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Synthesis and anti-leishmanial activity of heterocyclic betulin derivatives

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ABSTRACT

Betulin, a naturally occurring abundant triterpene is converted in four steps to 3,28-di-*O*-acetyllupa-12,18-diene. When various 4-substituted urazoles were oxidized to the corresponding urazines with iodobenzene diacetate in the presence of 3,28-di-*O*-acetyllupa-12,18-diene, new heterocyclic betulin derivatives were produced. These betulin derivatives were examined in a microplate assay at 50 μM for their ability to inhibit the growth of *Leishmania donovani* axenic amastigotes, a species that causes the fatal visceral leishmaniasis. GI_{50} (concentration for 50% growth inhibition) values of the most effective compounds were determined and their cytotoxicity on the human macrophage cell line THP-1 evaluated. The anti-leishmanial activity on *L. donovani* amastigotes growing in macrophages was also examined. The heterocycloadduct between 3,28-di-*O*-acetyllupa-12,18-diene and 4-methylurazine was the most effective derivative with an GI_{50} = 8.9 μM against *L. donovani* amastigotes.

1. Introduction

Leishmaniasis is a parasitic disease that affects millions of people in developing countries and has been designated as a neglected tropical disease by the World Health Organization. It is caused by the protozoan parasite *Leishmania* and transmitted by sand flies belonging to the genus *Phlebotomus* and *Lutzomyia* in the Old and New World, respectively.[1] Three main forms of leishmaniasis are commonly observed: (1) visceral leishmaniasis, a fatal disease where parasites invade the liver, spleen and bone marrow; (2) mucocutaneous leishmaniasis, a highly disfiguring, chronic disease of the nasopharynx and mucosal facial tissue and (3) cutaneous leishmaniasis, a self-limiting disease resulting in scarring at the site of skin lesion. First line drugs for treatment include pentavalent antimony compounds, pentamidine or amphotericin B. All these drugs are administrated by intravenous injection and require clinical supervision or hospitalization due the possibility of severe side effects. Liposomal encapsulated amphotericin B shows lower toxicity but is highly expensive. Several new drugs such as allopurinol, primaquine, imipramine and 3-chloroimipramine are being developed, however none of them are fully effective against *Leishmania*. Recently, miltefosine which can be administrated orally has shown promising anti-leishmanial activity and is currently in phase IV clinical studies, though the use of miltefosine in pregnant women is limited due to teratogenic effects.[2,3] Parasite resistance against pentavalent antimony drugs has become a serious problem and is present in approximately 65% of the patients with visceral leishmaniasis in India.[4]

Betulin (lup-20(29)-ene-3β,28-diol) **1** (Fig. 1) is a widespread compound found in the plant kingdom. It can be isolated up to 30% dry weight from the outer bark of white-barked birches of the genus *Betula* sp.[5] This pentacyclic triterpene can be converted to betulinic acid **2**,[6] which has shown anti-malarial,[7] anti-inflammatory[8] and especially cytotoxic activity against many tumor cell lines by inducing apoptosis.[9–11] Some betulin derivatives have also shown remarkable anti-HIV activity with new mechanisms of action.[12,13]

In a study by Chowdhury et al. dihydrobetulinic acid **3** was found to inhibit growth of *Leishmania donovani* promastigotes and amastigotes with an IC_{50} (50% inhibitory concentration) value of 2.6 and 4.1 μM, respectively.[14] It acts by targeting DNA topoisomerase I and II preventing DNA cleavage and formation of enzyme–DNA complex, ultimately inducing apoptosis. Leishmanial parasitic burden in golden hamsters was reduced by 92% after 6-week treatment with dihydrobetulinic acid **3** (10 mg/kg body weight). In a related study by Sauvain et al., a rare natural product, betulinic aldehyde **4**, obtained from *Doliocarpus dentatus* (Aubl.) showed weak in vitro activity against *Leishmania amazonensis* amastigotes with a survival index of 12% at 136 μM and 42% at 68 μM.[15] However, at these doses **4** also showed toxicity against peritoneal macrophages with survival indices of 70% and 80%, respectively. At a concentration of 34 μM, aldehyde **4** was ineffective against *L. amazonensis* as well as non-toxic to macrophages. In

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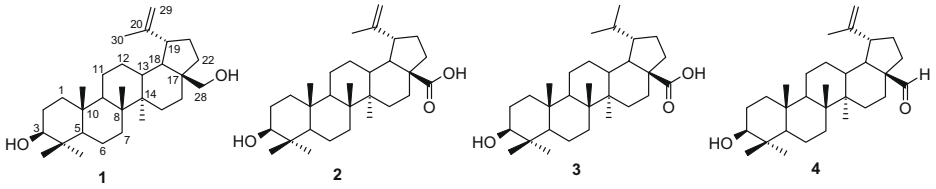


Figure 1. Chemical structures of betulin **1**, betulinic acid **2**, dihydrobetulinic acid **3** and betulinic aldehyde **4**.

another study, where anti-leishmanial activity of plethora of natural products was screened, betulinic acid **2** isolated in minute quantities from *Betula platyphylla* var. *japonica* was found to be weakly active against *Leishmania major* with an IC_{50} value of 88 μM.[16] It was also noted that in the triterpenes with ursane, oleanane or lupane skeletons, a carboxyl substituent was required for anti-leishmanial activity.

In our previous work, the hydroxy groups of C-3 and C-28, and the carbon–carbon double bond C-20–C-29 were used as a starting point to prepare a library of 24 triterpenoid derivatives for anti-leishmanial studies against *L. donovani* axenic amastigotes.[17] Betulonic acid was the most potent derivative giving a GI_{50} value of 14.6 μM.[17] To improve the aqueous solubility and convert our initial anti-leishmanial triterpenoids to more drug-like compounds we embarked on the synthesis of their heterocyclic derivatives and the evaluation of their leishmanicidal activity against *L. donovani* axenic amastigotes.

2. Results and discussion

2.1. Chemistry

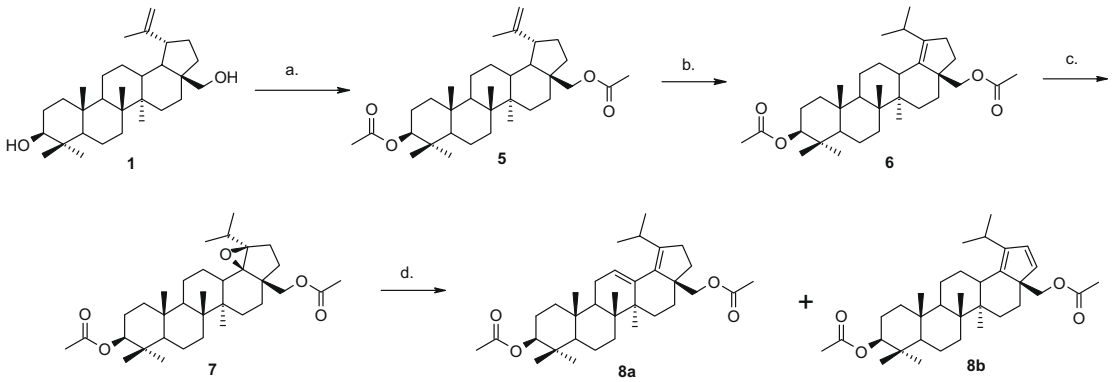
Betulin **1** was acetylated with excess of acetic anhydride to give 3,28-di-*O*-acetylbetulin **5** in 97% yield (Scheme 1). Treatment of **5** with hydrobromic acid (HBr) in the presence of acetic anhydride and acetic acid in toluene caused the migration of the C-20–C-29 double bond of **5** to the position C-18–C-19, and thus afforded 3,28-di-*O*-acetyllup-18-ene **6** in 42% yield.[18a,b] The C-18–C-19 double bond of 3,28-di-*O*-acetyllup-18-ene **6** was epoxidized with 3-chloroperoxybenzoic acid (*m*CPBA) in chloroform to yield the oxirane intermediate **7** in 65% yield. Treatment of 3,28-di-*O*-acetyl-18,19-epoxylupane **7** with pyridine *p*-toluenesulfonate (PPTS) in toluene gave a mixture (4:1) of conjugated dienes, 3,28-di-*O*-acetyllupa-12,18-diene **8a** and 3,28-di-*O*-acetyllupa-18,21-diene **8b**, in 68% yield.

Heterocycloadducts **9a**–**9l** were obtained in moderate yields from the reaction between a mixture of betulin-derived dienes **8a** and **8b** and urazines **16** (Scheme 2). Because urazines **16** are not generally stable for long period of time, they were synthesized in situ by oxidation of urazoles **15** with the hypervalent iodine reagent, (diacetoxyiodo)benzene.[19] Of the two dienes **8a** and **8b**, only the sterically least hindered **8a** took part in the hetero Diels–Alder reaction producing heterocyclic betulin derivatives **9a**–**9l**.

