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Heterocycle-fused Lupane Triterpenoids Inhibit *Leishmania donovani* Amastigotes

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Raisa Haavikko,[a] Abedelmajeed Nasereddin,[b] Nina Sacerdoti-Sierra,[b] Dmitry Kopelyanskiy,[b] Sami Alakurtti,[c] Mari Tikka,[a] Charles L. Jaffe[b] and Jari Yli-Kauhaluoma[a*]

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The synthesis of heterocyclic betulin derivatives and their activity against *Leishmania donovani* is reported. Betulonic acid was used as a versatile intermediate. Several different fused heterocycles were introduced at the 2,3-position of the lupane skeleton including isoxazole, pyrazine, pyridine, indole and pyrazole rings. Also 28-position was modified. Three compounds, **5**, **8** and **25**, showed low micromolar activity with IC_{50} values of 13.2, 4.3 and 7.2 μM, respectively. Compound **8** showed the best activity and selectivity, and its activity was tested on infected macrophages using a concentration, 5 μM, where no macrophage toxicity was exhibited. Interestingly, the activity of compound **8** on axenic amastigotes and *Leishmania*-infected macrophages was similar.

Introduction

Leishmaniasis is a spectrum of diseases caused by over 20 species of protozoan parasites belonging to the genus *Leishmania.* These diseases affect people in more than 88 countries. There are an estimated 1 - 2 million new cases every year, 12 million people currently infected, and 350 million people living in endemic areas at risk.[1,2] During past ten years leishmaniasis has spread considerably.[2] It is transmitted by bite of infected female *Phlebotomine* or *Lutzomyia* sandflies in the Old World and the New World, respectively.[3]

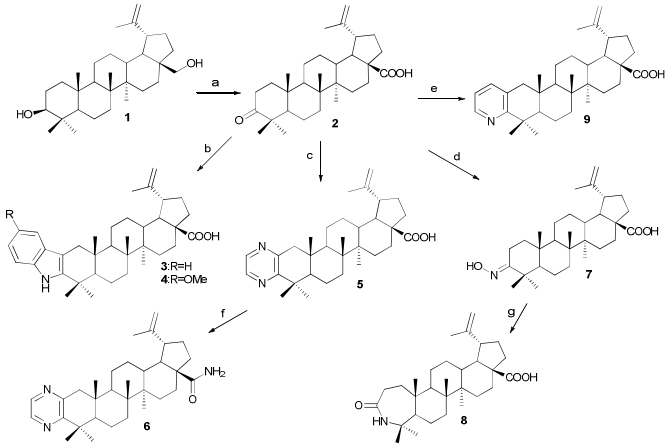
There are three major forms of leishmaniasis: cutaneous, mucocutaneous, and visceral disease.[2,4] Cutaneous leishmaniasis is the mildest form of this disease and is characterized by skin ulcers on exposed areas at the site of the sand fly bite. The ulcers generally self-heal leaving scars after a few months to years. In the mucocutaneous form, which is difficult to treat, disfiguring lesions destroy the mucous membranes of the nose, mouth and throat cavity. Finally, visceral leishmaniasis (VL), the most severe form of the disease, is fatal if untreated. VL causes fever, weight loss, anaemia, and enlargement of the spleen and liver. Several treatments exist for leishmaniasis, but most of them have adverse effects. Pentavalent antimonials, the first-line treatment for leishmaniasis, have lost their efficacy in some regions endemic for VL[5], and liposomal amphotericin B is highly expensive. These treatments are administrated by injection and require clinical supervision or hospitalization. Miltefosine, the first effective orally administrated drug for leishmaniasis, is contraindicated in women of child-bearing age due to teratogenic effects.[6] Hence, there is an urgent need to develop new, safe and effective treatments for these diseases.

Betulin is a plentiful naturally occurring lupane-type pentacyclic triterpene. Betulinic acid and other betulin derivatives show antiviral[7], anti-HIV[8], anti-inflammatory[9], anti-malarial[10], and anti-tumoral[11] effects. Previously our group has shown that heterocyclic betulin derivatives have an effect against *L. donovani* amastigotes, which cause VL.[12] In this study we describe a new set of heterocyclic betulin derivatives and their biological activity against *Leishmania donovani* amastigotes, as well as the structure-activity relationships of the compounds.

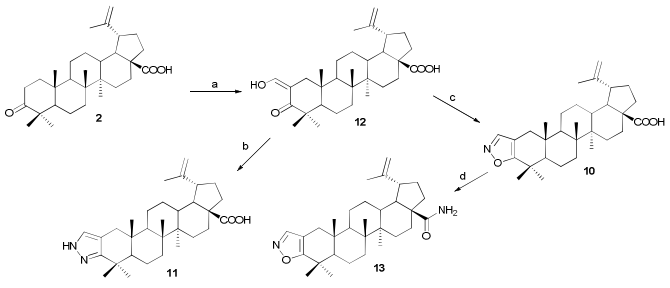
Results and discussion

Chemistry

First, betulin **1** was subjected to Jones oxidation, and the resulting betulonic acid **2** was used as a key intermediate for the synthesis of several heterocyclic adducts (Scheme 1). The indole derivatives **3-4** were prepared by the Fischer indole synthesis in 21-42% yields. Letting betulonic acid react with ethylenediamine in the presence of sulfur and morpholine gave lupa-2,20(29)-dieno[2,3-*b*]pyrazin-28-oic acid **5** in 68% yield.[13] This was treated with oxalyl chloride in dichloromethane (DCM) and the resulting acyl chloride was converted to the primary amide **6** quantitatively by aqueous ammonia in chloroform.[14] 3-Oximinolup-20(29)-en-28-oic acid **7** was obtained by refluxing betulonic acid in the presence of NH_2OH•HCl and pyridine in methanol. The oxime **7** was further converted to 4-aza-3-oxohomolup-20(29)-en-28-oic acid **8** in 33% yield in the Beckmann rearrangement reaction by treating it with trifluoroacetic anhydride (TFAA) in DCM. Lupa-2,20(29)-dieno[2,3-*b*]pyridin-28-oic acid **9** was obtained from the reaction of betulonic acid and propargylamine in the presence of Cu(I)Cl in ethanol in 11% yield.[15]

