Design, Synthesis and Biological Activity of C3 Hemisynthetic Triterpenic Esters as Novel Antitrypanosomal Hits

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Research for innovative drugs is crucial to contribute to parasitic infections control and eradication. Inspired by natural antiprotozoal triterpenes, a library of 12 hemisynthetic 3-*O*-arylalkyl esters was derived from ursolic and oleanolic acids through one-step synthesis. Compounds were tested on *Trypanosoma*, *Leishmania* and the WI38 cell line alongside with a set of triterpenic acids. Results showed that the triterpenic C3 esterification keeps the antitrypanosomal activity ($IC_{50} \approx 1.6$–5.5 μM) while reducing the cytotoxicity compared to parent acids. Unsaturation of the ester alkyl chain leads to an activity loss interestingly kept when a sterically hindered group replaces the double bond or shields the ester group. An ursane/oleanane C3 hydroxylation was the only important feature for antileishmanial activity. Two candidates, dihydrocinnamoyl and 2-fluorophenylpropionyl ursolic acids, were tested on an acute mouse model of African trypanosomiasis with significant parasitemia reduction at day 5 post-infection for the dihydrocinnamoyl derivative. Further evaluation on other alkyl/protective groups should be investigated both *in vitro* and *in vivo*.

1. Introduction

Neglected tropical diseases (NTDs) is a collective term qualifying a group of communicable diseases that prevail in tropical and subtropical conditions in 149 countries and affect more than one billion people.[1] They include Human African Trypanosomiasis (or African Sleeping Sickness, HAT) and Leishmaniasis caused by *Trypanosoma brucei* (*T. b*) and some twenty species of *Leishmania*, respectively.[2] Importantly, only 4% of newly approved drugs between 2000–2011 were dedicated to NTDs.[3]

T. b is present mainly in sub-Saharan Africa and is transmitted by a bite of an infected tsetse fly to a mammalian host. The disease progresses in two stages: at first, *T. b* resides in the blood and lymphatic system (hemolymphatic stage) before crossing the blood-brain-barrier and infecting the central nervous system (meningoencephalitic stage).[4] Currently, HAT treatments for stage 1 are pentamidine and suramin for *T. b gambiense* and *rhodesiense*, respectively, while at stage 2 of the disease, melarsoprol is used against *T. b rhodesiense* and eflornithine plus nifurtimox (NECT-nufurtimox-eflornithine combination therapy) for *T. b gambiense*. All these treatment options present strong limitations in terms of toxicity (e.g., suramin or the arsenical compound melarsoprol), difficulties in the administration (e.g., slow intravenous injection) or resistance issues (e.g., NECT).[5,6] In late 2018, a positive scientific opinion was given by EMA (European Medicines Agency) to fexinidazole and more recently (July 2021) by FDA (Federal Drug Administration)[7] as first all-oral treatment for both stage-1 and stage-2 of the *T. b. gambiense* form of sleeping sickness in patients over six years old weighing more than 20 kg, with non-neglectable label limitations.[8] Regardless of the successful efforts of the last decades in controlling HAT, these regions are still at risk of large-scale epidemic outbreaks. Leishmaniasis is also a vector-borne disease transmitted by sandflies and encompasses multiple clinical syndromes, most notably visceral, cutaneous and mucosal forms. Cutaneous leishmaniasis is caused by at least ten *Leishmania* species of the *mexicana* (*L. m*) and *braziliensis* (*L. b*) complexes. Parasites inoculation happens through a sand fly bite followed by a variable incubation period ranging from one week to many months.[9] It is estimated that between 600000 to 1 million new cases occur worldwide annually. Treatment of leishmaniasis caused by *L. mexicana*