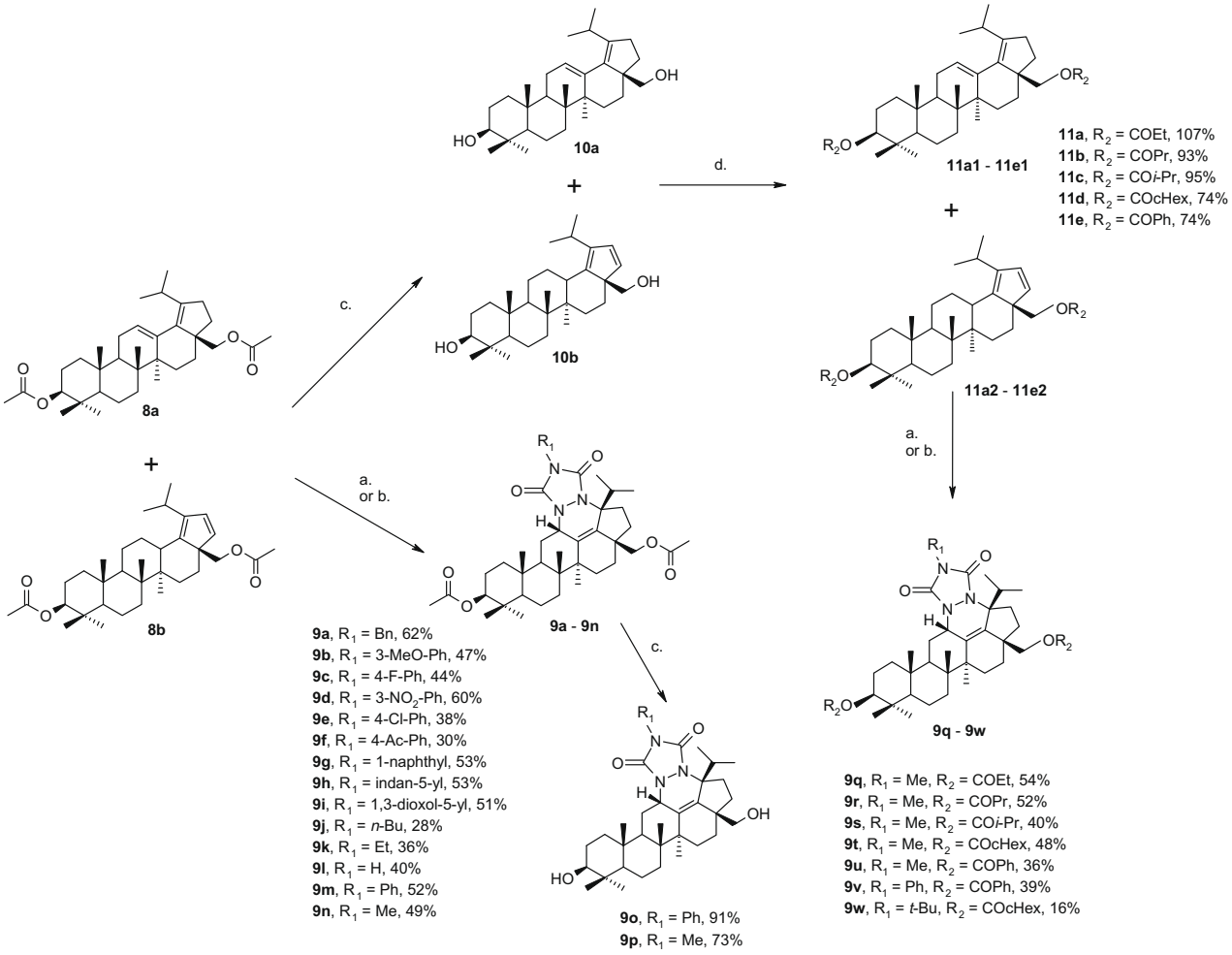
Reactions of 4-phenyl- or 4-methyl-1,2,4-triazoline-3,5-dione with a mixture of dienes **8a** and **8b** in toluene gave the corresponding heterocycles **9m** and **9n** in 52% and 49% yields, respectively (Scheme 2). Also in this case the only detected and isolated cycloadducts were formed from the diene **8a**. Treatment of the heterocycles **9m** and **9n** with NaOH in a mixture of MeOH and tetrahydrofuran (THF) (2:3) provided the diols **9o** and **9p** as the hydrolysis products in 91% and 73% yields, respectively. The [4+2] cycloaddition reactions between the mixture of dienes **8a**/**8b** and other less reactive dienophiles, such as maleic anhydride, *N*-phenylmaleimide, diethyl azodicarboxylate and dimethyl acetylenedicarboxylate were unsuccessful.

Treatment of the dienes **8a**/**8b** with NaOH in a mixture of MeOH and THF (2:3) gave a mixture of 3,28-dihydroxylupa-12,18-diene **10a** and 3,28-dihydroxylupa-18,21-diene **10b** in 85% yield. The subsequent acylation with various acyl chlorides in the presence of 4-(dimethylamino)pyridine (DMAP) as a nucleophilic catalyst and pyridine (py) in CH_2Cl_2 yielded a mixture of dienes **11a**–**11e**. Cycloaddition reactions of 4-methyl- or 4-phenyl-1,2,4-triazoline-3,5-dione with a mixture of dienes **11a**–**11e** provided the corresponding heterocycles **9q**–**9v** in moderate yields. When *tert*-butyl-substituted urazole **15l** was oxidized to the corresponding 4-substituted urazine **16** with (diacetoxyiodo)benzene in situ in the presence of **11d**, a new heterocycle **9w** was obtained.

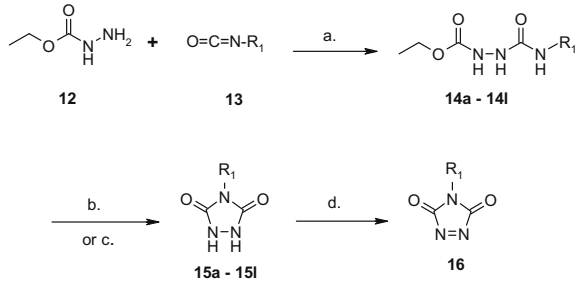
The dienophilic urazines **16** and their corresponding urazole precursors **15** were synthesized as follows (Scheme 3): Reaction of stoichiometric amount of ethyl hydrazinecarboxylate **12** and isocyanates **13** in toluene yielded carbethoxysemicarbazides **14** in excellent 90–99% yields.[20a,b] If the substituent of carbethoxysemicarbazides was aromatic (**14a**–**14i**), the cyclization to the corresponding urazoles **15a**–**15i** was carried out by heating **14a**–**14i** in aqueous 4 M KOH solution for 90 min. Acidification of this basic solution with aqueous HCl solution afforded urazoles **15a**–**15i** in



Scheme 1. Reagents and conditions: (a) Ac_2O (6 equiv), DMAP, py, CH_2Cl_2, rt, 17 h, 97%; (b) HBr, Ac_2O, AcOH, PhMe, rt, 21 d, 42%; (c) *m*CPBA, Na_2CO_3, $CHCl_3$, rt, 2 h, 65%; (d) PPTS, Ac_2O, PhMe, reflux, 3 h, 68%.



Scheme 2. Reagents and conditions: (a) 4-substituted urazole **15a**–**15l**, $PhI(OAc)_2$, CH_2Cl_2, THF, rt, 20 h; (b) 4-methyl- or 4-phenyl-1,2,4-triazoline-3,5-dione, CH_2Cl_2, THF, rt, 20 h; (c) NaOH, MeOH, THF, rt, 18 h, **10a** + **10b** 85%; (d) R_2Cl, DMAP, py, CH_2Cl_2, 40 °C, 20 h.



Scheme 3. Reagents and conditions: (a) PhMe, rt, 2 h to 80 °C, 2 h, 90–99%; (b) (i) 4 M KOH in H_2O, 70 °C, 90 min, (ii) 37% HCl **15a**–**15i**, 37–88%; or (c) (i) Na, EtOH, 85 °C, 24 h, (ii) 1.25 M HCl in EtOH **15j**–**15l**, 94–96%; (d) in situ $PhI(OAc)_2$, CH_2Cl_2, THF (for the substituents R_1, see Scheme 3).

moderate to good yields (37–88%). If the substituent R_1 was aliphatic (**14j**–**14l**), the cyclization reaction was carried out in a refluxing mixture of 4% sodium ethoxide in ethanol for 24 h. Acidification of the basic solution with HCl in ethanol afforded urazoles **15j**–**15l** in excellent yields (94–96%).

2.2. Biological assays

The anti-leishmanial activity of heterocyclic betulin derivatives **9a–9w** was screened using a fluorescent viability microplate assay with *L. donovani* axenic amastigotes and alamarBlue (resazurin) as previously described.[21,22] Initial screening was carried out by assessing the inhibition of amastigote growth at 50 μM betulin derivative. All compounds were tested at least twice in triplicate. Complete medium both with and without dimethyl sulfoxide (DMSO) was used as negative controls (0% inhibition of amastigote growth). Amphotericin B was included as a positive control on each plate and gave >90% inhibition at 1 μM. In some cases the GI_{50} was also determined. Screening for activity on amastigotes growing in macrophages was carried out as previously described using retinoic acid-treated human macrophage cell line THP-1 infected with *L. donovani* expressing the luciferase gene (*Ld*:pSSU-int/LUC) at a 3:1 parasite: macrophage ratio.[23] Compounds (at 50, 25 and 12.5 μM) to be tested were added for 48 h and the luminescence determined after adding luciferase substrate and measuring in a microplate reader. Cytotoxic effect of the compounds on THP-1 cells was assessed using the alamarBlue viability assay.

2.2.1. Microplate assay at 50 μM against *L. donovani* axenic amastigotes

Initial anti-leishmanial activity of heterocyclic compounds was measured against *L. donovani* axenic amastigotes at 50 μM on microplate assay. The screening results of the first batch (compounds **9a–9p**; Scheme 3) showed that small R_1 substituents at the nitrogen atom (**9k**, R_1 = Et and **9n**, R_1 = Me) gave the best anti-leishmanial activity. Therefore, a second batch of heterocyclic betulin derivatives **9q–9u** was synthesized so that acyl groups at

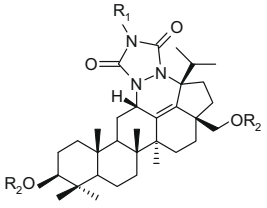
R_2 were altered and R_1 group was kept as methyl. In addition, two cycloadducts **9v** and **9w** with bulky R_1 and R_2 groups were synthesized.

When the R_1 and R_2 groups are bulky, such as phenyl and benzoyl (R_1, R_2) in **9v** and *tert*-butyl (R_1) in **9w**, practically no anti-leishmanial activity was noticed (Table 1). When bulky acyl groups in R_2 were replaced with sterically less demanding acetyl group and R_1 was kept aromatic (compounds **9a**–**9i** and **9m**) anti-leishmanial activity was improved. In general, different substituents at the aromatic ring had only minor effect on leishmanicidal activity, and among these derivatives, 1-naphthyl derivative **9g** and 1,3-dioxol-5-yl derivative **9i** showed the best inhibition activity at 58% and 52%, respectively. Phenyl derivative **9o**, with free hydroxy groups had slightly improved inhibition activity of 48% when compared to **9m** (36%).

However, when the aromatic group R_1 was replaced with less hindered aliphatic groups and the R_2 groups were kept as acetyl (compounds **9j**–**9l**), anti-leishmanial activity improved systematically. The *n*-butyl derivative **9j** displayed moderate activity, 49%, the ethyl derivative **9k** showed 88% inhibition and finally, the methyl derivative **9n** displayed 98% inhibition. Derivative **9l** with a hydrogen atom as R_1 displayed slightly decreased inhibition of 88%. Based on these results it seems that methyl group as R_1 gives the best anti-leishmanial activity.

Therefore, the effect to anti-leishmanial activity of different (acyl) groups at R_2 was studied, while keeping the R_1 group as methyl. Derivative **9p**, with hydroxy groups as R_2 had clearly decreased inhibition activity of 50% when compared to **9n** with acetyl groups. Dipropionate **9q** showed good leishmanicidal activity, 93% inhibition, but replacement of R_2 with bulkier ester groups resulted in weaker anti-leishmanial activity. Dibutyrate **9r** and diisobutyrate **9s** had weak anti-leishmanial activity of 26% and 20%, respectively, but dicyclohexanoate **9t** as well as dibenzoate **9u** had practically no activity.