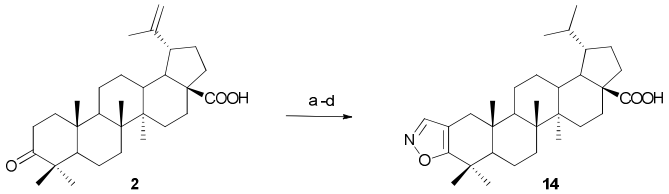


Scheme 1 Reagents and conditions: (a) Jones oxidation, $Na_2Cr_2O_7$, H_2SO_4, H_2O, acetone, rt, 21 h, 44%; (b) appropriate phenylhydrazine hydrochloride, HOAc, reflux, 3 h, 21-42%; (c) ethylenediamine, sulfur, morpholine, reflux, 21 h, 68%; (d) NH_2OH•HCl, pyridine, MeOH, reflux, 16 h, 84%; (e) propargylamine, Cu(I)Cl, EtOH, reflux, 17 h, 11%; (f) 1. oxalyl chloride, DCM, rt, 3 h 2. aqueous ammonia, DCM, rt, 1 h, quant.; (g) TFAA, DCM, rt, 20 h, 33%. DCM = dichloromethane, TFAA = trifluoroacetic anhydride.



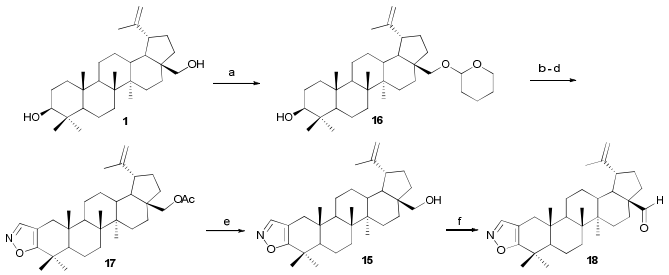
Scheme 2 Reagents and conditions: (a) ethyl formate, NaH, THF, rt, 16 h, 56%; (b) H_2NNH_2•H_2O, *p*-TsOH, PhMe, 150 °C, 17 h, 80%; (c) NH_2OH•HCl, HOAc, reflux, 3 h, 68%; (d) 1. oxalyl chloride, DCM, rt, 1 h; 2. aqueous ammonia, $CHCl_3$, rt, 30 min, quant. THF = tetrahydrofuran.

The corresponding isoxazole **10** and pyrazole **11** derivatives were synthesized via the 2-hydroxymethylene adduct **12** of betulonic acid **2** followed by the condensation/cyclization reaction with NH_2OH•HCl[16] or H_2NNH_2•H_2O[17] in 68% and 80% yields, respectively (Scheme 2). The carboxyl group of lupa-2,20(29)-dieno[2,3-*d*]isoxazol-28-oic acid **10** was converted to the primary amide functionality **13** as described above in case of compound **6**.[14]



Scheme 3 Reagents and conditions: (a) benzyl bromide, K_2CO_3, DMF, 55 °C, 22 h, 43%; (b) H_2, 10% Pd/C, EtOAc, rt, 72 h, quant.; (c) ethyl formate, NaH, THF, rt, 22 h, 56%; (d) NH_2OH•HCl, HOAc, reflux, 6 h, 90%. DMF = *N*,*N*-dimethylformamide.

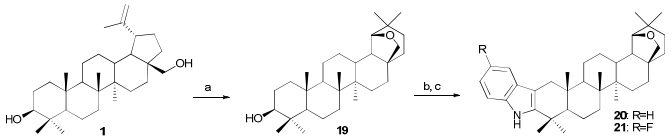
20(29)-Dihydrolup-2-en[2,3-*d*]isoxazol-28-oic acid **14** was obtained from benzyl betulonate in three steps (Scheme 3). First, the carbon-carbon double bond of benzyl betulonate was reduced under hydrogen atmosphere in the presence of palladium on carbon in ethyl acetate to give the corresponding dihydrobetulonic acid in 77% yield. The subsequent Claisen condensation with ethyl formate produced 2-(hydroxymethylene)-3-oxo-20(29)-dihydrolupen-28-oic acid in 56% yield. Finally, the treatment of the Claisen product with NH_2OH•HCl in acetic acid gave the target 20(29)-dihydrolup-2-en[2,3-*d*]isoxazol-28-oic acid **14** in 90% yield.



Scheme 4 Reagents and conditions: (a) pyridine *p*-toluenesulfonate, 3,4-dihydro-2*H*-pyran, DCM, rt, 18 h, 80%; (b) PCC, DCM, rt, overnight, 46%; (c) ethyl formate, NaH, THF, rt, 22 h, 52%; (d) NH_2OH•HCl, HOAc, reflux, 4 h, quant.; (e) *p*-TsOH, MeOH, reflux, 20 h, quant.; (f) IBX, THF, DMSO, rt, 3.5 h, 51%. PCC = pyridinium chlorochromate, IBX = 2-iodoxybenzoic acid, DMSO = dimethyl sulfoxide.