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consists of pentavalent antimonials as pentostam or glucantime, directly injected into the ulcer or intramuscularly.[10] In this context and given the severe therapeutic limitations such as reduced efficacy due to increased parasite resistance, innovative drugs with new modes of action are urgently needed for NTDs control and, possibly, eradication. In this sense, lead repurposing strategies were demonstrated to be efficient medicinal chemistry rationale for NTDs hit identification as demonstrated by the successful case of eflornithine.[11] The interest of pentacyclic triterpenes (PTs) in parasitology was recently emphasized in a review reporting some 85 active compounds on *Trypanosoma*, *Leishmania*, *Plasmodium* and *Nematoda*.[12] For instance, tingenin B, a PT quinone methide, has been reported as the most active PT against *T. b. brucei* and *T. cruzi* ($IC_{50}<$ 0.25 $\mu g\,mL^{-1}$) although it was found very cytotoxic.[13] In the oleanane PTs group, some saponin glycosides named maesabalides (isolated from *Maesa balansae*) were very active against *Leishmania infantum* ($IC_{50}=0.007–0.046\ \mu g\,mL^{-1}$).[14] In this context, very few aromatic ursane and oleanane derivatives were tested on *T. b* and *L. m* species such as *L. mexicana mexicana* (*L. mm*). In more recent research, novel compounds derived from medicinal plants by *in situ* hemisynthesis were reported, highlighting the successful input of medicinal chemistry to natural lead compounds.[15] Our approach towards the development of new antitrypanosomal drugs was based upon the known antitrypanosomal activity of pentacyclic triterpenic acids and some of their C3 esters. Recent investigations on traditionally used plants lead to the isolation of antitrypanosomal triterpenic C3 esters, 3-*O*-*p*-*E/Z*-coumaroyltormentic acids[16] characterized by a hydroxyl function at the C2 position and a (*E/Z*)-3-(4-hydroxyphenyl)acrylate group at C3 with an ursane skeleton (Supporting Information). This triterpenic mixture has shown attractive *in vitro* antitrypanosomal activity ($IC_{50}=$ 0.7 μM) and *in vivo* parasitemia reduction, yet with some bioavailability limitations.[16] Considering its promising features but also the difficulties linked to the plant material availability or the isolation process, our approach was to pursue a lead optimization strategy of the natural structure, regularly used in disease drug discovery. A bioisosteric replacement of the hydroxylic function with fluorine in the aromatic ring inspired the generation of some fluorine derivatives.[16] Those derivatives were tested alongside the non-halogenated ones to assess whether the functional groups or their position would be important for the antiparasitic activity. To possibly increase the stability of the triterpenic esters, sterically hindered esters were designed to disrupt esterase activity and delay esterase hydrolysis.[17] Therefore, a concise library of 3-*O*-ursane and oleanane derivatives were hemisynthesized. The purpose of this paper is to report on the synthesis, antiparasitic activity and make an assessment to structure-activity relationships (SAR) of a series of C3 esters with triterpenic scaffolds.

2. Results and Discussion

A first screening was done, initially, for a set of triterpenic acids (Figure 1) indicating ursolic acid **1 a** as the most active acid, followed by oleanolic acid **2 a**. Some other PT free acids (**1 b**, **2 b**) were indeed endowed with interesting antiparasitic profiles, but suffered from significant cytotoxicity (Table 1). A

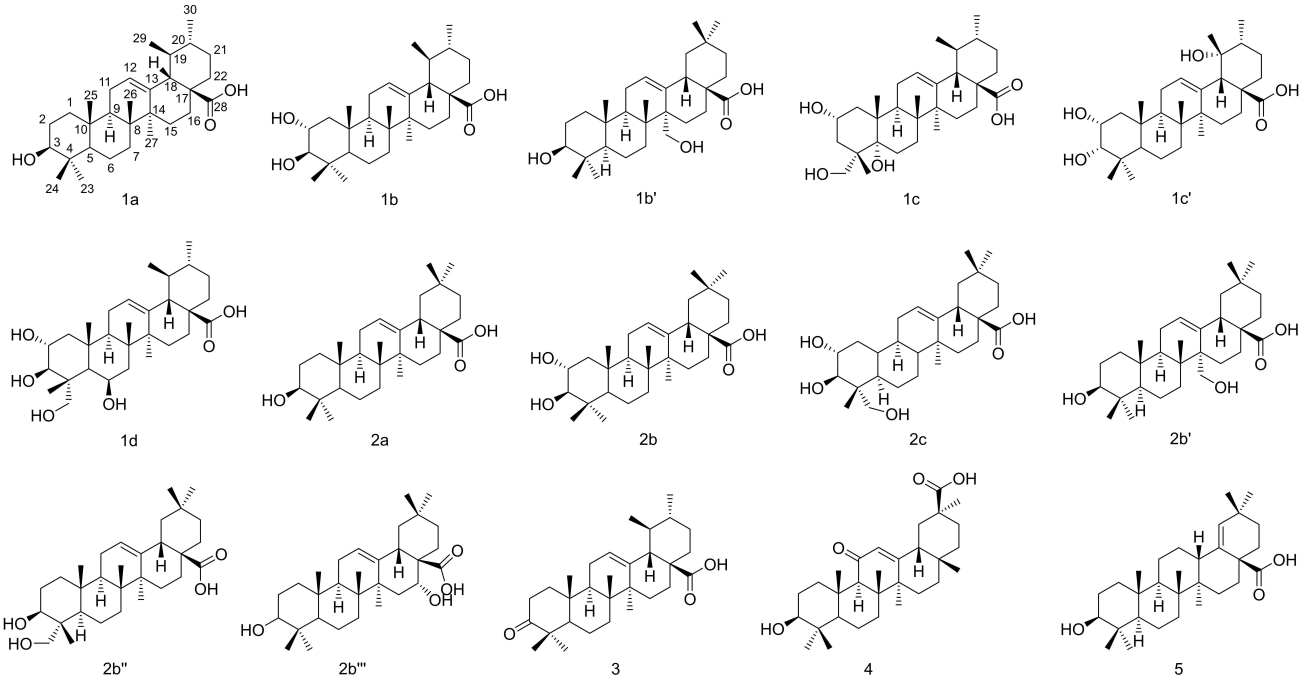


Figure 1. Pentacyclic triterpenic acids tested *in vitro* with ursane (**1 a**–**d**, **3**) and oleanane (**2 a**–**c**, **4**, **5**) skeletons. **1 a**: ursolic acid; **1 b**: corosolic acid; **1 b′**: (3β)-3,27-dihydroxyolean-12-en-28-oic acid; **1 c**: asiatic acid; **1 c′**: tormentic acid; **1 d**: madecassic acid; **2 a**: oleanolic acid; **2 b**: maslinic acid; **2 c**: arjunolic acid; **2 b′**: (3β)-3,27-dihydroxyolean-12-en-28-oic acid; **2 b″**: hederagenin; **2 b‴**: echinocystic acid; **3**: ursonic acid; **4**: 18β-glycyrrhetinic acid; **5**: (3β)-3-hydroxyolean-18-en-28-oic acid.