Table 1
Anti-leishmanial activity of heterocyclic betulin derivatives at 50 μM against *L. donovani* axenic amastigotes



Compound	R_1	R_2	Average inhibition[a] (%)	GI_{50}[b] (μM)
9v	Ph	COPh	0.0	
9w	*tert*-Bu	COcHex	4.8	
9a	Bn	Ac	25	
9b	3-MeO-Ph	Ac	44	
9c	4-F-Ph	Ac	48	
9d	3-NO_2-Ph	Ac	30	
9e	4-Cl-Ph	Ac	30	
9f	4-Ac-Ph	Ac	45	
9g	1-Naphthyl	Ac	58	
9h	Indan-5-yl	Ac	23	
9i	1,3-Dioxol-5-yl	Ac	52	
9m	Ph	Ac	36	
9o	Ph	H	48	
9j	*n*-Bu	Ac	49	
9k	Et	Ac	88	30
9n	Me	Ac	98	8.9
9l	H	Ac	88	26
9p	Me	H	50	
9q	Me	COEt	93	25
9r	Me	COPr	26	
9s	Me	CO*i*-Pr	19	
9t	Me	COcHex	5.5	
9u	Me	COPh	0.0	
Positive control[c]			95	
Negative control[d]			0.0	

[a] Determined at 50 μM concentration of betulin derivative with the exception of compounds **9r** and **9s** that were used at 15 μM due to their poor solubility.
[b] GI_{50} = concentration for 50% growth inhibition.
[c] Amphotericin B (1 μM).
[d] Culture medium and DMSO.

2.2.2. Determination of GI_{50} values, cytotoxicity and anti-leishmanial activity on *L. donovani* amastigotes growing in THP-1 cells

The most potent derivatives (**9k**, **9n**, **9l** and **9q**) on microplate assay at 50 μM against *L. donovani* axenic amastigotes were selected for further investigations, that is, determination of GI_{50} on axenic amastigotes, cytotoxicity for macrophages and anti-leishmanial activity against *L. donovani* amastigotes growing in THP-1 macrophages. The methyl derivative **9n** had the best GI_{50} value = 8.9 μM, while the other compounds showed slightly higher but very similar GI_{50} values: **9k** 30 μM, **9l** 26 μM and **9q** 25 μM (Table 1). Cytotoxicity of the derivatives was evaluated at 50 μM, 25 μM and 12.5 μM concentrations using the human macrophage THP-1 cell line (Table 2). Compound **9q** was non-toxic at all test concentrations while **9k** and **9l** only showed cytotoxicity at the highest concentration (50 μM) examined 48% and 97% growth inhibition, respectively, of THP-1 cell line. No significant cytotoxicity was observed at lower concentrations. Compound **9n** was cytotoxic at all concentrations examined still inhibiting THP-1 growth, 39%, even at 12.5 μM.

Finally, anti-leishmanial activity was tested on *L. donovani* amastigotes growing in macrophages at concentrations, which showed less than 40% cytotoxicity for the macrophage cell line THP-1. The compound **9q** showed activity on *L. donovani* amastigotes growing in macrophages similar to that seen with axenic amastigotes at 50 μM, 83% and 93%, respectively. Even at 25 μM concentration **9k**, **9l** and **9q** still showed good activity inhibiting amastigote growth in the macrophages 53%, 55% and 55%, respectively. Compound **9q** still displayed weak anti-parasitic activity, 18%, against amastigotes growing in macrophages at 12.5 μM. Compound **9n** showed good inhibition at 12.5 μM (60%), however even at this concentration it was also moderately cytotoxic (39%) to macrophage cell line.

3. Conclusion

A series of new heterocycles derived from the naturally occurring and abundant triterpene betulin was synthesized. We found

Table 2
Cytotoxicity and anti-leishmanial activity of the most potent betulin derivatives on macrophage cell line THP-1 and *L. donovani* amastigotes growing in THP-1 cells

Compound	50 μM	25 μM	12.5 μM
	Cytotoxic inhibition of THP-1 cell line growth (%)		
9k	48	13	8.5
9n	94	78	39
9l	97	2.4	0.0
9q	16	15	5.0
	Inhibition of L. donovani amastigote growth (%) in the macrophage cell line THP-1		
9k	nt[a]	53	0.0
9n	nt	nt	60
9l	nt	55	4.8
9q	83	55	18

[a] nt = not tested.

that small R_1 substituents at the nitrogen atom of the triazolo moiety as well as the least sterically hindered acyl groups at the R_2 positions in the betulin skeleton promoted anti-leishmanial activity. Also triazolo ring had beneficial effect on anti-leishmanial activity, as it was found out in our previous work that 3,28-di-*O*-acetylbetulin **5** was totally inactive. The most effective derivative against *L. donovani* amastigotes was the heterocycloadduct **9n** with a GI_{50} = 8.9 μM, however this compound showed some toxicity (39%) for the macrophage cell line at approximately the same concentration (12.5 μM). Several additional derivatives with slightly higher GI_{50}, ~25 μM, showed none or only limited toxicity for macrophages at this concentration, and demonstrated good activity against the intracellular parasites. New therapeutic agents for the leishmaniases are urgently needed. Most existing drugs are toxic or expensive and drug resistance by parasites causing fatal visceral disease has led to discontinued use of first-line drugs in some highly endemic regions of India. Heterocyclic betulin derivatives show promising activity against *L. donovani* that causes fatal visceral disease. Further studies to develop more potent betulin derivatives with leismanicidal properties but no toxicity for the human host macrophages are underway.

4. Experimental section

4.1. Chemistry

4.1.1. General procedures

Commercially available reagents were used without further purification and all of the solvents were HPLC grade. Anhydrous solvents were purchased from Sigma–Aldrich. All reactions in anhydrous solvents were performed in oven dried glassware under an inert atmosphere of anhydrous argon or nitrogen. Thin layer chromatography (TLC) was performed on E. Merck Silica Gel 60 aluminium packed plates, with visualization accomplished by UV illumination and staining with 5% H_2SO_4 in MeOH. The ^{1}H NMR spectra were measured on a Varian Mercury-VX 300 MHz or a Chemagnetics CMX 400 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 7.26 ppm as an internal standard or in DMSO-d_6 at 23 °C, solvent peak at 2.50 ppm as an internal standard). The ^{13}C NMR spectra were obtained on a Varian Mercury-VX 75 MHz or a Chemagnetics CMX 100 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 77.0 ppm as an internal standard or in DMSO-d_6 at 23 °C, solvent peak at 39.50 ppm as an internal standard). HPLC–MS and elemental analyses were performed to determine purity of all tested compounds. Purity of all tested compounds was >95%. Mass spectra were measured on a Bruker Daltonik Esquire-HPLC spectrometer, with XTerra MS RP18 column (4.6 × 30 mm, 2.5 μm) or on a JEOL JMS-AX505 (Tokyo, Japan) spectrometer with direct input and electron ionization (EI). Elemental analyses were performed by Robertson Microlit Laboratories, Madison, NJ, USA. Melting points were obtained with a Sanyo Gallenkamp apparatus without correction. The Fourier transform infrared (FTIR) spectra were recorded on a Bruker Vertex 70 spectrometer with Pike MIRacle diamond crystal or with a Bruker Equinox 55 spectrometer including IRScope II and diamond anvil.

4.1.2. Betulin (1)

Betulin **1** was isolated (95% purity) from the bark of birch (*Betula* sp.) by extraction and was obtained from UPM Kymmene (Lappeenranta, Finland). The crude betulin was recrystallized from 2-propanol/H_2O azeotrope to give **1** as a white solid:[5] R_f = 0.2 (EtOAc/*n*-hexane 1:4); mp: 252–253 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.75 (s, 3H), 0.82 (s, 3H), 0.96 (s, 3H), 0.97 (s, 6H), 1.02 (s, 3H), 1.68 (s, 3H), 2.38 (m, 1H), 3.18 (dd, J = 5.1, 10.8 Hz, 1H), 3.32 (d, J = 10.8 Hz, 1H), 3.79 (d, J = 10.8 Hz, 1H), 4.57 (s, 1H), 4.68 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 14.7, 15.3, 15.9, 16.1, 18.3, 19.1, 20.8, 25.2, 27.0, 27.3, 27.9, 29.1, 29.7, 33.9, 34.2, 37.1, 37.3, 38.7, 38.8, 40.9, 42.7, 47.7, 47.7, 48.7, 50.4, 55.2, 60.5, 79.0, 109.7, 150.4; IR: $\tilde{\nu}$ = 879, 1009, 1035, 1232, 1375, 1452, 1739, 2939, 3360; MS (EI, 70 eV) *m/z*: 442 $[M]^+$; Anal. Calcd for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38. Found: C, 81.40; H, 11.56.

4.1.3. 3,28-Di-*O*-acetylbetulin (5)

To a solution of **1** (15 g, 34 mmol), 4-(dimethylamino)pyridine (0.41 g, 3.4 mmol), and pyridine (25 mL) in CH_2Cl_2 (150 mL) was added acetic anhydride (19 mL, 200 mmol). The resulting mixture was stirred at room temperature for 17 h. The reaction mixture was then washed with 10% hydrochloric acid (200 mL), saturated aqueous $NaHCO_3$ solution (400 mL), water (100 mL) and dried with anhydrous Na_2SO_4. Removal of the solvent in vacuo gave **5** as a white solid (17 g, 97%): R_f = 0.6 (EtOAc/*n*-hexane 1:4); mp: 219–220 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.83 (s, 3H), 0.84 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.38 (s, 3H), 1.68 (s, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 2.39–2.48 (m, 1H), 3.84 (d, J = 11.0 Hz, 1H), 4.24 (d, J = 11.0 Hz, 1H), 4.46 (dd, J = 5.8, 10.2 Hz, 1H), 4.58 (s, 1H), 4.68 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.6, 16.0, 16.1, 16.4, 18.1, 19.0, 20.7, 21.0, 21.3, 23.6, 25.1, 27.0, 27.9, 29.5, 29.7, 34.1, 34.5, 37.0, 37.5, 37.7, 38.3, 40.8, 42.6, 46.2, 47.6, 48.7, 50.2, 55.3, 62.7, 80.8, 109.8, 150.1, 170.9, 171.6; IR: $\tilde{\nu}$ = 1243, 1731, 2951; MS (EI, 70 eV) *m/z*: 526 $[M]^+$; Anal. Calcd for $C_{34}H_{54}O_4$: C, 77.52; H, 10.33. Found: C, 77.46; H, 10.36.