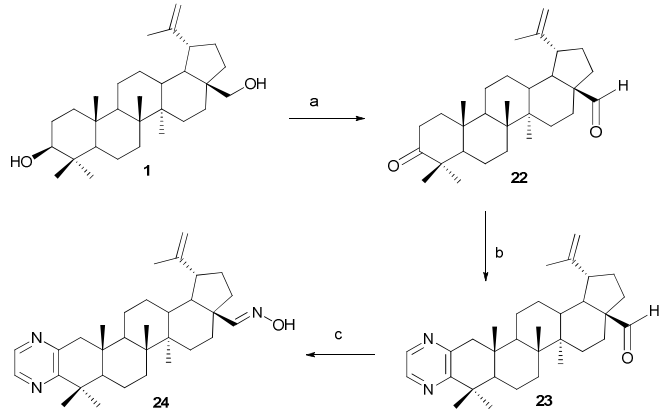
28-Hydroxylupa-2,20(29)-dieno[2,3-*d*]isoxazole **15** was synthesized from betulin **1** in five steps (Scheme 4). First, the betulin C-28 hydroxy group was protected as a tetrahydropyranyl ether **16** in 80% yield, and the resulting THP ether was oxidized to the THP-protected betulonic alcohol with PCC in DCM[18] in 46% yield. Subsequently, the same cascade of reactions as described above for lupa-2,20(29)-dieno[2,3-

d]isoxazol-28-oic acid **10** was used to produce the isoxazole-fused 28-*O*-acetyl triterpene **17** in 26% yield over two steps (Scheme 4). In acidic conditions of the cyclization reaction THP protecting group was cleaved and replaced with the acetoxy group. The acetoxy group was removed with *p*-TsOH in methanol in quantitative yield. Finally, 28-hydroxylupa-2,20(29)-dieno[2,3-*d*]isoxazole **15** was treated with 2-iodoxybenzoic acid in THF and DMSO to give 28-oxolupa-2,20(29)-dieno[2,3-*d*]isoxazole **18** in 51% yield.



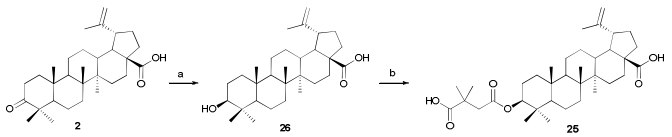
Scheme 5 Reagents and conditions: (a) formic acid, reflux, 45 min; 1 M KOH in EtOH, benzene, 30 min, 25%; (b) Jones oxidation, 21 h, 88%; (c) appropriate phenylhydrazine hydrochloride, HOAc, reflux, 2 h, 57-63%.

Allobetulin **19** was obtained in 25% yield by refluxing betulin **1** in formic acid followed by refluxing the resulting intermediate formate ester in ethanolic solution of KOH in benzene (Scheme 5). The indole derivatives of allobetulin **20-21** were obtained with the same methodology as described above for betulonic acid (cf. synthesis of compounds **3** and **4**) in 57-63% yields.



Scheme 6 Reagents and conditions: (a) PCC, DCM, rt, 1 h, 27%; (b) ethylenediamine, sulfur, morpholine, reflux, 2.5 h, 17%; (c) $NH_2OH{\bullet}HCl$, pyridine/EtOH (1:3), reflux, 16 h, 77%.

Betulonic aldehyde **22** was obtained from betulin **1** by PCC oxidation in DCM in 27% yield (Scheme 6). 28-Oxolupa-2,20(29)-dieno[2,3-*b*]pyrazine **23** was synthesized in 17% yield using the same methods as in the preparation of lupa-2,20(29)-dieno[2,3-*b*]pyrazin-28-oic acid **5**. It was further reacted with $NH_2OH{\bullet}HCl$ to give 28-oximinolupa-2,20(29)-dien[2,3-*b*]pyrazine **24** in 77% yield.



Scheme 7 Reagents and conditions: (a) $NaBH_4$, 2-propanol, rt; (b) 2,2-dimethylsuccinic anhydride, DIPEA, DMF 170 °C, 2 d, 5%. DIPEA = *N*,*N*-diisopropylethylamine.

3β-(3-Carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oic acid (bevirimat) **25** was synthesized from betulinic acid **26** by refluxing it in the presence of 2,2-dimethylsuccinic anhydride and DIPEA in DMF for 2 days in 5% yield (Scheme 7).

Biology and structure-activity relationships

Previously we found a set of heterocyclic betulin derivatives to have promising activity against axenic amastigotes of *L. donovani*,[12] and based on those results we synthesized a new set of fused heterocyclic adducts of betulin, betulinic acid and betulonic acid; and varied substituents at the position C-28 to explore effects of that position as well. Leishmanicidal activity of the modified compounds was assayed using the alamarBlue (AbD Serotec, Oxford, UK) viability assay on axenic amastigotes of *L. donovani* (Table 1).

Primary screen was performed at 50 μM concentration and compounds showing >70% inhibition were assayed at 15 μM concentration and finally most potent derivatives at 5 μM concentration (Table 1).

In the series of A-ring fused isoxazoles, the betulin-derived compound **15** had 57% inhibition of the growth at 50 μM concentration. The betulinic acid-derived isoxazole **10** inhibits 96% at 50 μM and 16% at 15 μM. Interestingly, the dihydrobetulinic acid-derived isoxazole **14** had only 3% inhibition at 50 μM. In our earlier studies we found a similar effect, but not this strong, between betulonic acid and dihydrobetulonic acid.[19] The primary amide derivative of the betulinic acid-derived isoxazole **13** inhibits 84% of the growth at 50 μM concentration, but only 17% at 15 μM concentration. On the other hand, the betulinic aldehyde-derived isoxazole **18** has lower activity (34%) at 50 μM but slightly better activity (21%) at 15 μM concentration compared to **13**. This may be due to solubility, as the aldehyde might not be completely soluble at high concentration. The least active isoxazole derivative, 28-*O*-acetylbetulin-derived isoxazole **17** inhibited only 6% of the growth at 50 μM concentration. It has been suggested that carboxyl group in triterpenoid skeleton enhances the observed antiprotozoal effects.[20] However, among these A-ring fused isoxazole derivatives of betulin, the compounds **18** and **13** were more active than the betulinic acid-derived isoxazole **10**. All isoxazole derivatives were less active than betulonic acid **2** (99% at 50 μM, 46% at 15 μM).