Table 1. IC_{50} (μM) ± SD on parasites and cells with relative selectivity indices (SI) of triterpenic acids and synthetic esters of at least 2 repetitions of 3 independent replicates.

	Biological activities expressed in IC_{50} (μM, Mean ± SD)			
ID	Antileishmanial[a]	Antitrypanosomal[b]	Cytotoxicity[c]	SI[d]
		Acids		
1a	7.03 ± 0.46	2.33 ± 0.22	10.08 ± 1.02	4.3
1b	14.23 ± 6.87	5.28 ± 1.25	18.34 ± 6.22	3.5
1b′	nd	44.37 ± 9.75	> 105.77	> 2.4
1c	55.23 ± 9.93	13.93 ± 3.79	75.66 ± 10.29	5.4
1c′	55.42 ± 3.14	22.72 ± 1.56	85.70 ± 2.13	3.8
1d	> 99.07	49.65 ± 2.81	> 190.00	> 3.8
2a	19.74 ± 2.03	5.83 ± 0.70	63.26 ± 0.74	10.9
2b	18.41 ± 0.51	6.73 ± 1.00	47.51 ± 8.86	7.1
2b′	nd	47.14 ± 16.31	> 105.77	> 2.2
2b″	23.71 ± 1.58	22.28 ± 3.18	94.49 ± 4.68	4.2
2b‴	62.63 ± 1.28	20.59 ± 1.60	55.43 ± 6.40	2.7
2c	nd	28.28 ± 5.48	> 60.00	> 2.1
3	83.28 ± 1.55	9.63 ± 0.67	> 109.48	> 11.4
4	> 106.20	71.75 ± 3.27	164.18 ± 10.32	2.3
5	68.42 ± 4.32	47.79 ± 18.43	29.09 ± 2.37	0.6
		Esters		
6a	> 84.97	3.50 ± 1.13	142.01 ± 3.45	40.7
6a′	> 82.39	2.45 ± 0.30	> 164.90	> 67.3
6a″	> 82.39	2.67 ± 0.83	> 164.90	> 61.5
6a‴	> 82.39	1.66 ± 0.38	> 131.92	> 79.5
6a⁗	> 82.92	2.17 ± 0.32	> 82.99	> 38.2
6b	> 85.20	66.19 ± 0.38	nd	
6c	> 100.25	5.02 ± 2.15	114.89 ± 4.74	22.9
6d	> 83.21	5.41 ± 0.14	> 83.28	> 15.4
7a	> 84.91	2.59 ± 0.87	23.54 ± 6.56	9.1
7a′	> 82.39	2.88 ± 1.11	16.33 ± 3.26	5.7
7a″	> 82.39	1.72 ± 0.07	20.74 ± 2.25	12.1
7a‴	> 82.39	4.63 ± 0.28	18.61 ± 4.47	4.0
		Control		
pentamidine	0.08 ± 0.03			
suramin		0.02 ± 0.00		
camptothecin			0.20 ± 0.27	