4.1.4. 3,28-Di-*O*-acetyllup-18-ene (6)

To a solution of HBr (47%, 250 g), acetic anhydride (100 g) and acetic acid (300 g) (resulting 14% HBr and 35% acetic acid) was added 3,28-di-*O*-acetylbetulin **5** (17 g, 33 mmol) in toluene (200 mL).[17] The reaction mixture was allowed to stand for 3 weeks at room temperature. The mixture was diluted with water (400 mL) and aqueous phase was separated and extracted with toluene (400 mL). Combined organic phases were washed with water (300 mL), saturated aqueous $NaHCO_3$ solution (600 mL) and dried with anhydrous Na_2SO_4. Solvent was removed in vacuo and resulting crude product was purified by flash chromatography on silica gel (hexane/EtOAc 8:1) to afford 3,28-di-*O*-acetyllup-18-ene **6** (7.4 g, 42%): R_f = 0.8 (EtOAc/*n*-hexane 1:4); mp: 202–203 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.83 (s, 3H), 0.84 (s, 3H), 0.88 (s, 3H), 0.91 (s, 3H), 0.97 (s, 3H), 1.00 (s, 3H), 1.05 (s, 3H), 2.03 (s, 3H), 2.04 (s, 3H), 2.24 (m, 2H), 2.41 (dd, J = 12.4 Hz, 1H), 3.13 (m, 1H), 4.00 (d, J = 4.4 Hz, 2H), 4.48 (dd, J = 6.2, 10.3 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.5, 16.5, 16.5, 16.8, 18.1, 21.0, 21.3, 21.5, 21.6, 22.1, 23.7, 26.6, 27.9, 28.2, 28.2, 29.3, 32.3, 34.6, 34.9, 37.1, 37.8, 38.6, 40.8, 40.9, 43.2, 51.0, 52.1, 55.5, 66.9, 80.9, 134.0, 143.6, 171.0, 171.4; IR: $\tilde{\nu}$ = 982, 1029, 1246, 1372, 1387, 1450, 1738, 2869, 2946, 2990; MS (EI, 70 eV) *m/z*: 526 $[M]^+$; Anal. Calcd for $C_{34}H_{54}O_4$: C, 77.52; H, 10.33. Found: C, 77.74; H, 10.61.

4.1.5. 3,28-Di-*O*-acetyl-18,19-epoxylupane (7)

To a solution of 3,28-di-*O*-acetyllup-18-ene **6** (4.91 g, 9.33 mmol) in $CHCl_3$ (120 mL) was added Na_2CO_3 (4.94 g, 46.7 mmol) and *m*-chloroperoxybenzoic acid (mCPBA 70%, 3.69 g, 14.9 mmol), and the resulting mixture was stirred at room temperature for 2 h.[17] The reaction mixture was washed with water (150 mL), saturated aqueous $NaHSO_3$ solution (150 mL), saturated aqueous $NaHCO_3$ solution (150 mL) and dried with anhydrous Na_2SO_4. Solvent was removed in vacuo, and the resulting crude product was crystallized from EtOH (175 mL) to afford 3,28-di-*O*-acetyl-18,19-epoxylupane **7** (3.31 g, 65%): R_f = 0.7 (EtOAc/*n*-hexane 1:4); mp: 210–212 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.81 (s, 3H), 0.86 (s, 3H), 1.00 (s, 3H), 1.02 (s, 3H), 1.03 (s, 3H), 1.04 (s,

3H), 1.07 (s, 3H), 1.08 (s, 3H), 2.02 (s, 6H), 3.85 (d, J = 10.7 Hz, 1H), 4.45 (dd, J = 7.4, 8.8 Hz, 1H), 4.48 (d, J = 10.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.4, 16.4, 16.7, 16.7, 18.1, 18.8, 19.9, 20.9, 21.3, 21.4, 22.6, 23.6, 23.7, 26.3, 26.6, 27.9, 28.5, 29.9, 34.1, 37.1, 37.7, 38.1, 38.5, 41.1, 43.1, 45.6, 51.3, 55.5, 66.7, 75.5, 77.7, 80.8, 170.9, 171.3; IR: $\tilde{\nu}$ = 982, 1244, 1732, 2940; MS (EI, 70 eV) m/z: 542 $[M]^+$; Anal. Calcd for $C_{34}H_{54}O_5$: C, 75.23; H, 10.03. Found: C, 75.29; H, 10.29.

4.1.6. 3,28-Di-*O*-acetyllupa-12,18-diene (8a) and 3,28-di-*O*-acetyllupa-18,21-diene (8b)

To a solution of 3,28-di-*O*-acetyl-18,19-epoxylupane **7** (2.00 g, 3.68 mmol) and *p*-toluenesulfonic acid (0.42 g, 2.21 mmol) in toluene (80 mL) was added acetic anhydride (0.560 mL, 5.90 mmol).[17] The resulting mixture was refluxed for 4 h and then washed with saturated aqueous $NaHCO_3$ solution (150 mL), water (100 mL) and dried with anhydrous Na_2SO_4. Solvent was removed in vacuo, and the resulting crude product was purified by flash chromatography on silica gel (hexane/EtOAc 12:1) and crystallized from EtOH (20 mL) to give a mixture (4:1) of 3,28-di-*O*-acetyllupa-12,18-diene **8a** and 3,28-di-*O*-acetyllupa-18,21-diene **8b** (1.31 g, 68%): R_f = 0.6 (EtOAc/*n*-hexane 1:4); **8a**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.03 (s, 3H), 2.05 (s, 3H), 2.30 (dd, J = 4.1, 9.6 Hz, 2H), 3.85 (q, J = 11.0, 39.0 Hz, 2H), 4.50 (dd, J = 1.2, 7.4 Hz, 1H), 5.31 (t, J = 3.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.1, 16.8, 17.6, 18.2, 21.1, 21.3, 21.4, 21.8, 22.6, 23.2, 23.6, 26.4, 27.5, 28.1, 28.4, 31.5, 34.2, 34.5, 36.8, 37.7, 38.7, 38.9, 44.1, 47.5, 51.4, 55.4, 66.1, 80.8, 122.9, 134.7, 136.6, 145.5, 171.0, 171.5.

4.1.7. General procedure for the synthesis of bis[acetyloxy]dihydro[1,2,4]triazolo[1,2-*a*]-pyridazine-1,3-diones by generating urazine in situ (9a–9l) and (9w): heterocycloadduct (9f)

Solution of *p*-acetyl-4-phenylurazole **15f** (50 mg, 0.23 mmol) and iodobenzene diacetate (74 mg, 0.23 mmol) in an anhydrous THF/CH_2Cl_2 mixture (4 mL, 1:1) was stirred under inert atmosphere for 15 min.[19] To the formed red solution was added a mixture (4:1) of 3,28-di-*O*-acetyllupa-12,18-diene **8a** and 3,28-di-*O*-acetyllupa-18,21-diene **8b** (100 mg, 0.19 mmol) in THF/CH_2Cl_2 mixture (4 mL, 1:1). The reaction mixture was stirred at room temperature for 24 h. Solvent was evaporated in vacuo, and purification of crude product by flash chromatography on silica gel (hexane/EtOAc 4:1→1:1) afforded **9f** (43 mg, 30%): R_f = 0.3 (EtOAc/*n*-hexane 1:2); mp: 127–128 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.87 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.12 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.60 (s, 3H), 2.53 (m, 1H), 2.77 (m, 2H), 4.00 (q, J = 14.1, 25.1 Hz, 2H), 4.49 (t, J = 8.1 Hz, 1H), 4.76 (t, J = 10.1 Hz, 1H), 7.71 (d, J = 7.5 Hz, 2H), 8.02 (d, J = 7.8 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.2, 19.4, 20.3, 20.9, 21.2, 22.4, 23.0, 23.4, 26.0, 26.6, 27.8, 29.3, 33.1, 33.6, 33.9, 37.1, 37.8, 37.9, 38.0, 38.4, 43.1, 43.5, 49.4, 53.0, 55.6, 68.1, 73.4, 80.5, 125.1, 128.9, 135.5, 135.6, 136.1, 139.4, 146.7, 148.8, 170.9, 171.0, 197.1; IR: $\tilde{\nu}$ = 1029, 1242, 1391, 1699, 2941; MS (ESI) m/z: 742.5 $[M+H]^+$; Anal. Calcd for $C_{44}H_{59}N_3O_7$: C, 71.23; H, 8.02; N, 5.66. Found: C, 70.43; H, 6.83; N, 5.04.

4.1.7.1. Heterocycloadduct (9a). Yield 62%: R_f = 0.6 (EtOAc/*n*-hexane 1:2); mp: 114–115 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.88 (s, 3H), 0.91 (s, 3H), 0.99 (s, 3H), 1.05 (s, 3H), 2.05 (s, 3H), 2.07 (s, 3H), 2.41 (m, 1H), 2.76 (m, 2H), 3.95 (q, J = 8.7, 19.5 Hz, 2H), 4.50 (t, J = 8.0 Hz, 1H), 4.64 (m, 1H), 4.65 (q, J = 19.8, 34.2 Hz, 2H), 7.26–7.44 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.0, 19.1, 20.2, 20.9, 21.2, 22.1, 22.9, 23.1, 25.9, 27.8, 29.2, 33.3, 33.6, 37.2, 37.7, 37.8, 37.9, 38.3, 42.5, 43.1, 43.2, 49.4, 52.5, 55.6, 68.0, 72.9, 80.5, 127.7, 128.5, 128.6, 135.5, 136.2, 139.4, 148.2, 150.4, 170.8, 171.0; IR: $\tilde{\nu}$ = 1028, 1241, 1441, 1736, 2940; MS (ESI) m/z: 714.4 $[M+H]^+$; Anal. Calcd for $C_{43}H_{59}N_3O_6$: C, 72.34; H, 8.33; N, 5.89. Found: C, 70.62; H, 7.52; N, 5.05.