The A-ring fused pyrazine derivative of betulinic acid **5** showed 93% at 50 μM, 80% at 15 μM, and 20% inhibition at 5 μM concentration, whereas for the corresponding primary amide **6**, we observed inhibition of 95% at 50 μM, and 35% at 15 μM concentration, and for its 28-oximino derivative **24** only 3% at 50 μM. Interestingly, the A-ring fused pyridine derivative of betulinic acid **9** inhibited 88% at 50 μM, but only 26% at 15 μM concentration. Here, with pyrazine derivatives, we could see the importance of carboxyl group for antileishmanial activity.

The A-ring fused 5'-methoxyindole derivative of betulinic acid **4** was the most active indole derivative. At 5 μM, the lowest concentration tested, inhibition was 14%, whereas the corresponding unsubstituted indole derivative **3** inhibited only 5%. Also with the related indole derivatives the importance of carboxyl group can be seen as the A-ring fused indole derivative of allobetulin **20** and the corresponding 5'-fluoroindole derivative **21** did not have activity at all. One factor affecting this might be the reduced solubility; allobetulin derivatives are not that soluble under the assay conditions. In addition, the A-ring fused pyrazole derivative of betulinic acid **11** and betulinic acid **26** precipitated at 50 μM in these assays.

4-Aza-3-oxohomobetulinic acid **8** displayed very good activity (98.2%) at 50 μM concentration and even at 5 μM concentration (inhibition 52.0%), whereas the A-ring fused pyrazole derivative of betulinic acid **11** displayed moderate 60.2% inhibition at 50 μM concentration. In addition, potent anti-HIV betulinic acid derived compound **25**, bevirimat, displayed very good inhibition: 100% inhibition at 50 μM concentration and 59.0% at 5 μM concentration. The best compounds after primary screen were compounds **5**, **8** and **25** that significantly inhibited parasite growth when tested at lower concentrations. The IC_{50} values for **5**, **8** and **25** were 13.2, 4.3 and 7.2 μM, respectively, with the compound **8** showing the best activity. Cytotoxicity IC_{50} values of **8** and **25** against THP-1 cell line were 55.5 and 54.0 μM, respectively. The compound **25** (bevirimat) showed the highest activity among the compounds tested in this study. It showed 100% inhibition at 50 μM, 69% at 15 μM, and 59% at 5 μM. Interestingly, bevirimat **25** also showed good activity against HIV-infected patients in a recent phase II study.[21] Only 4-aza-3-oxohomobetulinic acid **8** showed a similar level of activity with 98% inhibition at 50 μM, 75% at 15 μM, and 52% at 5 μM. The third most active compound was the A-ring fused pyrazin derivative of betulinic acid **5**. Cytotoxicity (Table 2) using the human macrophage cell line THP-1 was determined for **8** and **25**, and found to be similar (IC_{50} ca. 50 μM) for both compounds. Compound **8** had the best selectivity index (IC_{50} THP-1 / IC_{50} axenic amastigotes; SI =12.9), and its activity was tested on *L. donovani* infected macrophages at low 5 μM concentration, where no macrophage toxicity was observed. Interestingly, activity of the compound **8** against *Leishmania* infected macrophages (54.0 ± 4.8% inhibition) was similar to that seen for axenic amastigotes (52.0 ± 1.2% inhibition).

Table 1 Activity of the compounds against axenic amastigotes of *Leishmania donovani*.

Compound	% inhibition ± s.e [$]			IC_{50} μM ± s.e. [**]
	50 μM	15 μM μM±s.e	5 μM	
2	98.6±0.1	45.9±0.7	-	
3	23.4±2.2	27.4±2.4	5.0±4.4	
4	29.3±4.1	17.5±2.7	14.0±2.0	
5	92.7±0.1	79.7±0.4	20.3±0.4	13.2±1.4
6	94.9±0.2	35.2±1.4	-	
8	98.2 ±0.1	75.2±1.1	52.0±1.2	4.3±0.4
9	87.9±0.5	25.6±1.5	-	
10	95.7±0.3	15.6±1.0	-	
11	60.2±0.7*	-	-	
13	84.3±0.8	16.7±2.0	-	
14	2.4±3.4	-	-	
15	56.9±0.4	-	-	
17	6.1±3.7	-	-	
18	34.2±2.0	21.1±1.1	-	
20	19.8±2.4	18.5±0.8	-	
21	6.8±0.7	10.2±4.1	0.2±1.3	
24	2.8±4.5	-	-	
25	100.4±0.1	69.1±3.1	59.0±0.4	7.2±0.2
26	61.1±0.6*	-	-	
Amphotericin B	-	-	99.9±0.3	
Medium alone	0.0±4.6	1.5±3.8	0.0±0.9	

*Precipitates at 50 μM, see crystals; [$]Average inhibition of triplicates. ** Average two experiments; Amphotericin B is a positive control and was tested at 1 μM.

Table 2 Toxicity for THP-1 macrophages, and activity against *Leishmania donovani* infected macrophages (iMΦ).

Compound	Toxicity THP-1 cells[$] IC_{50} (μM ± s.e)	SI*	% Inhibition ± s.e of parasites in iMΦ 5 μM **
8	55.5±1.8	12.9	54.0±4.8
25	54.0±1.7	7.5	nd
Amphotericin B	nd	nd	96.7±0.7

[$] Average two experiments; *Selectivity Index = IC_{50} THP-1 / IC_{50} Axenic amastigotes; ** Average three experiments; nd – not done; Amphotericin B is a positive control and was tested on infected macrophages at 1 μM