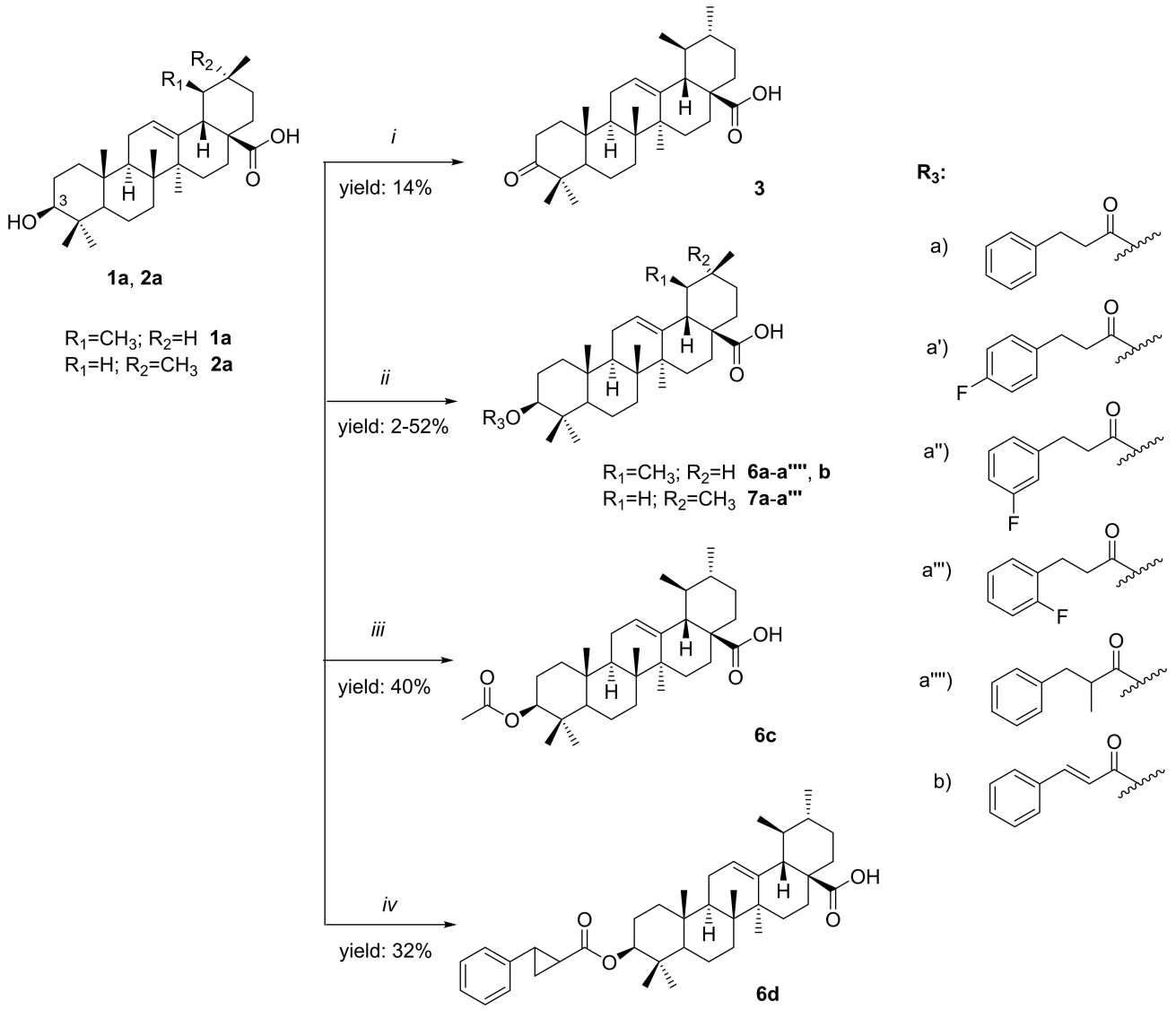
[a] *Leishmania mexicana mexicana* (promastigotes); [b] *Trypanosoma brucei brucei* (bloodstream form); [c] human-diploid fibroblast cells (WI38); [d] Selectivity index (SI) = IC_{50} on WI38cell line/IC_{50} on *T. bb.*

specifically designed library was then generated (Scheme 1) with ursane (**6a**–**d**) and oleanane (**7a**–**a‴**) skeletons, maintaining the phenylpropyl ester chain (as the natural active models) or adding a halogen to the phenyl group. Antiparasitic activity investigations and structural effects on the biological activity were analyzed to explore a potential activity/selectivity improvement alongside a chemical and more effective drug production.

2.1. Oleanane and Ursane C3 Hemisynthetic Triperpenic Esters

The 12 triterpenic esters library was readily synthesized in one step syntheses with, generally, a low yield (not over 52% in the best cases). The different *in vitro* biological activities and their selectivity indexes are tabulated in Table 1. As a general rule, most synthesized compounds offered low activity scores against *L. mm.* However, several compounds gave high activity against *T. bb* along with very attractive selectivity indices (SI) for compounds in the ursane series (**6a**-**a‴**), giving compounds fulfilling the criteria for hit selection.[18] The oleanane series of 3-*O*-esters (**7a**–**a‴**), while performing adequately in terms of pharmacological scores, led to discouraging results for the selectivity index. It was decided, therefore, to halt any further investigations of these oleanane analogues. On the contrary, the ursane series (**6a**--**d**) showed interesting activity and low cytotoxicity in most of the cases. On the aromatic moiety, a fluoro substituent was implemented in the three positions available, that is, *para* (**6a′**), *meta* (**6a″**) and *ortho* (**6a‴**). The best profile was achieved with the *ortho*-substitution (**6a‴**). The dihydrocinnamoyl group (**6a**) displayed interesting data both in terms of efficacy and selectivity and was chosen as one of the lead compounds for further structural improvements to possibly increase the metabolic stability (ester linkage) introducing elements of steric crowding to the two central methylene moieties.

This led to an α-methyl derivative (**6a⁗**) at the cost of introducing an additional carbon, and a *trans*-cyclopropane derivative (**6d**) which can be considered as a bioisoster of the cinnamoyl ester side chain. The synthetic ursonic acid (**3**) showed a significant activity decrease compared to the parent acid (**1a**) and the 3-phenylproprionate derivative (**6a**) (Supporting Information). This supports the hypothesis of a hydroxylic



Scheme 1. Synthesis of ursane and oleanane esters tested in vitro (**3**, **6a**–**d**, **6**–**7a**–**a**‴ and **6a**⁗). Conditions: ***i***) PCC, $(CH_3)_2CO$, DCM; ***ii***) DMAP, DCC, R_3OH, Tol, reflux, 24 h; ***iii***) DMAP, $C_{10}H_9ClO$, Tol, reflux, 22 h; ***iv***) C_2H_3ClO, Py, 60 °C, 1 h.

group or an ester function at C3 to be important for the trypanocide activity. Further efforts are needed to expand the 3-*O*-derivatives library and improve the synthetic yields.