4.1.7.2. Heterocycloadduct (9b). Yield 47%: R_f = 0.6 (EtOAc/*n*-hexane 1:2); mp: 122–123 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.87 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.12 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.50 (m, 1H), 2.77 (m, 2H), 3.81 (s, 3H), 3.99 (q, J = 14.4, 25.7 Hz, 2H), 4.49 (t, J = 8.0 Hz, 1H), 4.75 (t, J = 9.9 Hz, 1H), 6.88 (dd, J = 2.4, 8.4 Hz, 1H), 7.08 (m, 2H), 7.33 (t, J = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.2, 19.4, 20.3, 20.9, 21.2, 22.4, 23.0, 23.1, 26.0, 27.8, 29.2, 29.3, 33.2, 33.6, 33.8, 37.1, 37.8, 37.9, 38.4, 43.1, 43.4, 49.4, 52.8, 55.4, 55.6, 68.1, 73.2, 80.5, 111.3, 114.0, 118.1, 129.5, 132.6, 135.5, 139.4, 147.3, 149.4, 169.9, 170.9, 171.0; IR: $\tilde{\nu}$ = 1008, 1241, 1393, 1697, 2941; MS (ESI) m/z: 730.4 $[M+H]^+$; Anal. Calcd for $C_{43}H_{59}N_3O_7$: C, 70.75; H, 8.15; N, 5.76. Found: C, 67.65; H, 7.20; N, 5.51.

4.1.7.3. Heterocycloadduct (9c). Yield 44%: R_f = 0.7 (EtOAc/*n*-hexane 1:2); mp: 127–128 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.12 (s, 3H), 1.55 (s, 3H), 2.05 (s, 3H), 2.09 (s, 3H), 2.50 (m, 1H), 2.78 (m, 2H), 4.00 (q, J = 13.2, 24.5 Hz, 2H), 4.50 (t, J = 8.1 Hz, 1H), 4.75 (t, J = 9.9 Hz, 1H), 7.12 (t, J = 8.4 Hz, 2H), 7.48 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.2, 19.4, 20.3, 20.9, 21.2, 22.3, 22.9, 23.1, 26.0, 27.8, 29.2, 33.2, 33.6, 33.8, 37.1, 37.8, 37.9, 38.0, 38.4, 43.1, 43.4, 49.4, 52.8, 55.6, 68.1, 73.2, 80.5, 115.6, 115.9, 127.5, 127.6, 127.6, 135.5, 139.4, 147.1, 149.2, 159.9, 170.8, 171.0; IR: $\tilde{\nu}$ = 1028, 1241, 1512, 1696, 2942; MS (ESI) m/z: 718.4 $[M+H]^+$; Anal. Calcd for $C_{42}H_{56}FN_3O_6$: C, 70.27; H, 7.86; N, 5.85. Found: C, 68.82; H, 7.11; N, 5.08.

4.1.7.4. Heterocycloadduct (9d). Yield 60%: R_f = 0.5 (EtOAc/*n*-hexane 1:2); mp: 135–136 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.88 (s, 3H), 0.97 (s, 3H), 1.03 (s, 3H), 1.12 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.53 (m, 1H), 2.78 (m, 2H), 4.00 (q, J = 14.4, 25.4 Hz, 2H), 4.50 (t, J = 8.3 Hz, 1H), 4.77 (t, J = 9.9 Hz, 1H), 7.61 (t, J = 8.3 Hz, 1H), 7.98 (dd, J = 1.2, 7.2 Hz, 1H), 8.18 (dd, J = 2.1, 8.1 Hz, 1H), 8.53 (t, J = 2.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.1, 19.4, 20.3, 20.9, 21.2, 22.4, 22.9, 23.1, 26.0, 27.8, 29.2, 33.1, 33.5, 33.9, 37.1, 37.8, 37.9, 38.1, 38.4, 43.1, 43.4, 49.4, 53.0, 55.6, 68.1, 73.4, 80.4, 120.4, 122.0, 129.5, 131.0, 133.0, 135.3, 139.3, 146.3, 148.3, 148.4, 170.8, 171.0; IR: $\tilde{\nu}$ = 749, 1030, 1244, 1351, 1535, 1701, 2945; MS (ESI) m/z: 745.4 $[M+H]^+$; Anal. Calcd for $C_{42}H_{56}N_4O_8$: C, 67.72; H, 7.58; N, 7.52. Found: C, 64.48; H, 7.41; N, 6.80.

4.1.7.5. Heterocycloadduct (9e). Yield 38%: R_f = 0.7 (EtOAc/*n*-hexane 1:2); mp: 104–106 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85 (s, 3H), 0.86 (s, 3H), 0.95 (s, 3H), 1.01 (s, 3H), 1.10 (s, 3H), 2.03 (s, 3H), 2.08 (s, 3H), 2.50 (m, 1H), 2.76 (m, 2H), 3.99 (q, J = 11.3, 25,8 Hz, 2H), 4.48 (t, J = 8.5 Hz, 1H), 4.74 (t, J = 9.3 Hz, 1H), 7.26–7.59 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.2, 19.4, 20.3, 20.9, 21.2, 22.4, 23.0, 23.1, 26.0, 27.8, 29.3, 33.2, 33.6, 33.8, 37.1, 37.8, 37.9, 38.0, 38.4, 43.1, 43.4, 49.4, 52.9, 55.6, 68.1, 73.3, 80.5, 123.6, 125.7, 127.8, 129.7, 132.8, 134.4, 135.5, 139.4, 146.8, 148.9, 170.9, 171.0; IR: $\tilde{\nu}$ = 1028, 1242, 1409, 1699, 1732, 2943; MS (ESI) m/z: 734.4 $[M+H]^+$; Anal. Calcd for $C_{42}H_{56}ClN_3O_6$: C, 68.69; H, 7.69; N, 5.72. Found: C, 66.46; H, 6.68; N, 4.48.

4.1.7.6. Heterocycloadduct (9g). Yield 53%: R_f = 0.3 (EtOAc/*n*-hexane 1:2); mp: 145–147 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.98 (s, 3H), 1.00 (s, 3H), 1.15 (s, 3H), 1.19 (s, 3H), 2.02 (s, 3H), 2.10 (s, 3H), 2.48 (m, 1H), 2.76 (m, 1H), 2.88 (m, 1H), 4.02 (q, J = 12.4, 23.9 Hz, 2H), 4.48 (t, J = 7.8 Hz, 1H),

4.83 (q, J = 9.9, 20.7 Hz, 1H), 7.45–7.58 (m, 4H), 7.68 (dd, J = 7.2, 26.4 Hz, 1H), 7.91 (t, J = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.6, 18,1, 19.4, 19.5, 20.3, 20.9, 21.2, 22.3, 23.1, 26.0, 27.9, 29.3, 33.2, 33.7, 37.1, 37.8, 37.9, 38.4, 43.2, 43.5, 49.5, 52.8, 53.0, 55.6, 67.6, 68.1, 73.2, 73.6, 80.5, 122.1, 125.3, 126.4, 127.1, 128.5, 129.9, 130.2, 134.5, 135.6, 139.5, 147.9, 148.1, 149.6, 150.3, 170.9, 171.1; IR: $\tilde{\nu}$ = 769, 1028, 1243, 1693, 2942; MS (ESI) m/z: 750.4 [M+H]$^+$; Anal. Calcd for $C_{46}H_{59}N_3O_6$: C, 73.67; H, 7.93; N, 5.60. Found: C, 70.84; H, 6.29; N, 4.29.

4.1.7.7. Heterocycloadduct (9h). Yield 53%: R_f = 0.5 (EtOAc/*n*-hexane 1:2); mp: 151–153 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.87 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.11 (s, 3H), 2.07 (s, 3H), 2.09 (s, 3H), 2.48 (m, 1H), 2.77 (m, 2H), 2.90 (q, J = 7.2, 14.4 Hz, 4H), 3.99 (q, J = 13.8, 24.6 Hz, 2H), 4.49 (t, J = 8.4 Hz, 1H), 4.74 (t, J = 9.9 Hz, 1H), 7.18–7.29 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.2, 19.4, 20.3, 20.9, 21.2, 22.3, 23.0, 23.1, 25.5, 25.6, 26.0, 27.8, 29.3, 32.5, 32.8, 33.2, 33.6, 33.7, 37.1, 37.8, 37.9, 38.4, 43.1, 43.4, 49.4, 52.7, 55.6, 68.0, 73.1, 80.5, 122.1, 124.0, 124.5, 129.4, 135.5, 139.4, 144.1, 145.1, 147.7, 149.8, 170.8, 171.0; IR: $\tilde{\nu}$ = 1028, 1241, 1693, 1738, 2908; MS (ESI) m/z: 740.5 [M+H]$^+$; Anal. Calcd for $C_{45}H_{61}N_3O_6$: C, 73.04; H, 8.31; N, 5.68. Found: C, 72.00; H, 8.11; N, 5.39.

4.1.7.8. Heterocycloadduct (9i). Yield 51%: R_f = 0.3 (EtOAc/*n*-hexane 1:2); mp: 117–119 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H), 0.87 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.11 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.48 (m, 1H), 2.77 (m, 2H), 3.99 (q, J = 12.9, 23.7 Hz, 2H), 4.49 (t, J = 8.0 Hz, 1H), 4.73 (t, J = 9.9 Hz, 1H), 5.98 (s, 2H), 6.84 (d, J = 9.3 Hz, 1H), 6.93 (d, J = 8.2 Hz, 1H), 6.95 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.2, 19.4, 20.3, 20.9, 21.2, 22.3, 23.0, 23.1, 26.0, 27.8, 29.2, 29.3, 33.2, 33.6, 33.7, 37.1, 37.8, 37.9, 38.4, 43.1, 43.4, 49.4, 52.8, 55.6, 68.1, 73.2, 80.5, 101.5, 107.5, 108.1, 119.9, 125.0, 135.5, 139.4, 147.1, 147.5, 147.8, 149.6, 170.9, 171.0; IR: $\tilde{\nu}$ = 1032, 1242, 1693, 2941; MS (ESI) m/z: 744.4 [M+H]$^+$; Anal. Calcd for $C_{43}H_{57}N_3O_8$: C, 69.42; H, 7.72; N, 5.65. Found: C, 67.49; H, 6.55; N, 3.87.

4.1.7.9. Heterocycloadduct (9j). Yield 28%: R_f = 0.4 (EtOAc/*n*-hexane 1:3); mp: 92–94 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.84 (s, 3H), 0.86 (s, 3H), 0.92 (s, 3H), 1.00 (s, 3H), 1.05 (s, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 2.41 (m, 1H), 2.73 (m, 2H), 3.49 (m, 2H), 3.95 (m, 2H), 4.47 (m, 1H), 4.65 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.6, 16.2, 16.5, 18.1, 19.1, 19.3, 19.9, 20.3, 20.9, 21.2, 22.2, 23.0, 23.2, 26.0, 27.9, 29.3, 30.1, 33.3, 33.5, 33.6, 37.2, 37.8, 37.8, 37.9, 38.4, 38.8, 43.1, 43.3, 49.4, 52.5, 55.7, 68.1, 72.8, 80.5, 135.6, 139.5, 148.5, 150.6, 171.0, 171.8; IR: $\tilde{\nu}$ = 1030, 1244, 1369, 1453, 1693, 1741, 2875, 2942; MS (ESI) m/z: 680.4 [M+H]$^+$; Anal. Calcd for $C_{40}H_{61}N_3O_6$: C, 70.66; H, 9.04; N, 6.18. Found: C, 69.56; H, 8.91; N, 5.65.