Experimental section

Chemistry

1'*H*-Lupa-2,20(29)-dieno[3,2-*b*]indol-28-oic acid. (3) Betulonic acid (0.10 g, 0.22 mmol) and the corresponding phenylhydrazine hydrochloride (0.35 g, 0.24 mmol) were dissolved in acetic acid (10 mL) and refluxed (130 °C) for 3 h. Water was added and the resulting mixture was extracted with Et_2O. The organic phase was washed with water and brine, dried over anhydrous Na_2SO_4, and the solvents were evaporated. The crude product was purified with SiO_2 column chromatography (25-50% EtOAc/*n*-hexane) to give a yellowish solid (49 mg, 42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (s, 1H), 7.37 (m, 1H), 7.29 (m, 1H), 7.08 (m, 2H), 4.79 (s, 1H), 4.65 (s, 1H), 3.08 (m, 1H), 2.83 (d, J = 15.0 Hz, 1H), 2.38–2.09 (m, 4H), 1.73 (s, 3H), 1.68–1.31 (m, 12H), 1.28 (s, 3H), 1.17 (s, 3H), 1.05 (s, 3H), 1.04 (s, 3H), 0.88 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.7, 150.4, 140.9, 136.2, 128.4, 121.0, 119.0,

117.9, 110.3, 109.8, 107.1, 56.5, 53.3, 49.4, 49.4, 47.0, 42.5, 40.8, 38.7, 38.3, 37.1, 34.1, 33.6, 32.2, 31.6, 30.9, 29.9, 25.7, 23.1, 22.6, 21.5, 19.4, 19.2, 16.3, 15.9, 14.8; FTIR (ν, cm^{-1}): 738, 885, 907, 1459, 1693, 2873, 2843; HRMS: *m/z* calcd for $C_{36}H_{50}NO_2$ 528.3842, found 528.3838 $[M+H]^+$. NMR spectral data is consistent with those previously reported.[22]

Lupa-2,20(29)-dieno[2,3-*b*]pyrazin-28-oic acid. (5) A mixture of betulonic acid (0.20 g, 0.44 mmol), 1,2-diaminoethane (130 mg, 2.0 mmol), sulfur (130 mg, 4.1 mmol) and morpholine (4 mL) was refluxed for 21 h. Water was added and the resulting mixture was extracted with CH_2Cl_2. The organic phase was washed with 1 M hydrochloric acid, water, a saturated aqueous solution of $NaHCO_3$, water and brine, dried over anhydrous Na_2SO_4 and evaporated. The crude product was purified with SiO_2 column chromatography (20-50% EtOAc/*n*-hexane) to give a white crystalline solid (147 mg, 68%).[13] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (d, J = 2.4 Hz, 1H), 8.29 (d, J = 2.4 Hz, 1H), 4.76 (s, 1H), 4.64 (s, 1H), 3.05 (m, 2H), 2.46 (d, J = 16.5 Hz, 1H), 2.29 (m, 2H), 1.30 (s, 3H), 1.72 (s, 3H), 1.27 (s, 3H), 1.04 (s, 3H), 1.03 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.5, 160.0, 151.0, 150.5, 142.6, 141.5, 110.0, 56.6, 53.2, 49.4, 49.0, 48.7, 47.1, 42.7, 40.8, 39.7, 38.7, 37.3, 37.0, 33.6, 32.4, 31.7, 30.8, 30.0, 25.7, 24.2, 21.6, 20.3, 19.7, 16.4, 15.9, 14.9; FTIR (ν, cm^{-1}): 878, 1107, 1381, 1408, 1686, 2869, 2943; HRMS: *m/z* calcd for $C_{32}H_{47}N_2O_2$ 491.3638, found 491.3637 $[M+H]^+$. ^{1}H NMR spectral data is consistent with those previously reported.[23]

Lupa-2,20(29)-dieno[2,3-*d*]pyrazin-28-amide. (6) A mixture of lupa-2,20(29)-dieno[2,3-*d*]pyrazin-28-oic acid (141 mg, 0.28 mmol), oxalyl chloride (44 mg, 0.34 mmol), and a drop of DMF in dry THF (10 mL) was stirred at room temperature for 2 h. The solvent was evaporated, and the residue was dissolved in EtOAc. The organic phase was washed with a saturated aqueous solution of $NaHCO_3$, water and brine, dried over anhydrous Na_2SO_4 and evaporated. The resulting crude lupa-2,20(29)-dieno[2,3-*b*]pyrazin-28-oyl chloride was dissolved in $CHCl_3$ (5 mL), and a water solution of 25% ammonia (2 mL) was added to the mixture. The resulting solution was stirred at room temperature for 30 min and evaporated to dryness to give a white crystalline solid (137 mg, quant).[14] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 (s, 1H), 8.29 (d, J = 2.4 Hz, 1H), 5.30 (br s, 2H), 4.77 (s, 1H), 4.64 (s, 1H), 3.72 (q, J = 7.0 Hz, 1H), 3.12 (d, J = 17.0 Hz, 2H), 2.67–2.40 (m, 2H), 2.08–1.74 (m, 5H), 1.71 (s, 3H), 1.67–1.35 (m, 13H), 1.31 (s, 3H), 1.30 (s, 3H), 1.04 (s, 3H), 1.03 (s, 3H), 0.82 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 182.4, 161.4, 152.3, 152.0, 143.8, 142.3, 110.0, 57.1, 54.3, 51.2, 50.1, 48.1, 43.7, 41.9, 40.62, 39.4, 39.1, 37.9, 34.6, 34.3, 31.9, 31.8, 30.7, 27.0, 24.4, 22.8, 21.2, 19.6, 16.6, 16.3, 15.0; FTIR (ν, cm^{-1}): 886, 1107, 1184, 1402, 1665, 2869, 2948, 3044, 3129; HRMS: *m/z* calcd for $C_{32}H_{48}N_3O$ 489.3797, found 490.3796 $[M+H]^+$.

