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Research Article

Effective Approach to Semi-Synthesis of Lupane and Ursane Brominated Derivatives and its Effects on Viability of *Leishmania amazonensis*

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Submitted: 09 October 2017

Accepted: 30 November 2017

Published: 02 December 2017

ISSN: 2378-9336

Keywords

- Triterpenes
- Ursolic acid
- Betulinic acid
- Bromination reaction
- *Leishmania amazonensis*

Abstract

Pentacyclic triterpenes, especially ursolic and betulinic acid, are extracted eco-friendly from natural sources and are biologically active compounds. Semi-synthesis tools have been employed in order to improve their biological activities. The bromination reaction could influence in the conformational structure of triterpenes skeleton and consequently, in their biological activity. In this work, we aim to survey and discuss an effective approach for semi-synthesis reactions to obtain betulinic acid (BA) and ursolic acid (UA) brominated derivatives and to evaluate their effects on viability of promastigote forms of *Leishmania amazonensis*. The bromination reaction protocol using *N*-bromosuccinimide (NBS) reagent was adapted for both triterpenes, aiming to obtain UA C12-Br and BA C30-Br modifications. The evaluation of leishmanicidal potential was performed using promastigotes forms of *Leishmania (Leishmania) amazonensis* and the viability was measured by MTT assay. According to spectroscopic data, the semi-synthesis of brominated triterpene derivatives using the methodology proposed was successfully accomplished. Bromination of the triterpene structures resulted in an increased antileishmanial activity comparing with the natural precursors, UA and BA. This report brings forth the discussion about the UA bromolactonization and BA C30-Br derivative semi-synthesis and characterization, and reports for the first time the activity of these derivatives on the viability of *L. amazonensis*, causative agent of leishmaniasis, a neglected tropical disease.

ABBREVIATIONS

BA: Betulinic Acid; UA: Ursolic Acid; OA: Oleanolic Acid; NBS: *N*-bromosuccinimide; FBS: Fetal Bovine Serum; Hepes: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonicacid; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethyl Sulfoxide; PBS: Phosphate Buffered Saline; OD: Optical Density; EtOAc: Ethyl Acetate; THF: Tetrahydrofuran; AIBN: Azobisisobutyronitrile; IR: Infrared Spectroscopy; NMR: Nuclear Magnetic Resonance; GC/MS: Gas Chromatography/ Mass Spectrometry.

INTRODUCTION

Triterpenes are one of the most numerous and diversified groups of natural products, having a wide range of applications in the several sectors, such as pharmaceutical, cosmetics, food, and industrial biotechnology. This class of secondary plant metabolites comprises complex molecules that are for the most part beyond the reach of chemical synthesis [1].

Pentacyclic triterpenes, specially, are a class of C_{30} isoprenoid compounds, which are produced biosynthetically by the folding and cyclization of squalene. The most important triterpenoid structures are lupane, ursane and oleanane, and their main representatives are betulinic acid (BA), ursolic acid (UA), and oleanolic acid (OA), respectively [1,2]. In the challenges of drug discovery, an advantage of lupane, ursane and oleanane groups is the fact that they display several biological and pharmacological activities while being devoid of prominent toxicity [2].

Bromine is a halogen, bulky and electronegative atom, which could influence in the conformational structure of triterpenes skeleton and consequently, in their biological activity. Therefore, the aim of this work was to survey and discuss an effective approach for semi-synthesis reactions to obtain betulinic acid (BA) and ursolic acid (UA) brominated derivatives and to evaluate their effects on the viability of *Leishmania (L.) amazonensis* promastigotes. This species of protozoan parasite induce distinct host responses. It causes the entire spectrum of leishmaniasis, including tegumentar cutaneous, mucocutaneous and diffuse cutaneous leishmaniasis (LCD). LCD is severe disease and often difficult to treat, which ranges from small cutaneous nodules to gross mucosal tissue destruction [3,4]. The treatment of leishmaniasis is extremely limited and first-line compounds

consists of pentavalent antimonials, sodium stibogluconate and meglumine antimoniate. The second line of drugs includes pentamidine and amphotericin B. Drugs such miltefosine, paromomycin and liposomal amphotericin B are currently options in the short term in some countries. Conventional treatment has several side effects and restrictions, in addition to other limitations such as high cost, requirement of parenteral administration and increasing drug resistance [4].

In Brazil this disease is predominantly caused by *L. braziliensis* and *L. amazonensis*, it is still persists in the country with a high number of cases are found; the anual burden is estimated at around 21.500 cases per year. These limitations together with the fact that leishmaniasis is one of the most neglected tropical diseases in terms of drug discovery [5], there is an urgent need of increased efforts towards the development of new effective drugs.