4.1.7.10. Heterocycloadduct (9k). Yield 36%: R_f = 0.3 (EtOAc/*n*-hexane 1:3); mp: 197–202 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.83 (s, 3H), 0.84 (s, 3H), 0.91 (s, 3H), 0.99 (s, 3H), 1.04 (s, 3H), 2.02 (s, 3H), 2.05 (s, 3H), 2.44 (m, 1H), 2.72 (m, 2H), 3.53 (m, 2H), 4.92 (m, 2H), 4.47 (m, 1H), 4.63 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.3, 16.2, 16.5, 18.1, 19.1, 19.2, 20.2, 20.9, 21.2, 22.2, 22.9, 23.1, 25.9, 27.8, 29.3, 33.3, 33.4, 33.6, 33.9, 37.1, 37.8, 37.9, 38.4, 43.1, 43.3, 49.4, 52.4, 55.6, 68.0, 72.8, 80.5, 135.6, 139.4, 148.4, 150.6, 170.8, 171.0; IR: $\tilde{\nu}$ = 758, 987, 1031, 1225, 1244, 1381, 1453, 1690, 1740, 2878, 2945; MS (ESI) m/z: 652.5 [M+H]$^+$; Anal. Calcd for $C_{38}H_{57}N_3O_6$: C, 70.01; H, 8.81; N, 6.45. Found: C, 69.97; H, 8.74; N, 6.23.

4.1.7.11. Heterocycloadduct (9l). Yield 40%: R_f = 0.2 (EtOAc/*n*-hexane 1:1); mp: 119–120 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85 (s, 3H), 0.86 (s, 3H), 0.91 (s, 3H), 0.99 (s, 3H), 1.05 (s, 3H), 2.04 (s, 3H), 2.07 (s, 3H), 2.43 (m, 1H), 2.68 (m, 2H), 3.96 (q, J = 17.4, 28.5 Hz, 2H), 4.49 (dd, J = 6.0, 10.2 Hz, 1H), 4.62 (t, J = 10.0 Hz, 1H), 9,13 (br s, NH, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.2, 16.2, 16.5, 18.1, 19.0, 19.3, 20.3, 20.9, 21.3, 22.2, 22.7, 23.2, 26.0, 27.9, 29.2, 33.1, 33.6, 37.1, 37.8, 38.0, 38.4, 43.0, 43.3, 49.4, 52.7, 55.7, 60.4, 68.1, 72.7, 80.6, 135.5, 139.2, 148.2, 150.3, 170.9, 171.1; IR: $\tilde{\nu}$ = 1028, 1241, 1367, 1693, 2942; MS (ESI) m/z: 624.4 [M+H]$^+$; Anal. Calcd for $C_{36}H_{53}N_3O_6$: C, 69.31; H, 8.56; N, 6.74. Found: C, 67.70; H, 8.03; N, 5.00.

4.1.7.12. Heterocycloadduct (9w). Yield 16%: R_f = 0.8 (EtOAc/*n*-hexane 1:3); mp: 145–147 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.83 (s, 3H), 0.87 (s, 3H), 0.92 (s, 3H), 0.96 (s, 3H), 0.98 (s, 3H), 1.00 (s, 3H), 1.06 (s, 3H), 1.62 (s, 9H), 2.29 (m, 2H), 2.41 (m, 1H), 2.72 (m, 1H), 3.94 (dd, J = 3.6, 14.8 Hz, 2H), 4.48 (t, J = 7.4 Hz, 1H), 4.60 (t, J = 9.3 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.4, 16.5, 18.1, 19.4, 19.5, 20.4, 22.4, 23.2, 23.5, 25.3, 25.5, 25.7, 25.8, 25.9, 27.9, 28.7, 28.8, 28.9, 29.0, 29.1, 29.3, 33.1, 33.6, 33.8, 37.2, 37.3, 37.9, 38.0, 38.5, 43.3, 43.4, 43.6, 49.5, 52.4, 55.7, 57.6, 67.2, 73.2, 79.9, 135.4, 139.7, 149.1, 152.0, 175.7, 176.2; IR: $\tilde{\nu}$ = 1131, 1165, 1389, 1689, 1727, 2932; Anal. Calcd for $C_{50}H_{77}N_3O_6$: C, 73.58; H, 9.51; N, 5.15. Found: C, 72.70; H, 9.55; N, 4.52.

4.1.8. General procedure for the synthesis of bis[acetyloxy]dihydro[1,2,4]triazolo[1,2-*a*]-pyridazine-1,3-diones from the commercially available urazines (9m and 9n) and (9q–9v): heterocycloadduct (9n)

A mixture of 3,28-di-*O*-acetyllupa-12,18-diene **8a**, 3,28-di-*O*-acetyllupa-18,21-diene **8b** (100 mg, 0.19 mmol together) [or a mixture of 3,28-di-*O*-acyllupa-12,18-diene **11a1**–**11e1**, 3,28-di-*O*-acyllupa-18,21-diene **11a2**–**11e2** in case of compounds **9q**–**9v**] and 4-methyl-1,2,4-triazoline-3,5-dione (32 mg, 0.29 mmol) in anhydrous toluene (5 mL) was stirred at room temperature for 22 h. Solvent was removed in vacuo, and the resulting crude product was purified by flash chromatography on silica gel (hexane/EtOAc 2:1) to afford adduct **9n** (60 mg, 49%): R_f = 0.1 (EtOAc/*n*-hexane 1:2); mp: 218 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.83 (s, 3H), 0.84 (s, 3H), 0.90 (s, 3H), 0.99 (s, 3H), 1.03 (s, 3H), 2.02 (s, 3H), 2.05 (s, 3H), 2.42 (m, 1H), 2.69 (m, 2H), 3.01 (s, 3H), 3.94 (q, J = 11.4, 24.0 Hz, 2H), 4.47 (t, J = 8.3, 1H), 4.63 (t, J = 10.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2, 16.5, 18.1, 19.0, 19.2, 20.2, 20.9, 21.2, 22.2, 22.8, 23.1, 24.7, 24.8, 25.9, 27.8, 29.2, 33.2, 33.6, 37.1, 37.8, 37.9, 38.3, 43.0, 43.3, 49.4, 52.5, 55.6, 68.1, 72.7, 80.5, 135.6, 139.3, 148.5, 150.5, 170.8, 171.0; IR: $\tilde{\nu}$ = 1029, 1223, 1691, 1728, 2910; MS (ESI) m/z: 638.4 [M+H]$^+$; Anal. Calcd for $C_{37}H_{55}N_3O_6$: C, 69.67; H, 8.69; N, 6.59. Found: C, 66.36; H, 7.94; N, 5.90.

4.1.8.1. Heterocycloadduct (9m). Yield 52%: R_f = 0.5 (EtOAc/*n*-hexane 1:4); mp: 164 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85 (s, 3H), 0.87 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.11 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.50 (m, 1H), 2.78 (m, 2H), 3.94 (q, J = 10.8, 32.4 Hz, 2H), 4.49 (t, J = 8.4, 1H), 4.75 (t, J = 10.0 Hz, 1H), 7.32 (ttt, J = 1.6, 7.4 Hz, 1H), 7.43 (t, J = 7.8 Hz, 2H), 7.50 (dd, J = 1.2, 8.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.2, 16.3, 16.6, 18.1, 19.2, 19.4, 20.3, 21.0, 21.0, 21.3, 22.4, 23.0, 23.2, 26.0, 27.9, 29.3, 33.2, 33.6, 33.8, 37.2, 37.8, 38.0, 38.4, 43.1, 43.4, 49.4, 52.8, 55.6, 60.4, 68.1, 73.2, 80.5, 125.8, 127.7, 128.9, 131.6, 135.5, 139.4, 147.3, 149.5, 170.9, 171.1; IR: $\tilde{\nu}$ = 756, 1028, 1241, 1391, 1694, 2941; MS (ESI) m/z: 700.4 [M+H]$^+$; Anal. Calcd for $C_{42}H_{57}N_3O_6$: C, 72.07; H, 8.21; N, 6.00. Found: C, 71.85; H, 8.37; N, 5.96.

4.1.8.2. Heterocycloadduct (9q). Yield 54%: R_f = 0.3 (EtOAc/*n*-hexane 1:3); mp: 133–139 °C; ^{1}H NMR (300 MHz, $CDCl_3$):

δ = 0.82 (s, 3H), 0.85 (s, 3H), 0.90 (s, 3H), 0.99 (s, 3H), 1.03 (s, 3H), 1.12 (s, 3H), 2.40 (m, 6H), 2.71 (m, 2H), 3.01 (s, 3H), 3.97 (m, 2H), 4.48 (m, 1H), 4.63 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 9.1, 9.2, 16.2, 16.5, 18.1, 19.0, 19.1, 20.2, 22.2, 22.8, 23.1, 24.7, 25.9, 27.5, 27.8, 27.9, 29.1, 33.1, 33.6, 33.6, 37.1, 37.7, 37.8, 37.9, 38.4, 43.1, 43.3, 49.4, 52.5, 55.6, 67.8, 72.7, 80.2, 135.5, 148.5, 150.5, 174.1, 174.5; IR: $\tilde{\nu}$ = 748, 1010, 1082, 1185, 1272, 1392, 1464, 1698, 1738, 2877, 2944; MS (ESI) *m/z*: 666.5 [*M*+H]$^+$; Anal. Calcd for $C_{39}H_{59}N_3O_6$: C, 70.34; H, 8.93; N, 6.31. Found: C, 69.42; H, 8.74; N, 6.71.

4.1.8.3. Heterocycloadduct (9r). Yield 52%: R_f = 0.3 (EtOAc/*n*-hexane 1:3); mp: 106–114 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.83 (s, 3H), 0.85 (s, 3H), 0.91 (s, 3H), 0.99 (s, 3H), 1.03 (s, 3H), 2.28 (m, 4H), 2.44 (m, 1H), 2.69 (m, 2H), 3.01 (s, 3H), 3.95 (m, 2H), 4.49 (m, 1H), 4.64 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.7, 16.3, 16.5, 18.1, 18.4, 18.5, 19.0, 19.2, 20.2, 22.2, 22.8, 23.2, 24.8, 25.9, 27.8, 29.1, 33.1, 33.6, 36.1, 36.6, 37.1, 37.7, 37.8, 37.9, 38.4, 43.1, 43.3, 49.4, 52.5, 55.6, 67.6, 72.7, 80.1, 135.5, 139.3, 148.6, 150.6, 173.3, 173.7; IR: $\tilde{\nu}$ = 750, 987, 1182, 1258, 1394, 1467, 1700, 1737, 1796, 2877, 2945, 2967; MS (ESI) *m/z*: 694.5 [*M*+H]$^+$; Anal. Calcd for $C_{41}H_{63}N_3O_6$: C, 70.96; H, 9.15; N, 6.05. Found: C, 69.89; H, 9.07; N, 6.06.