4-Aza-3-oxohomolup-20(29)-en-28-oic acid. (8) A mixture of betulonic acid (0.20 g, 0.44 mmol), hydroxylamine hydrochloride (290 mg, 4.2 mmol), dry pyridine (5 mL) and methanol (8 mL) was refluxed for 16 h. Water was added, and the precipitated 3-oximinolup-20(29)-en-28-oic acid **7** was filtered and collected (173 mg, 84%). 3-Oximinolup-20(29)-en-28-oic acid **7** (86 mg, 0.18 mmol) was dissolved in CH_2Cl_2 (5 mL), and the resulting solution cooled to the ice-water bath temperature. Trifluoroacetic anhydride (1.0 mL, 7.1 mmol) was added to this solution and the resulting mixture was stirred at room temperature for 20 h. The reaction mixture was washed with water, a saturated aqueous solution of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified with SiO_2 column chromatography (0-10% MeOH/CH_2Cl_2) to yield a white crystalline solid (28 mg, 33%).[16] ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.39 (s, 1H), 4.73 (s, 1H), 4.61 (s, 1H), 2.99 (m, 1H), 2.59–2.41 (m, 1H), 2.42–2.15 (m, 4H), 2.13–1.94 (m, 2H), 1.69 (s, 3H), 1.57–1.33 (m, 12H), 1.31 (s, 3H), 1.23 (s, 3H), 1.03 (s, 3H), 0.98 (s, 3H), 0.97 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.3, 177.5, 150.6, 109.9, 56.7, 56.5, 56.5, 53.2, 51.2, 49.3, 47.7, 42.8, 41.0, 40.5, 39.5, 38.8, 37.3, 33.9, 33.4, 32.3, 32.0, 30.8, 29.8, 27.2, 26.1, 22.7, 22.3, 19.6, 18.5, 16.1, 14.8, 14.7; FTIR (ν, cm^{-1}): 731, 883, 1185, 1374, 1454, 1628, 1691, 2938, 3250; HRMS: *m/z* calcd for $C_{30}H_{48}NO_3$ 470.3634, found 470.3630 $[M+H]^+$.

1'*H*-Lup-20(29)-eno[2,3-*b*]pyridin-28-oic acid. (9) A mixture of betulonic acid (100 mg, 0.22 mmol), propargylamine (24 mg, 0.44 mmol), Cu(I)Cl (5.0 mg, 0.050 mmol) and ethanol (5 mL) was refluxed for 17 h. The resulting solution was filtered, evaporated, and the crude product was purified with SiO_2 column chromatography (10-20% EtOAc/*n*-hexane) to yield a crystalline solid (12 mg, 11%).[15] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.47 (m, 1H), 7.27 (m,1H), 7.02 (dd, J = 7.6, 4.8 Hz, 1H), 4.75 (s, 1H), 4.62 (s, 1H), 3.10 (m, 1H), 2.74 (d, J = 15.9 Hz, 1H), 2.32 (m, 3H), 2.03 (m, 2H), 1.70 (s, 3H), 1.67–1.36 (m, 13H), 1.32 (s, 3H), 1.27 (s, 3H), 1.02 (s, 3H), 0.99 (s, 3H), 0.78 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 180.3, 163.6, 150.8, 146.8, 138.4, 130.3, 121.1, 109.7, 56.6, 53.8, 49.5, 49.0, 47.12 46.1, 42.7, 40.8, 39.6, 38.7, 37.2, 36.4, 33.7, 32.5, 31.6, 30.9, 30.0, 25.8, 24.2, 21.7, 20.4, 19.6, 16.0, 15.9, 14.8; FTIR (ν, cm^{-1}): 1012, 1045, 1110, 1132, 1184, 1457, 2856, 2927, 2959; HRMS: *m/z* calcd for $C_{33}H_{48}NO_2$ 490.3685, found 490.3683 $[M+H]^+$.

2-(Hydroxymethylene)-3-oxolup-20(29)-en-28-oic acid. (12) A mixture of betulonic acid (0.200 g, 0.440 mmol), NaH (60% dispersion in mineral oil, 0.490 g, 12.8 mmol) and dry THF (10 mL) was cooled to the ice-water bath temperature. To this solution ethyl formate (0.749 g, 10.1 mmol) was added, the resulting mixture was warmed to room temperature and stirred overnight. A saturated aqueous solution of NH_4Cl was added, and the resulting mixture was extracted with EtOAc, washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified with SiO_2 column chromatography (10-50% EtOAc/*n*-hexane) to yield a white crystalline solid (127 mg, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.85 (d, J = 2.6 Hz, 1H), 9.88 (br s, 1H), 8.58 (d, J = 2.6 Hz, 1H), 4.75 (s, 1H), 4.62 (s, 1H), 3.01 (m, 1H), 2.31 (m, 3H), 2.09–1.80 (m, 3H), 1.70 (s, 3H), 1.46 (m, 16H), 1.18 (s, 3H), 1.08 (s, 3H), 1.00 (s, 3H), 0.99 (s, 3H), 0.83 (s, 3H).

Lupa-2,20(29)-dieno[2,3-*d*]isoxazol-28-oic acid. (10) A mixture of 2-(hydroxymethylene)-3-oxolup-20(29)-en-28-oic

acid (0.091 g, 0.18 mmol), hydroxylamine hydrochloride (0.036 g, 0.52 mmol) and acetic acid (10 mL) was refluxed for 3 h. Water was added, and the resulting mixture was extracted with EtOAc, washed with a saturated aqueous solution of $NaHCO_3$, water, and brine, dried over anhydrous Na_2SO_4, and evaporated to give a white solid (59 mg, 68%).[12] ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.92 (br s, 1H), 7.97 (s, 1H), 4.75 (s, 1H), 4.63 (s, 1H), 3.09–2.92 (m, 1H), 2.47 (d, J = 15.1 Hz, 1H), 2.36–2.19 (m, 3H), 2.06–1.88 (m, 3H), 1.70 (s, 3H), 1.60–1.33 (m, 15H), 1.28 (s, 3H), 1.19 (s, 3H), 1.01 (s, 3H), 0.99 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.3, 173.0, 150.3, 150.3, 109.8, 108.9, 56.4, 53.6, 49.2, 49.1, 46.9, 42.5, 40.8, 39.0, 38.5, 37.0, 35.9, 34.8, 33.4, 32.1, 30.6, 29.8, 28.7, 25.5, 21.4, 21.2, 19.4, 18.8, 16.1, 15.8, 14.7; FTIR (ν, cm^{-1}): 733, 881, 1181, 1375, 1454, 1695, 2875, 2940; HRMS: *m/z* calcd for $C_{31}H_{46}NO_3$ 480.3478, found 480.3478 $[M+H]^+$. NMR spectral data is consistent with those previously reported.[24]

