrocinnamoyloxy-13*β*,28-oxidoolean-16α,28α-diol (9). A white amorphous powder. FABMS (negative ion mode) *m/z*: 1511 $[M - H]^-$. IR (KBr): ν_{max} 3422, 2934, 1720, 1636, 1268, 1078 cm^{-1}. $[\alpha]^{18}_D$ −47.2° (*c* 0.53, pyridine). λ_{max} = 218.0 nm. ^1H NMR and ^{13}C NMR data are listed in Tables 2 and 3 in Supporting Information. Anal. ($C_{74}H_{112}O_{32}$): C, 58.31%; H, 7.65%; (calculated C, 58.72%; H, 7.46%).

Acid Hydrolysis. Compound **2** (86 mg, 0.057 mmol) was dissolved in 10 mL of 2 N HCl solution (H_2O−MeOH 1:1), and then the mixture was refluxed with stirring for 3 h. After evaporation of the methanol *in vacuo*, the aqueous solution was extracted with EtOAc (3 × 4 mL). The combined organic layers were washed with H_2O and then evaporated to dryness to give an amorphous powder that was subjected to HPLC purification yielding compound **11** (*m/z* 701 $[M + H]^+$) (28 mg, 0.040 mmol, 70%). Conditions were the following: column Hypersil C18-BDS, 5 μm, 21.2 mm × 250 mm, 30 mL/min, H_2O−MeCN 20:80 to 100% MeCN in 30 min, UV detection at 275 nm. By the same method compounds **2** (86 mg, 0.057 mmol), **3** (120 mg, 0.078 mmol), **4** (124 mg, 0.082 mmol), **5** (96 mg, 0.064 mmol), and **6** (93 mg, 0.060 mmol) were hydrolyzed, affording aglycones **12** (*m/z* 723 $[M + H]^+$) (40 mg, 0.055 mmol, 71%), **13** (*m/z* 701 $[M + H]^+$) (42 mg, 0.060 mmol, 73%), **14** (*m/z* 765 $[M + H]^+$) (33 mg, 0.043 mmol, 67%), and **15** (*m/z* 743 $[M + H]^+$) (30 mg, 0.041 mmol, 68%). ^{13}C NMR data are listed in Table 4 in Supporting Information.

Compounds. Compounds for biological testing were prepared in 100% dimethyl sulfoxide (DMSO, Sigma) at 20 mg/mL. The reference drugs sodium stibogluconate (Pentostam, GSK) and amphotericin B (Fungizone, Squibb) were diluted in physiological saline.

Parasites. A laboratory strain of *L. infantum* (MHOM/MA(BE)/67), known to be sensitive to the available anti-leishmanial reference drugs, was obtained from the Institute of Tropical Medicine in Antwerp, Belgium. Maintenance in the laboratory was done by serial passage in the golden hamster (*Mesocricetus auratus*).

Drug Sensitivity Assay on Primary Mouse Macrophages. The *in vitro* sensitivity of amastigotes to the test compounds was determined in primary mouse peritoneal macrophages (MPΦ). MPΦ were induced in mice by intraperitoneal administration of 2% potato starch and harvested about 24 h later in RPMI-1640 medium. For the drug sensitivity assay, the compound stock solutions were appropriately diluted to obtain a final in-test dose range between 0.5 and 32 μg/mL. Assays were performed in triplicate in 96-well tissue culture plates, each well containing the compound dilutions together with 3×10^4 macrophages and 3×10^5 parasites/well. After 5 days of incubation at 37 °C, intracellular amastigote burdens were microscopically assessed after Giemsa staining. The results are expressed as percent reduction of parasite burden compared to that of untreated control wells.

Cytotoxicity against Mammalian Cells. Human lung fibroblasts (MRC-5) were cultured in 96-well tissue culture plates in MEM + 5% heat-inactivated fetal calf serum (FCS) at about 5000 cells/well. Assays were performed in triplicate, and cell viability was compared to untreated controls after 7 days of incubation after addition of the fluorescent dye Alamar Blue.[13] The results are expressed as percent reduction in overall cell viability compared to untreated control wells, and a CC_{50} (50% cytotoxic concentration) was estimated from a log−concentration plot (Graph Pad Prism software).