4.1.8.4. Heterocycloadduct (9s). Yield 52%: R_f = 0.3 (EtOAc/*n*-hexane 1:3); mp: 134–142 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.82 (s, 3H), 0.85 (s, 3H), 0.90 (s, 3H), 0.99 (s, 3H), 1.03 (s, 3H), 1.14 (s, 3H), 1.16 (s, 3H), 2.51 (m, 2H), 2.67 (m, 2H), 3.01 (s, 3H), 3.95 (m, 2H), 4.46 (m, 1H), 4.63 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.3, 16.5, 18.0, 18.8, 19.0, 19.1, 19.2, 20.3, 22.2, 22.8, 23.1, 24.7, 25.9, 27.8, 28.8, 33.0, 33.5, 33.7, 34.0, 34.3, 37.1, 37.6, 37.9, 37.9, 38.4, 43.3, 49.4, 52.5, 55.6, 67.4, 72.7, 79.9, 135.5, 139.5, 148.5, 150.5, 176.6, 177.1; IR: $\tilde{\nu}$ = 749, 1158, 1192, 1391, 1260, 1468, 1698, 1734, 1755, 2876, 2943, 2972; MS (ESI) *m/z*: 694.6 [*M*+H]$^+$; Anal. Calcd for $C_{41}H_{63}N_3O_6$: C, 70.96; H, 9.15; N, 6.05. Found: C, 70.71; H, 9.19; N, 6.33.

4.1.8.5. Heterocycloadduct (9t). Yield 48%: R_f = 0.3 (EtOAc/*n*-hexane 1:3); mp: 161–175 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.83 (s, 3H), 0.87 (s, 3H), 0.91 (s, 3H), 1.00 (s, 3H), 1.04 (s, 3H), 2.30 (m, 2H), 2.49 (m, 1H), 2.70 (m, 2H), 3.02 (s, 3H), 3.95 (m, 2H), 4.48 (m, 1H), 4.63 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.4, 16.5, 18.1, 19.1, 19.3, 20.3, 22.3, 22.9, 23.2, 24.8, 25.4, 25.5, 25.7, 25.8, 25.9, 27.9, 28.8, 28.9, 29.0, 29.2, 33.0, 33.6, 33.7, 37.1, 37.6, 37.9, 38.0, 38.4, 43.2, 43.3, 43.4, 43.5, 49.4, 52.7, 55.6, 67.2, 72.8, 79.8, 135.5, 139.4, 148.6, 150.6, 175.7, 176.1; IR: $\tilde{\nu}$ = 748, 1037, 1132, 1169, 1246, 1392, 1465, 1699, 1731, 2856, 2935; Anal. Calcd for $C_{47}H_{71}N_3O_6$: C, 72.93; H, 9.24; N, 5.43. Found: C, 72.68; H, 9.26; N, 5.18.

4.1.8.6. Heterocycloadduct (9u). Yield 36%: R_f = 0.3 (EtOAc/*n*-hexane 1:3); mp: 175–183 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.92 (s, 3H), 0.96 (s, 3H), 1.03 (s, 3H), 1.06 (s, 3H), 1.09 (s, 3H), 2.56 (m, 1H), 2.71 (m,2H), 3.04 (s, 3H), 4.27 (m, 2H), 4.71 (m, 1H), 7.50 (m, 6H), 8.04 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.8, 16.8, 18.4, 19.4, 19.6, 20.7, 22.6, 23.2, 23.5, 25.1, 26.1, 28.3, 28.9, 33.9, 33.3, 34.1, 37.4, 37.9, 38.2, 38.5, 38.7, 43.8, 43.8, 49.7, 52.9, 56.0, 60.6, 67.9, 73.1, 81.4, 128.6, 128.7, 129.8, 129.8, 130.2, 131.1, 133.0, 133.5, 136.1, 139.6, 148.9, 150.9, 166.4, 166.9; IR: $\tilde{\nu}$ = 711, 1110, 1273, 1452, 1466, 1697, 1718, 1755, 2875, 2945; Anal. Calcd for $C_{47}H_{59}N_3O_6$: C, 74.08; H, 7.80; N, 5.51. Found: C, 72.98; H, 7.85; N, 5.45.

4.1.8.7. Heterocycloadduct (9v). Yield 39%: R_f = 0.7 (EtOAc/*n*-hexane 1:3); mp: 192–204 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.94 (s, 3H), 0.98 (s, 3H), 1.04 (s, 3H), 1.09 (s, 3H), 1.17 (s, 3H), 2.61 (m, 1H), 2.81 (m, 2H), 4.29 (m, 2H), 4.80 (m, 2H), 7.52 (m, 11H), 8.05 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.5, 16.5, 18.1, 19.3, 19.5, 20.4, 22.5, 23.0, 23.2, 25.9, 28.0, 28.6, 33.0, 33.6, 34.0, 37.2, 37.9, 38.0, 38.2, 38.4, 43.5, 43.6, 49.4, 52.8, 55.7, 67.6, 73.2, 81.1, 125.7, 127.7, 128.3, 128.4, 128.8, 129.5, 129.5, 129.9, 130.8, 131.6, 132.7, 133.2, 135.7, 139.4, 147.3, 149.4, 166.6; IR: $\tilde{\nu}$ = 711, 756, 969, 1026, 1069, 1110, 1273, 1392, 1415, 1452, 1503, 1601, 1702, 1758, 2875, 2944, 2963; Anal. Calcd for $C_{52}H_{61}N_3O_6$: C, 75.79; H, 7.46; N, 5.10. Found: C, 75.61; H, 7.52; N, 4.97.

4.1.9. General procedure for the synthesis of 3-hydroxy-8a-(hydroxymethyl)dihydro[1,2,4]-triazolo[1,2-*a*]pyridazine-1,3-diones generating urazine in situ (9o and 9p): heterocycloadduct (9o)

To a mixture of **9m** (50 mg, 0.071 mmol) in a solution of MeOH/THF 2:3 (1.4 mL) was added 1 M NaOH solution (0.30 mL), the and reaction mixture was stirred at room temperature for 19 h. Water (10 mL) was added and the precipitated product was filtered and dried in vacuum oven to give **9o** (40 mg, 91%): R_f = 0.5 (EtOAc/*n*-hexane 1:1); mp: 154–156 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.79 (s, 3H), 0.94 (s, 3H), 0.98 (s, 3H), 0.99 (s, 3H), 1.12 (s, 3H), 2.56 (m, 1H), 2.71 (m, 2H), 3.20 (dd, *J* = 5.2, 10.7 Hz, 1H), 3.47 (d, *J* = 10.4 Hz, 1H), 3.61 (d, *J* = 10.7 Hz, 1H), 4.73 (t, *J* = 10.0 Hz, 1H), 7.29–7.50 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.1, 16.5, 18.3, 19.2, 19.5, 20.3, 22.6, 23.0, 26.1, 26.9, 27.8, 28.4 32.6, 33.7, 34.3, 37.5, 37.8, 38.0, 38.5, 38.9, 43.5, 45.1, 49.6, 53.0, 55.6, 67.4, 73.5, 78.8, 125.8, 127.7, 128.9, 131.7, 134.8, 140.2, 147.4, 149.3; IR: $\tilde{\nu}$ = 744, 1035, 1427, 1689, 2932, 3498; MS (ESI) *m/z*: 616.4 [*M*+H]$^+$; Anal. Calcd for $C_{38}H_{53}N_3O_4$: C, 74.11; H, 8.67; N, 6.82. Found: C, 73.94; H, 8.39; N, 6.62.

4.1.9.1. Heterocycloadduct (9p). Yield 73%: R_f = 0.1 (EtOAc/*n*-hexane 1:1); mp: 130–131 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.79 (s, 3H), 0.90 (s, 3H), 0.93 (s, 3H), 0.95 (s, 3H), 0.98 (s, 3H), 1.00 (s, 3H), 1.02 (s, 3H), 1.05 (s, 3H), 2.52 (m, 1H), 2.65 (m, 2H), 3.03 (s, 3H), 3.20 (dd, *J* = 5.2, 10.4 Hz, 1H), 3.44 (d, *J* = 10.4 Hz, 1H), 3.57 (d, *J* = 10.4 Hz, 1H), 4.63 (t, *J* = 9.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.1, 16.5, 18.3, 19.0, 19.4, 20.2, 22.4, 22.9, 24.7, 26.1, 26.9, 27.9, 28.3, 32.6, 33.6, 34.1, 37.5, 37.6, 37.9, 38.5, 38.8, 43.4, 45.1, 49.5, 52.7, 55.6, 67.3, 72.8, 78.8, 134.9, 140.1, 148.6, 150.5; IR: $\tilde{\nu}$ = 728, 1046, 1468, 1678, 2939, 3438; MS (ESI) *m/z*: 554.4 [*M*+H]$^+$; Anal. Calcd for $C_{33}H_{51}N_3O_4$: C, 71.57; H, 9.28; N, 7.59. Found: C, 64.03; H, 7.39; N, 5.77.

4.1.10. 3,28-Dihydroxylupa-12,18-diene (10a) and 3,28-dihydroxylupa-18,21-diene (10b)

A mixture of 3,28-di-*O*-acetyllupa-12,18-diene **8a** and 3,28-di-*O*-acetyllupa-18,21-diene **8b** (9.45 g, 18.0 mmol), aqueous 2 M NaOH (40 mL), MeOH (80 mL) and THF (120 mL) was stirred at room temperature for 18 h. The reaction mixture was acidified with aqueous 18% HCl solution (pH ~5.5) and diluted with water (900 mL). The formed crude product precipitate was filtered and dried in vacuum oven for 3 h to give a mixture of **10a** and **10b** (6.50 g, 85%, purity 72%) and it was used without further purification in the next reaction step. Compound **10a**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.79 (s, 3H), 1.00 (s, 3H), 1.26 (s, 3H), 2.28–2.39 (m, 1H; m, 2H), 2.91 (m, 1H), 3.42 (m, 2H), 5.35 (m, 1H).