MATERIALS AND METHODS

Synthesis of bromine derivatives

Betulinic acid (BA) was obtained from barks of *Platanus acerifolia*, collected in Bento Gonçalves, Rio Grande do Sul State, Brazil (29°10'40.43"S 51°34'2.2"W). Ursolic acid (UA) was isolated from apple pomace (*Malus domestica*); a by-product of juice manufacture Tecnovin Ltd., that was kindly donate by this industry, situated in this same city. The compounds UA and BA was extracted of these natural sources and identified according as previously reported [6,7].

Both UA and BA 0.25 g were suspended in dry pyridine (2.75mL) and acetic anhydride (6.85mL). After stirring at room temperature for 24 hours, the reactions were treated with HCl (30%). The acylated products obtained were purified by column chromatography using a gradient of cyclohexane and dichloromethane as eluents (Yield: 36-37%). An excess of NBS (0.9mmol) was added to a solution of acylated UA and BA (0.3mmol) in 11mL of chloroform, under N_2 atmosphere. The reactions were stirred at 60°C for 72 hours, and every 24 hours, more NBS (0.9mmol) was added. The mixtures were washed with brine (20mL) and extracted with ethyl acetate/chloroform (1:1) (100mL), the organic layer was dried with Na_2SO_4 and the solvent was evaporated under reduced pressure (40 °C). The products were purified by silica gel column chromatography, using as eluent cyclohexane and dichloromethane in gradient system.

The identification of compounds was carried on using infrared spectroscopy (IR) (PerkinElmer FT-IR System Spectrum BX instrument), ^1H NMR and ^{13}C NMR (Varian 400 spectrometer, in $CDCl_3$), and gas chromatography/mass spectrometry (GC/MS) (Thermoelectron Trace GC Ultra – DSQ spectrometer).

In vitro anti-Leishmania amazonensis activity

Promastigotes *Leishmania (Leishmania) amazonensis* (IFLA/67/BR/PH8) were cultured *in vitro* at 23°C in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20mM Hepes (N- 2-hydroxyetrylpiperazine-N'-2-ethanesulfonic acid), and 50 mg/mL of gentamycin and subcultured every 96 hours. The cytotoxicity of compounds was determined by measurement of viability of promastigote forms using MTT assay [8]. For these experiments, promastigotes in stationary phase were seeded at $1x10^6$ parasite/100µL/well in 96-well plates in complete RPMI medium, with compounds at final concentration of 100µM, solubilized in DMSO (0.6%). Pentamidine (9µM) was used as reference antileishmanial agent. Promastigotes were incubated at 23°C for 72 hours. Afterwards, 10µL of a MTT solution (5mg/mL in phosphate buffered saline - PBS) was added to each well and incubated for further 4 hours at 23°C. Subsequently, the plate was centrifuged at 1.500 rpm for 10 minutes and 100µL of DMSO was added to each well and the plate was incubated for 1 hour at room temperature. The OD was measured at 540 nm. The results are expressed as percentage of inhibition of parasites growth, compared with controls.

RESULTS AND DISCUSSION

Taking into account that natural products remain a potential source of novel therapeutic agents [9], using eco-friendly raw material, our research group obtained BA from barks falling spontaneously of *Platanus acerifolia*, and UA from apple pomace (juice industrial waste). Since these compounds are effectively extracted from natural sources, chemical modifications by semi-synthesis processes have been performed in order to improve their biological activity [6,10-12].

In this work, our group synthesized the brominated derivatives of UA and BA through allylic bromination, using NBS reagent (Sigma-Aldrich®) and heating as initiator. The reaction protocol was adapted for both triterpenes. Primarily, the hydroxyl group at C-3 of BA and UA was acetylated (acetic anhydride/pyridine). The corresponding acylated compounds (0.3mmol) were subjected to bromination reaction, using NBS (0.9mmol) dissolved in $CHCl_3$ (11mL) under N_2 atmosphere. The mixture was maintained at 60°C, and every 24 hours more NBS (0.9mmol) was added, until 72 hours of reaction (Figure 1). Then, the mixtures were washed with brine (20mL), extracted with EtOAc/$CHCl_3$ (100mL), dried with Na_2SO_4, and the solvent was evaporated under reduced pressure. The dried products ("A" and "B" – Table 1) were purified by silica gel column chromatography, using as eluent cyclohexane and dichloromethane in gradient system. The UA-bromolactone (A) was given with 88% of yield and brominated-BA (B) with 28.5%.