2.2. *In Vitro* Antiprotozoal Evaluation and Structural Effects on the Biological Activity of Triterpenic Acids and 3-*O*-Triterpenic Esters

As shown in Table 1, **1a** and **2a** remained the most active triterpenic acids against *T. bb* in their series, while activities in the same range were also observed for the α-2-hydroxylated derivatives, corosolic (**1b**) and maslinic acids (**2b**). Concerning other 3-OH pentacyclic acids, the C-α-16/21/23/27 hydroxylation showed deleterious effects on antitrypanosomal activities, with a significant decrease for one additional hydroxyl group at C27 (**1b**′, **2b**′), two at C2/21 (**1c**′) and C2/23 (**2c**), or three at C2/6/23 (**1d**). Previous literature data reported the carboxylic group at C17 to be important for the (**1a**) antitrypanosomal activity, underlying a replacement with an aldehyde group (uvaol) would lead to an activity reduction more evident when the carboxylic group is replaced by a methyl one (α-amyrin). Hoet *et al.* reported an activity loss on *T. bb* when (**2a**) carboxylic group at C17 is replaced with a CH_3 group. The presence of a ketone or the double bond shift also induced a significant activity decrease, as observed for ursonic (**3**), 18β-glycyrrhetinic (**4**) and morolic (**5**) acids.[19] Similarly, **1a** with a keto function at C3, was reported to lose the antitrypanosomal activity for *T. cruzi* parasites.[20] For oleanane derivatives, the activity decrease was also associated with a reduction in selectivity. The importance of the hydroxylic group at C3 has been reported for the antitrypanosomal action for both **1a** and **2a** and the ursane- and oleanane-type pentacyclic triterpenes.[21] A clear counteracting of anti-*T. bb* activity loss

happens when the adjacent carbon, C4, is substituted with a polar group.[20] This indicates that the substituent's nature and orientation effect on the activity might depend on parasite strain as demonstrated for a tri-esterified oleanane derivative, 3β-acetoxy-27-trans-caffeoyloxyolean-12-en-28-oic acid methyl ester. The latter displayed high selective antitrypanosomal activity on *T. bb* but was inactive on *T. b. rhodesiense* subspecies.[22] The activity of all tested 3-*O*-ursane esters, except the cinnamic one (**6b**), was similar to **1a** with enhanced selectivity for aromatic esters (**6a**–**a**′′′′). For 3-*O*-oleanane derivatives, activity remained similar as for **2a** except for hydrocinnamic (**7a**) and *para*/*meta*-fluorophenylpropionic derivatives (**7a**′–**a**′′) showing a significantly increased activity but also higher cytotoxicity, leading to similar selectivity. C3 acetylation (**6c**) was shown to induce a decrease in activity smaller than described in the literature. Cunha *et al.* reported a loss in activity against *T. cruzi* of both **1a** and **2a** after acetylation.[20] As for the sleeping sickness parasite, single C3 acetylation decreased the activity on *T. cruzi* trypomastigotes compared to unesterified acids, with **1a** also being the most active derivative. The number and position of polar groups affect antitrypanosomal activities, potentially due to steric hindrance and modified conformation. In this paper, we showed that the C3 aromatic esterification mainly modified the cytotoxicity of ursane and oleanane derivatives, giving more selective or active compounds, respectively. Concerning the antileishmanial activity of the free acids tested, the most active compound was **1a** ($IC_{50} = 7.03 \pm 0.46$ μM), significantly more active than **2a** ($IC_{50} = 19.74 \pm 2.03$ μM) ($p < 0.01$). Unlike antitrypanosomal activity, all tested esters were inactive ($IC_{50} >$ 50 μM). According to the literature, a C3 acetylation or C3 fatty acid chain (11C) esterification did not affect the activity, whereas a slight decrease in IC_{50} was observed with 17C chain esterification on *L. amazonensis* promastigotes.[23] Some tested unesterified acids were already evaluated on other *Leishmania* species responsible for visceral (*L. donovani*, *L. infantum*) or cutaneous (*L. amazonensis*, *L tropica*, *L. braziliensis*) leishmaniasis. Activities were highly species-dependent, but **1a** remained the most active among the tested compounds. Noteworthy, esterification may keep the activity and decrease the cytotoxicity, but it also influences the solubility and lipophilicity, depending on the nature of the aryl/alkyl group, as well as introducing a hydrolysable function. These problems should be addressed with appropriate formulations for further *in vivo* investigation.