4.1.11. General procedure for the synthesis of mixture of lupa-12,18-diene-3,28-diol and lupa-18,21-diene esters (11a–11e): 3,28-di-*O*-butyryllupa-12,18-diene (11b1) 3,28-di-*O*-butyryllupa-18,21-diene (11b2)

Mixture of 3,28-dihydroxylupa-12,18-diene **10a** and 3,28-dihydroxylupa-18,21-diene **10b** (1.50 g, 3.40 mmol), DMAP (1.25 g, 10.2 mmol) and butyryl chloride (1.09 g, 10.2 mmol) in

anhydrous pyridine (35 mL) was heated with stirring at 40 °C for 20 h. EtOAc (250 mL) was added, and the formed precipitate was filtered off. The filtrate was washed with aqueous HCl solution (18%, 4 × 250 mL), dried with Na_2SO_4 and evaporated in vacuo to provide mixture of **11b1** and **11b2** (1.84 g, 93%). Compound **11b1**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.87 (s, 3H), 0.97 (s, 3H), 2.30 (m, 2H), 2.90 (m, 1H), 3.85 (m, 2H), 4.52 (m, 1H), 5.31 (m, 1H).

4.1.11.1. 3,28-Di-*O*-propionyllupa-12,18-diene (11a1) and 3,28-di-*O*-propionyllupa-18,21-diene (11a2). Yield 88%. Compound **11a1**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.87 (s, 3H), 0.97 (s, 3H), 0.99 (s, 3H), 1.13 (s, 3H), 1.14 (s, 3H), 2.32 (m, 2H), 2.92 (m, 1H), 3.88 (m, 2H), 4.51 (m, 1H), 5.31 (s, 1H).

4.1.11.2. 3,28-Di-isobutyryllupa-12,18-diene (11c1) and 3,28-di-*O*-isobutyryllupa-18,21-diene (11c2). Yield 95%. Compound **11c1**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.14 (s, 3H), 1.17 (s, 3H), 2.37 (m, 2H), 2.55 (m, 2H), 2.90 (m, 1H), 3.84 (m, 2H), 4.50 (m, 1H), 5.31 (m, 1H).

4.1.11.3. 3,28-Di-*O*-cyclohexanoyllupa-12,18-diene 11d1 and 3,28-di-*O*-cyclohexanoyllupa-18,21-diene (11d2). Yield 74%. Compound **(11d1)**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (s, 3H) 0.88 (s, 3H), 0.97 (s, 3H), 0.99 (s, 3H), 1.26 (s, 3H), 2.32 (m, 4H), 2.86 (m, 1H), 3.87 (m, 2H), 4.49 (m, 1H), 5.36 (m, 1H).

4.1.11.4. 3,28-Di-*O*-benzoyllupa-12,18-diene (11e1) and 3,28-di-*O*-benzoyllupa-18,21-diene (11e2). Yield 74%. Compound **11e1**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.95 (s, 3H), 1.03 (s, 3H), 2.37 (m, 2H), 2.95 (m, 1H), 4.11 (m, 2H), 5.74 (m, 1H), 5.37 (m, 1H), 7.49 (m, 6H), 8.16 (m, 4H).

4.1.12. General procedures for the synthesis of *N*-substituted 1,2,4-triazolidine-3,5-diones (15)[18]

4.1.12.1. Aryl isocyanates (13a–13i): *p*-Acetyl-4-phenyl-1-carbethoxysemicarbazide (14f). To a solution of ethyl hydrazinecarboxylate **12** (275 mg, 2.64 mmol) in anhydrous PhMe (5 mL) was added dropwise 4-acetylphenyl isocyanate **13f** (425 mg, 2.64 mmol) in anhydrous PhMe (5 mL). The reaction mixture was stirred at room temperature for 2 h and then at 80 °C for 2 h. The formed precipitate was filtered and dried in vacuum oven to give *p*-acetyl-4-phenyl-1-carbethoxysemicarbazide **14f** (630 mg, 90%). It was used without further purification in the next reaction step. **14a**, R_1 = Bn;[18b] **14b**, R_1 = 3-MeO-Ph;[18b] **14c**, R_1 = 4-F-Ph;[18b] **14d**, R_1 = 3-NO_2-Ph;[18b] **14e**, R_1 = 3-Cl-Ph, 92%; **14g**, R_1 = 1-naphthyl;[18b] **14h**, R_1 = indan-5-yl;[18b] **14i**, R_1 = 1,3-dioxol-5-yl.[18b]

4.1.12.2. *p*-Acetyl-4-phenylurazole (15f). *p*-Acetyl-4-phenyl-1-carbethoxysemicarbazide **14f** (300 mg, 1.13 mmol) was reacted with aqueous 4 M KOH solution (0.570 mL, 2.26 mmol) at 70 °C for 1.5 h. Remaining precipitate was filtered off, and filtrate was cooled to room temperature and acidified with concentrated HCl (10 drops). Formed precipitate was filtered and dried in desiccator to give *p*-acetyl-4-phenylurazole **15f** (162 mg, 65%). Compound **15a**, R_1 = Bn[18b]; **15b**, R_1 = 3-MeO-Ph, 88%; **15c**, R_1 = 4-F-Ph, 73%; **15d**, R_1 = 3-NO_2-Ph, 37%; **15e**, R_1 = 3-Cl-Ph, 68%; **15g**, R_1 = 1-naphthyl[18b]; **15h**, R_1 = indan-5-yl, 92%; **15i**, R_1 = 1,3-dioxol-5-yl, 37%.

4.1.12.3. Alkyl isocyanates (13j– 13l): 4-butyl-1-carbethoxysemicarbazide (14j). To a solution of ethyl hydrazinecarboxylate **12** (2.10 g, 20.2 mmol) in anhydrous PhMe (70 mL) was added dropwise butyl isocyanate **13j** (2.00 g, 20.2 mmol) in anhydrous PhMe (5 mL). The reaction mixture was stirred at room temperature for 2 h and then at 80 °C for 2 h. The reaction mixture was cooled to room temperature, and the formed precipitate was filtered, washed with PhMe (60 mL) and dried in vacuum oven to provide 4-butyl-1-carbethoxysemicarbazide **14j** (3.94 g, 96%). It was used without further purification in the next reaction step. Compound **14k**, R_1 = Et, 99%; **14l**, R_1 = *t*-Bu, 97%.

4.1.12.4. 4-Butylurazole (15j). Sodium metal (1.02 g, 44.3 mmol) was reacted with EtOH (40 mL, 99.5%). After 1 h, 4-butyl-1-carbethoxysemicarbazide **14j** (2.00 g, 9.84 mmol) was added and the resulting brown reaction mixture was heated at 85 °C for 24 h. The reaction mixture was acidified with 1.25 M HCl in EtOH (50 mL). The formed precipitate was filtered off, and the filtrate was evaporated to dryness in vacuo to give 4-butylurazole **15j** (1.44 g, 93%). Compound **15k**, R_1 = Et, 96%; **15l**, R_1 = *t*-Bu, 95%; **15m**, R_1 = H, urazole.

4.2. Biology

4.2.1. Parasite and cell culture

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[20] in complete RPMI 1640 containing 20% fetal calf serum, pH 5.5. Stably transfected *L. donovani* promastigotes expressing the firefly luciferase gene (*Ld:pSSU-int/LUC*) were cultured in Medium-199 adjusted to pH 6.8 and supplemented with L-glutamine (2 mM), adenosine (100 μM), folic acid (23 μM), 1 × BME vitamin mix, 10% fetal calf serum, penicillin G (100 IU), streptomycin (100 μg/mL) and hygromycin B (100 μg/mL). The human leukemia monocyte cell line (THP-1) was cultured in complete RPMI-1640 supplemented with antibiotics (100 IU penicillin G and 100 mg/mL streptomycin), 2 mM L-glutamine and fetal calf serum (10% v/v).

4.2.2. Axenic amastigote viability assay

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.[20] Standardization and optimization of the assay for axenic amastigotes have been described elsewhere.[21] Compounds to be assayed were diluted to twice the final concentration 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). 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.

4.2.3. Screening on infected macrophages

THP-1 cells in the log-phase of growth were differentiated by incubation for 3 days in complete RPMI-1640 containing 1 μM retinoic acid (Sigma–Aldrich, St. Louis, MO).[22] Excess retinoic acid was removed by washing the cells three times with RPMI-1640 (250*g*, 10′, 4 °C) and the treated macrophages suspended in complete RPMI-1640 and transferred to 75 mL tissue culture flasks (Costar Brand, NUNC™, Denmark). Stationary-phase *Ld*:pSSU-int/LUC promastigotes were added to the treated macrophages (3:1 parasite/macrophage ratio) and incubated in a 5% CO_2 incubator for 16 h at 37 °C to allow for infection and differentiation of the *Leishmania* into intracellular amastigotes. Any remaining extracellular parasites were removed by centrifugation 4 - 5 times (210*g*, 8′, 4 °C). This was validated by phase

microscopy. Infected THP-1 cells in complete RPMI-1640 were counted and aliquoted (1×10^5 cells in 50 μL/well) in triplicate into opaque 96-well flat bottom plates (Costar Brand, NUNC™, Denmark). Test substances diluted in complete RPMI-1640 containing 1% DMSO (10 μM, 50 μL/well) were added to the infected cells. The cultures were incubated for 48 h (37 °C, 5% CO_2). Cells were lysed by the addition of Steady-Glo® Luciferase Assay substrate (100 μL/well, Promega, MT, USA) to each well and the luminescence measured after 10 min using a microplate reader (Luminometer Mithras LB940, Berthold Technologies, Germany). Complete medium both with and without DMSO was used as negative controls (0% inhibition). Amphotericin B (Sigma–Aldrich, St. Louis MO) was included as a positive control on each plate and gave >90% inhibition at 1 μM.

4.2.4. Toxicity assay

Effect of the compounds on human cells was assessed using the alamarBlue viability assay. Compounds to be tested were diluted in the complete medium containing 1% DMSO (10 μM) and aliquoted in triplicate (125 μL/well) into 96-well flat-bottom plates. THP-1 macrophages in complete RPMI-1640 were added (8×10^5 cells/mL, 125 μL/well) to the plates and incubated for 48 h (37 °C, 5% CO_2). The viability indicator alamarBlue (25 μL) was added, the plates incubated for an additional 3 h and the fluorescence read as described above. Complete medium both with and without DMSO was used as negative controls (0% inhibition).

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Supplementary data

Supplementary data (^1H and ^{13}C NMR spectra of compounds **9a**–**9w**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.003.

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