1'*H*-Lup-20(29)-eno[3,2-*c*]pyrazol-28-oic acid. (11) A mixture of 2-(hydroxymethylene)-3-oxolup-20(29)-en-28-oic acid (53 mg, 0.11 mmol), hydrazine hydrate (16 mg, 0.31 mmol) and toluene (20 mL) was refluxed at 150 °C under the Dean-Stark conditions overnight. After cooling the reaction mixture to room temperature, solvent was evaporated, and the resulting crude product was purified with SiO_2 column chromatography (1-10% EtOAc/*n*-hexane) to give a white crystalline solid (42 mg, 80%).[17] ^{1}H NMR (300 MHz, CD_3OD) δ 7.16 (s, 1H), 4.72 (s, 1H), 4.60 (s, 1H), 3.04 (m, 1H), 2.64 (d, J = 14.8 Hz, 1H), 2.46–2.18 (m, 2H), 1.91 (m, 2H), 1.70 (s, 3H), 1.65–1.33 (m, 11H), 1.26 (s, 3H), 1.17 (s, 3H), 1.04 (s, 3H), 1.03 (s, 3H), 0.80 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 178.8, 150.8, 149.9, 133.2, 112.4, 109.0, 56.3, 53.8, 49.4, 49.2, 47.3, 42.5, 40.8, 38.6, 36.9, 36.5, 33.6, 33.4, 32.1, 30.6, 30.1, 29.8, 25.8, 22.8, 21.4, 19.1, 18.4, 15.3, 15.2, 14.0; FTIR (ν, cm^{-1}): 883, 960, 1086, 1184, 1370, 1452, 1643, 1695, 2869, 2943; *m/z* calcd for $C_{31}H_{47}N_2O_2$: 479.3638; found 479.3638 $[M+H]^+$. NMR spectral data is consistent with those previously reported.[24]

Biology

L. donovani (MHOM/SD/1962/1S-Cl2d) was used in all bioassays. Axenic amastigotes were grown at 37 °C in a 5% CO_2 incubator as described in complete RPMI 1640 containing 20% fetal calf serum, pH 5.5. Screening of the compounds for leishmanicidal activity was carried out using the alamarBlue (AbD Serotec, Oxford, UK) viability assay similar to that reported for leishmanial promastigotes. Standardization and optimization of the assay for axenic amastigotes has been described elsewhere.[25] Compounds to be assayed were diluted to twice the final concentration used in the assays in the complete amastigote medium, containing 1% DMSO, and were aliquoted in triplicate (125 µL/well) into 96-well flat-bottom plates (Nunc, Roskilde, Denmark). IC_{50} was determined using serial two-fold dilutions of the test compounds from 50 to 0.4 µM. Amastigotes (5.0×10^5 cells/mL; 125 µL/well) were added to each well and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The alamarBlue viability indicator was added (25 µL/well) and the plates incubated for an additional 24 h at which time the fluorescence (λ_{ex} = 544 nm; λ_{em} = 590 nm) was measured in a microplate reader (Fluoroskan Ascent FL, Finland). Complete medium both with and without DMSO was used as negative controls (0% inhibition of amastigote growth). Amphotericin B (Sigma-Aldrich, St. Louis MO), a drug used to treat VL, was included as a positive control on each plate and gave >90% inhibition of parasite growth at 1 µM. Toxicity was measured on the human leukaemia monocyte cell line (THP-1 6.4×10^4 cells/well) using the alamarBlue viability indicator as previously described.[26] IC_{50} was determined using serial two-fold dilutions of the test compounds in triplicate from 500 to 0.25 µM. Inhibition of intracellular amastigote growth in infected THP-1 cells (1.0×10^5 cells/well) was carried out using transgenic *Ld*:pSSU-int/LUC promastigotes that express luciferase essentially as previously described.[26] Amphotericin B (1 µM) was included as a positive control on each plate. Complete medium both with and without DMSO was used as negative controls. Calculation of the IC_{50}'s and statistical analysis were carried out using GraphPad Prism Vesion 6.0b (GraphPad Software, Inc. San Diego, CA).

Conclusions

A set of betulin, betulinic acid and dihydrobetulinic acid derivatives, including eight new A-ring fused heterocycles, was synthesized and tested against *L. donovani*. Two heterocyclic compounds **5**, **8**, and potent anti-HIV drug candidate **25**, had significant inhibition on parasite growth even at 5 µM concentration. Compound **8** had the best selectivity index, and showed similar good activity on *Leishmania*-infected macrophages and axenic amastigotes. Further improvement and optimization are needed to get more potent betulin derivatives against *L. donovani*.

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Notes and references

[a] Faculty of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki, Viikinkaari 5 E (P. O. Box 56), FI-00014 University of Helsinki, Helsinki, Finland. Tel: +358-9-191 59170; E-mail: jari.yli-kauhaluoma@helsinki.fi
[b] Department of Microbiology and Molecular Genetics, IMRIC, Hebrew University-Hadassah Medical School, P. O. Box 12272, Jerusalem 91120, Israel. Tel: +97226757435; E-mail: cjaffe@cc.huji.ac.il
[c] VTT Technical Research Centre of Finland, P. O. Box 1000, FI-02044 VTT (Espoo), Finland

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‡CLJ holds the Michael and Penny Feiwel Chair of Dermatology.

1 J. Alvar, I. D. Velez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin and M. den Boer, *PLoS One*, 2012, **7**, e35671 (DOI: 10.1371/journal.pone.0035671).