2.3. *In Vivo* Antitrypanosomal Evaluation of Promising C3 Triterpenic Esters

The range of active compounds was assessed according to their *in vitro* antitrypanosomal profile, leading to the selection of two hits for the in vivo test: the hydrocinnamic (**6a**) and the *ortho*-fluorophenylpropionic (**6a**′′′) esters of **1a**. Ursolic acid **1a** was also tested as comparative corresponding 3-OH triterpenic acid. All compounds were evaluated for their absence of toxicity in an acute mice model. No compound induced any acute symptoms at the highest tested cumulative dose of 100 mg kg^{-1} by the IP injection route. In the antitrypanosomal assay realized with a single dose of compounds, no parasitemia difference between each treated group was observed at the end of the experiment (Figure 2), neither any survival impact, except for positive controls. On day 4, no important parasitemia reduction was seen, whereas at day 5, we observed a significant ($p \leq 0.05$) reduction compared to vehicle-treated mice with an estimated mean inhibition percentage of 61.2 ± 27.8% for treatment with **6a** (Figure 3). These SEM values emphasized the high parasitemia variability even with a very aggressive strain, very likely to be linked to the adaptive immune response. **1a** and **2a**, indeed, already showed immunomodulatory effects with pro- or anti-inflammatory activities associated with dose and environment.[24] This work also showed that the tested compounds were not effective enough to control or delay parasite growth. However, an earlier and/or longer treatment with a more appropriate dose should be tested for the hydrocinnamic

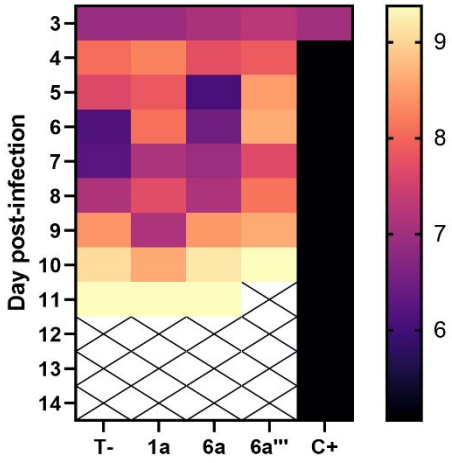


Figure 2. *Trypanosoma brucei brucei* growth expression heat map, showing the parasitemia (log(*T. bb*)/mL) between day 3–14 post-infection after administration of **1a** (50 mg kg^{-1}), **6a** (50 mg kg^{-1}), **6a‴** (40 mg kg^{-1}) in comparation with T-(vehicle) and C + (suramin) (0.5 mg kg^{-1}). For this experiment, 7 mice were used as negative control (T-); 4 mice as positive control (C +); 5 mice for **1a**, 5 mice for **6a**, 6 mice for **6**′′′. The chromatic scale represents equivalent log number of organisms per milliliter of blood.

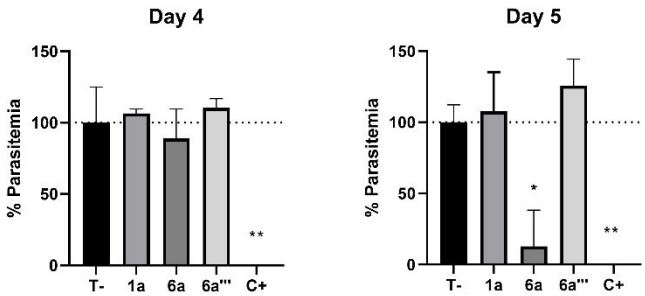


Figure 3. Estimated parasitaemia (%) day 4 and 5 post-infection (Mean ± SEM) normalised with T-. For this experiment, 7 mice were used as negative control (T-); 4 mice as positive control (C +); 5 mice for **1a**, 5 mice for **6a**, 6 mice for **6**′′′. Non-parametric Mann–Whitney in one tailed (in comparison to negative control). * = $p < 0.05$, ** = $p < 0.01$.

derivative **6a** to further investigate the observed parasitemia decrease. Triterpenic esters **6a**, **6a'''** and **1a** were tested here in an acute African trypanosomiasis *in vivo* model for the first time. The used 427 strain is known to induce acute disease with rapid exponential parasite growth, only controlled by highly potent drugs, as observed with suramin given at a 100-fold lower dose. Further investigation of **1a** and derivatives on other less aggressive models to assess compounds potential are needed, for example on the GVR35 strain.

3. Conclusion

Among the tested PT acids, none was more active against the evaluated parasites than ursolic acid **1a**, with the highest activity against *Trypanosoma brucei brucei*. Esterification with a lipophilic aromatic chain on C3 did not appear to improve antileishmanial activities for which the presence of a C3 hydroxyl group appeared to be important. Concerning the antitrypanosomal activity, C3 triterpenic esterification has a positive impact on the cytotoxicity of triterpenes while keeping the activity in close range of the parent acids. Different improvements were observed on ursane and oleanane skeletons: 3-*O*-aromatic ursane derivatives showed an increased selectivity, whereas some oleanane derivatives showed increased activity. According to hydroxyl and ester positions, some intramolecular hydrogen bonds can occur, affecting biological activities, as well as a steric hindrance and preferred conformation modifying target interaction. Adding a steric shield in the proximity of the ester function or a bioisoster replacement of the double bond in the alkyl chain showed a positive effect on the antitrypanosomal activity and cytotoxicity which can potentially increase the ester stability, too. Other esterified derivatives with increased hydroxylation numbers on the aromatic ring should be tested to assess the antiparasitic potential of both combined features. In this work, new antiparasitic scaffolds and activities were reported for known acids/esters as well as for newly hemisynthesized esters. Some displayed interesting in vitro antitrypanosomal activity and the in vivo potential has to be further investigated. The influence of different alkyl/protective groups should be evaluated to continue structure-activity relationships studies.