BALB/C Mouse Model with *L. infantum*. Male BALB/C mice were infected with 10^7 amastigotes of *L. infantum* in the lateral tail vein on day 0 and randomly allocated into groups of three animals each. The amastigotes for infection were obtained from the spleen of a heavily infected donor hamster. The test compounds were dissolved in isotonic saline solution at 2 mg/mL and filter-sterilized through a 0.22 μm filter (Millipore). By further dilution in saline, formulations for injection were prepared at lower concentrations. Sodium stibogluconate (Pentostam 100 mg/mL, equivalent to 32 mg of Sb^V/mL) and amphotericin B (Fungizone, 50 mg of soluble powder) were dissolved in saline to prepare a 50 mg/mL stock solution. Fresh solutions were made before each experiment. The test compounds were administered on day 0 as a single subcutaneous dosing, and autopsy was performed at 7 days post-treatment (dpt) for determination of amastigote burdens in the liver. Impression smears from the livers were prepared and stained with 10% Giemsa for microscopic enumeration of the number of amastigotes per liver cell. The active dose (ED_{90}) was defined as the dose level that reduced total amastigote burdens in the liver by at least 90%.

Supporting Information Available: ^1H NMR and ^{13}C NMR data for the chemical derivatives used in the biological evaluation (Tables 2−4) and elemental analysis results. This

material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Maes, L.; Germonprez, N.; Van Puyvelde, L.; Van Tri, M.; Ngoc Ninh, T.; De Kimpe, N. Antiprotozoal saponins. International Patent WO 00/38700, July 2000.

(2) *Pham Hoang Ho, Cay co Vietnam* (an illustrated Flora of Vietnam); NHA XUAT BAN TRE, Vietnam, 1999; Vol. 1, p 675.

(3) *Vo Van Chi, Tu dien cay thuoc Vietnam*; NHA XUAT BAN Y HOC, Vietnam, 1997; p 489.

(4) Maes, L.; Vanden Berghe, D.; Germonprez, N.; Quirijnen, L.; Cos, P.; De Kimpe, N.; Van Puyvelde, L. In vitro and in vivo activity of a triterpenoid saponin extract (PX-6518) from the plant *Maesa balansae* against visceral Leishmania species. *Antimicrob. Agents Chemother.* **2004**, *48*, 130−136.

(5) Germonprez, N.; Van Puyvelde, L.; Maes, L.; De Kimpe, N. New pentacyclic triterpene saponins with strong antileishmanial activity from the leaves of *Maesa balansae*. *Tetrahedron* **2004**, *60*, 219−228.

(6) Desjeux, P. The increase in risk factors for leishmaniasis worldwide. *Trans. R. Soc. Trop. Med. Hyg.* **2001**, *95*, 239−243.

(7) Guerin, P.; Olliaro, J. P.; Sundar, S.; Boelaert, M.; Croft, S. L.; Desjeux, P.; Wasunna, M. K.; Bryceson, A. D. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect. Dis.* **2002**, *2*, 494−501.

(8) Ridoux, O.; Di Giorgio, C.; Delmas, F.; Elias, R.; Mshvildadze, V.; Dekanosidze, G.; Kemertelidze, E.; Balansard, G.; Timon-David, P. In vitro anti-leishmanial activity of three saponins isolated from ivy, α-hederin, *β*-hederin and hederacolchiside A_1, in association with pentamidine and amphotericin B. *Phytother. Res.* **2001**, *15*, 298−301.

(9) Okunji, C. O.; Iwu, M. M.; Jackson, J. E.; Tally, J. D. Biological activity of saponins from two Dracaena species. *Adv. Exp. Med. Biol.* **1996**, *404*, 415−428.

(10) Plock, A.; Sokolowska-Kohler, W.; Presber, W. Application of flow cytometry and microscopical methods to characterize the effect of herbal drugs on Leishmania spp. *Exp. Parasitol.* **2001**, *97*, 141−153.

(11) Del Rayo Camancho Corona, M.; Croft, S. L.; Phillipson, J. D. Natural sources of antiprotozoal drugs. *Curr. Opin. Anti-Infect. Invest. Drugs* **2000**, *2*, 47−62.

(12) Hostettmann, K.; Marston, A. Saponins. In *Chemistry & Pharmacology of Natural Products*; Cambridge University Press: London, 1995; pp 239−284.

(13) O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **2000**, *267*, 5421−5426.

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