2 WHO, *Leishmaniasis*, World Health Organization, http://www.who.int/leishmaniasis/en/index.html .

3 World Health Organization, *Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010*, N°949, 2010.

4. P. Desjeux, *Comp. Immunol. Microbiol. Infect. Dis.*, 2004, **27**, 305-318 (DOI: 10.1016/j.cimid.2004.03.004).

5 S. L. Croft, S. Sundar and A. H. Fairlamb, *Clin. Microbiol. Rev.,* 2006, **19**, 111-126 (DOI:10.1128/CMR.19.1.111-126.2006).

6 T. P. C. Dorlo, M. Balasegaram, J. H. Beijnen and P. J. de Vries, *J. Antimicrob. Chemother.,* 2012, **67**, 2576-2597 (DOI:10.1093/jac/dks275).

7 T. Kanamoto, Y. Kashiwada, K. Kanbara, K. Gotoh, M. Yoshimori, T. Goto, K. Sano and H. Nakashima, *Antimicrob. Agents Chemther.,* 2001, **45**, 1225-1230.

8 J. F. Mayaux, A. Bousseau, R. Pauwels, T. Huet, Y. Henin, N. Dereu, M. Evers, F. Soler, C. Poujade, E. De Clercq and J. B. Le Pecq, *Proc. Natl. Acad. Sci.,* 1994, **91**, 3564-3568.

9 P. K. Mukherjee, K. Saha, J. Das, M. Pal and B. P. Saha, *Planta Med.,* 1997, **63**, 367-369.

10 J. C. P. Steele, D. C. Warhurst, G. C. Kirby and M. S. J. Simmonds, *Phytother. Res.,* 1999, **13**, 115-119.

11 E. Pisha, H. Chai, I. S. Lee, T. E. Chagwedera, N. R. Farnsworth, G. A. Cordell, C. W. W. Beecher, H. H. S. Fong, A. D. Kinghorn, D. M. Brown, M. C. Wani, M. E. Wall, T. J. Hieken, T. K. Das Gupta and J. M. Pezzuto, *Nat. Med.,* 1995, **1**, 1046-1051 (DOI:doi:10.1038/nm1095-1046).

12 S. Alakurtti, T. Heiska, A. Kiriazis, N. Sacerdoti-Sierra, C. L. Jaffe and J. Yli-Kauhaluoma, *Bioorg. Med. Chem.,* 2010, **18**, 1573-1582 (DOI:10.1016/j.bmc.2010.01.003).

13 J.-F. Li, Y. Zhao, M.-M. Cai, X.-F. Li and J.-X. Li, *Eur. J. Med. Chem.,* 2009, **44**, 2796-2806 (DOI:10.1016/j.ejmech.2008.12.024).

14 O. B. Flekhter, E. I. Boreko, L. R. Nigmatullina, E. V. Tret'yakova, N. I. Pavlova, L. A. Baltina, S. N. Nikolaeva, Savinova. O. V., V. F. Eremin, F. Z. Galin and Tolstikov G. A., *Russ. J. Bioorg. Chem.,* 2004, **30**, 80-88.

15 G. Abbiati, A. Arcadi, G. Bianchi, S. Di Giuseppe, F. Marinelli and E. Rossi, *J. Org. Chem.,* 2003, **68**, 6959-6966 (DOI:10.1021/jo0347260).

16 H. Parra-Delgado, C. M. Compadre, T. Ramírez-Apan, M. J. Muñoz-Fambuena, R. L. Compadre, P. Ostrosky-Wegman and M. Martínez-Vázquez, *Bioorg. Med. Chem.,* 2006, **14**, 1889-1901 (DOI:10.1016/j.bmc.2005.10.038).

17 F. Fernández, O. Caamaño, M. D. García, I. Alkorta and J. Elguero, *Tetrahedron,* 2006, **62**, 3362-3369 (DOI:10.1016/j.tet.2006.01.059).

18 Y. Wei, C. -M. Ma and M. Hattori, *Eur. J. Med. Chem.,* 2009, **44**, 4112-4120 (DOI:10.1016/j.ejmech.2009.05.002).

19 S. Alakurtti, P. Bergström, N. Sacerdoti-Sierra, C. L. Jaffe and J. Yli-Kauhaluoma, *J. Antibiot.,* 2010, **63**, 123-126 (DOI:10.1038/ja.2010.2).

20 M. del Rayo Camacho, R. Mata, P. Castaneda, G. C. Kirby, D. C. Warhurst, S. L. Croft and J. D. Phillipson, *Planta Med.,* 2000, **66**, 463-468 (DOI:10.1055/s-2000-8598).

21 P. F. Smith, A. Ogundele, A. Forrest, J. Wilton, K. Salzwedel, J. Doto, G. P. Allaway and D. E. Martin, *Antimicrob. Agents Chemother.,* 2007, **51**, 3574-3581 (DOI:10.1128/AAC.00152-07).

22 V. Kumar, N. Rani, P. Aggarwal, V. K. Sanna, A. T. Singh, M. Jaggi, N. Joshi, P. K. Sharma, R. Irchhaiya and A. C. Burman, *Bioorg. Med. Chem. Lett.,* 2008, **18**, 5058-5062 (DOI:http://dx.doi.org/10.1016/j.bmcl.2008.08.003).

23 M. Urban, J. Sarek, M. Kvasnica, I. Tislerova and M. Hajduch, *J. Nat. Prod.,* 1997, **70**, 526-532 (DOI:10.1021/np060436d).

24 J. Xu, Z. Li, J. Luo, F. Yang, T. Liu, M. Liu, W. -. Qiu and J. Tang, *J. Med. Chem.,* 2012, **55**, 3122-3134 (DOI:10.1021/jm201540h).

25 O. Shimony and C. L. Jaffe, *J. Microbiol. Methods,* 2008, **75**, 196-200 (DOI:http://dx.doi.org/10.1016/j.mimet.2008.05.026).

26 C. Reichwald, O. Shimony, U. Dunkel, N. Sacerdoti-Sierra, C. L. Jaffe and C. Kunick, *J. Med. Chem.,* 2008, **51**, 659-665 (DOI:-10.1021/jm7012166).