Experimental Section

Reagents and HPLC-MS conditions

Commercial reagents and solvents were obtained from AvaChem Scientific, VWR Chemicals, Extrasynthese, Phytolab, Sigma-Aldrich and Acros Organics in the grades puriss, p.a or purum and were used without further purification. The identification of hemisynthesized compounds was realized on a Bruker Avance spectrometer for ^{1}H NMR and ^{13}C NMR recorded in MeOD or $CDCl_3$. The processing and evaluation of the spectra were performed using the program MestReNova 6.0. The resonance multiplicity is indicated as s (singlet), d (doublet), t (triplet) and m (multiplet) or a combination of them, and the coupling constants (*J*) are given in Hz. The mass spectra were obtained with an LC–HRMS/MS system consisting of a Thermo Accela pump, autosampler, photodiode array detector and Thermo Scientific LTQ Orbitrap XL mass spectrometer. High-resolution MS and MS^2 of the major peaks were analysed with an APCI or ESI source in the negative or positive modes. The following inlet conditions were applied: capillary temperature 250 °C, APCI vaporizer temperature 400 °C, sheath gas flow 25.00 u.a., auxiliary gas flow 25.00 u.a., sweep gas flow 5.00 u.a and discharge current 5 μA; with capillary voltage and tube lens of −10 V and −125 V or 9 V and 70 V in negative and positive mode, respectively. Data acquisition and processing were performed with Xcalibur software 2.2.

General Chemistry

Syntheses of 6a–b, 6d and 7a–a'''. Hemi-synthesis was adapted from Lee *et al.*[25] and summarized in Scheme 1. A flame-dried 50 mL round-bottomed flask, equipped with stirring bar, reflux condenser with a septum cap, argon inlet (through septum cap) and syringe inlet (through another septum cap), was charged with 25 mL of dry toluene, 1.3 equiv. of DCC, 0.04 equiv. of 4-(dimethylamino) pyridine, and 2.2 equiv. of cinnamic or hydrocinnamic acids (**6a**, **6b**, **7a**) or *ortho*/*meta*/*para* fluorophenylpropionate acid isomers (**6a'**–**6a'''** and **7a'**–**7a'''**) or *trans*-2-phenyl-1-cyclopropanecarbonyl chloride (**6d**). Then, 1 equiv. of purchased triterpenic acid was added. The resulting solution was brought to 80 °C and stirred for 24 h. After the reaction was completed, the syringe apparatus was removed and the reaction mixture was cooled to room temperature. Stirring was continued for 30 min, at which time no DCC was detected by TLC analysis (10% EtOAc-Toluene). Further TLC analysis in two solvent systems revealed the formation of the desired ester ($0.55 < R_f < 0.7$ in 15% EtOAc-Toluene). The mixture was washed 3 times with AcOEt/water and the organic phase was dried over $MgSO_4$ and evaporated. The residue was taken up in a minimal amount of toluene and purified through a silica gel column (Merck, silica gel 60, 0.065–2 mm) slurry-packed in toluene. Elution was performed with a gradient of toluene-ethyl acetate. Recrystallization from methanol afforded the pure desired compounds. When necessary, an additional purification step by semi-preparative HPLC was performed with a Phenomenex Luna C18 column (250 × 10 mm^2 with 5 μm as particle size) on a Shimadzu Prominence system (LC20-AP pumps and SPD-20AV UV/VIS detector) with 100% methanol at 3 ml min^{-1}. The purity of each compound is available in the Supporting Information and was assessed at 210 nm with the analytical column (250 × 4 mm^2), a flow rate of 1 ml min^{-1} and a binary solvent system composed with acetonitrile and Milli-Q water as followed: 50% acetonitrile 0–2 min, 100% acetonitrile 27–42 min, 50% acetonitrile 43–50 min.

Syntheses of 3 and 6c. The hemi-synthesis of 3-oxo-urs-12-en-28-oic acid (**3**) and 3-acetyl ursolic acid (**6c**) was adapted from Daneshtalab *et al.*[26] and Gnoatto et al.,[27] respectively (Scheme 1) and results compared to literature data. 3-Oxo-urs-12-en-28-oic acid (**3**): to a cold solution of 4.1 mmol ursolic acid (**1a**) in 5 ml acetone-DCM was added 12.5 mmol of pyridinium chlorochromate (PCC) in a cold solution of acetone-DCM. The reaction mixture was stirred overnight on an ice-water bath, concentrated and washed 3 times with water and DCM. 3-Acetyl ursolic acid (**6c**): 1 equiv. of ursolic acid (**1a**) was dissolved in dry toluene with 8 equiv. of pyridine, and 6 equiv. of acetyl chloride were added step-wise. The reaction mixture was stirred in an oil bath at 60 °C for about 1 h under a nitrogen atmosphere, neutralized with 1 M HCl and washed 3 times with water-DCM. The organic phases of **3** and **6c** were dried over $MgSO_4$, filtered and evaporated. Synthesized esters were purified on a silica gel flash column (0.063–0.2 mm) with *n*-hexane-acetone (95:5) and dichloromethane-methanol (8:2) for **3** and **6c**, respectively.

In Vitro Studies

Trypanosoma brucei brucei (strain 427) bloodstream forms were cultivated *in vitro* in HMI-9 medium (Gibco) containing 10% heat-inactivated fetal bovine serum, 150 mM L-cysteine and 20 mM β-mercaptoethanol (Sigma-Aldrich) 507 with 5% CO_2 at 37 °C. *Leishmania mexicana mexicana* (MHOM/BZ/84/BEL46) promastigotes were cultivated in SDM-79 medium (Gibco) supplemented with 15% heat-inactivated fetal bovine serum and 5 mg L^{-1} hemin (Sigma-Aldrich) with 5% CO_2 at 28 °C. The human normal fibroblast cell line, WI38 (ATCC Number CCL-75), was cultivated *in vitro* in DMEM medium containing 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 UI ml^{-1} to 100 μg mg^{-1}) (Sigma-Aldrich) with 5% CO_2 at 37 °C. *In vitro* tests were performed through the AlamarBlue assay.[28] The cytotoxicity test was based on the mitochondrial activity assessment with the MTT tetrazolium salt dye.[29] All pure compounds were solubilized in DMSO at a 10 mg ml^{-1} stock solution and tested in 96-well microtiter plates in eight serial three-fold dilutions (concentration range: 50–0.02 μg ml^{-1} on parasites and 100–0.05 μg ml^{-1} on cells). Pentamidine isethionate salt (antileishmanial drug, Sigma-Aldrich, 98% purity), suramin sodium salt (antitrypanosomal commercial drug, Sigma-Aldrich > 99% purity), and camptothecin (cytotoxic commercial drug, Sigma-Aldrich, 95% purity) were used as positive controls at an initial concentration of 10, 1, 0.1 or 25 μg ml^{-1}, respectively. Tests were performed at least three times in duplicate or two times in triplicate for antiparasitic and cells assays respectively. The activities are defined with the IC_{50} values calculated on the inhibition sigmoidal curves and selectivity indices (SI) were obtained as followed:

$$SI = (\text{IC50 on mammalian cells})/(\text{IC50 on protozoan})$$

In Vivo Acute Toxicity and Antitrypanosomal Assay

In vivo acute toxicity and antitrypanosomal tests were performed according to DNDi guidelines on female NMRI mice (6 weeks, 25.9 ± 3.5 g or 7–8 weeks, 28.2 ± 3.6 g respectively, Envigo).[18] Evaluated pure compounds **1a**, **6a** and **6a'''** and positive controls were solubilized in 10% Tween80/EtOH (7:3) in sterile water and given intraperitoneally (IP). For acute toxicity evaluation, the highest tolerated dose was assessed on two mice groups receiving four increasing doses every two hours (10, 15, 25 and 50 mg kg^{-1}) or four times 20 mg kg^{-1} doses for the *ortho*-fluorophenylpropionate ursolic acid (**6a'''**). Mice were carefully observed after each injection and each day until autopsy after 48 h. The body weight and haematocrit were monitored and main organs (heart, liver, lungs, kidneys and spleen) weights were compared to vehicle control mice ones. For antitrypanosomal activity evaluation, groups of at least 5 infected mice (with 10^4 *T. bb* on day 0) were treated with 50 mg kg^{-1} ursolic acid (**1a**), 50 mg kg^{-1} ursolic acid phenylpropionate (**6a**) or 40 mg kg^{-1} *ortho*-fluorophenylpropionic ursolic acid (**6a'''**) IP during 5 consecutive days (day 3 to 7 post-infection). The negative control (7 mice) received the vehicle and positive controls were given suramin (4 mice) at 0.5 mg kg^{-1} day^{-1}. Parasitaemia was estimated with the Herbert and Lumsden matching method almost every day and compared to negative control mice as survival evolution.[30] All animal studies were designed according to the internationally recognized guidelines and approved by the Ethical Committee for the animals used at the Health Sciences Sector of the Catholic University of Louvain (2017/UCL/MD/017).

Statistical Analysis

In vitro data were analysed by Graphpad Prism statistical software and presented as the mean ± standard deviation. Differences between two independent experiments were analysed by the non-parametric Mann-Whitney test or the Wilcoxon signed-rank test for comparison to a highest tested concentration and between several comparing groups by the non-parametric ANOVA (Kruskal-Wallis and Dunn's post-test) in two-tailed. *In vivo* results were presented as mean ± standard error of the mean and analysed by Mann-Whitney test in one-tailed. Statistical significance for all statistical tests was set at $p \leq 0.05$.

Supporting Information

Molecular characterization and spectral information of all synthetic molecules are reported. Synthetic scope and limitation are described. All the statistical IC_{50} and SI analyses obtained in this work are also available.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: antitrypanosomal drugs · biological activity · in vivo studies · synthesis design · terpenoids

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