Few papers devoted to bromination of triterpenes are available. Some merely mention the reaction or apply it as a means to obtain a different final compound, and do not describe the full identification of the brominated compounds neither evaluate the biological activity, especially for lupane derivatives [13,14]. Tkachev et al. (1994), discussed the bromination in the study of oxidation of UA and acetyl ursolic acid, since the researchers proposal was to synthesize a bromolactone. Prior to this publication, it was believed that acetyl ursolic acid could not be brominated, unlike acetyloleanolic acid, since previous reactions gives only unsaturated ethers without mixtures of bromo-derivatives. The assumption made by the researchers is that different behavior of the ursane bromolactone may be caused by severe strain in the molecule due to the non-bonded interaction of axial bromine at C-12 atom and the methyl group C-29. This possibility leads to changed relative rates of two competing reactions – bromine substitution and proton H-12 elimination. Interestingly, in this study, after several approaches

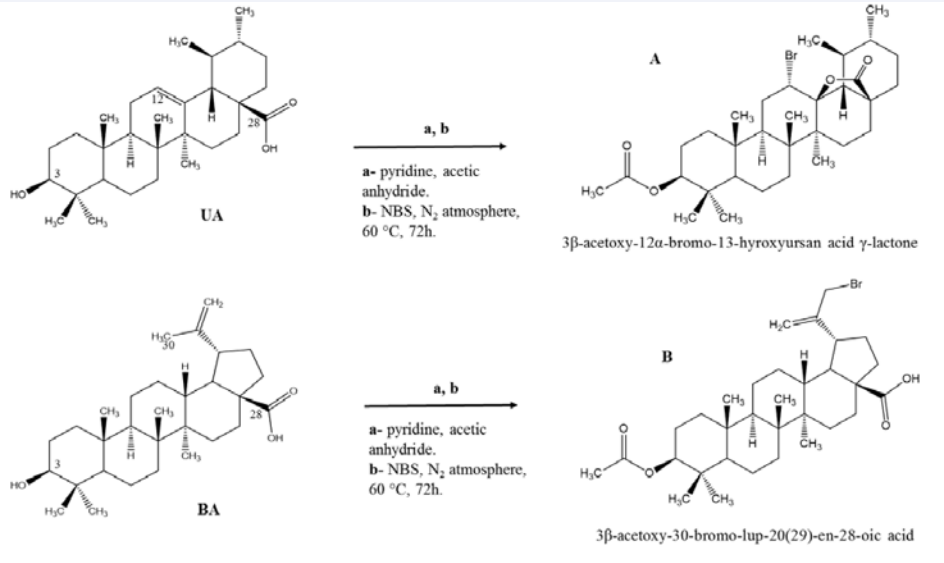


Figure 1 Synthesis route of 3β-acetoxy-12α-bromo-13-hydroxyursan acid γ-lactone (A) and 3β-acetoxy-30-bromo-lup-20(29)-en-28-oic acid (B).

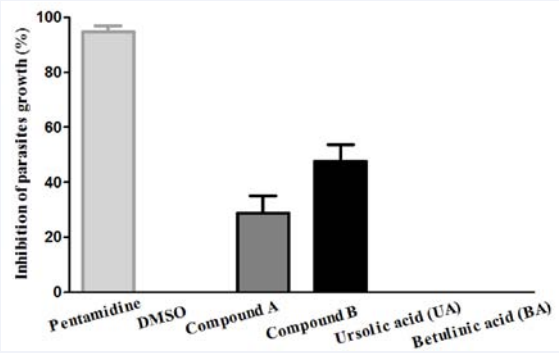


Figure 2 Anti-*L. (L.) amazonensis* activity of UA and BA bromine derivatives: 3β-acetoxy-12α-bromo-13-hydroxyursan acid γ-lactone **(A)** and 3β-acetoxy-30-bromo-lup-20(29)-en-28-oic acid **(B)**.Promastigotes were treated with 100 μM of all compounds shown, exception of Pentamidine (9 μM).

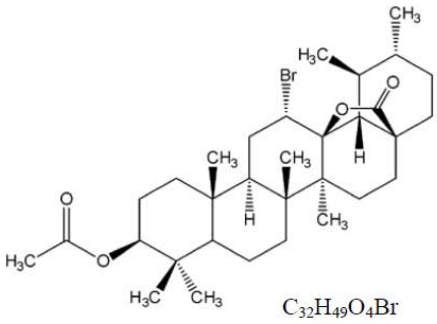
in reactions, the UA bromolactone was achieved with low yields (utmost 14%), using excess of *N*-bromosuccinimide (NBS) in a mixture of THF and water at 10-12°C for 48 h [15]. Recently, a paper of semi-synthesis of taraxerane triterpenoids from OA demonstrated diverse bromination reactions, and it was proposed suitable mechanisms to explain the formation of derivatives. The OA-bromolactone was obtained using Br_2/CCl_4 (80% yield), NBS/AIBN (9% yield) and NBS/AIBN/H_2O (55% yield). The proposed mechanism for its formation, when water is added, is based on the 12α-bromonium ion at C-12 and C-13, which evolved to a 12α-bromo derivative by the attack of the carboxyl group at C-28 to form the lactone ring at C-13 [16].

The carboxyl group at C-28 is the essential condition for the bromolactonization at the C-13 position in ursane derivatives since lactones are cyclic esters formed by intramolecular esterification of the corresponding hydroxycarboxylic acids; α-lactones and β-lactones are very reactive, making their isolation difficult, and as consequence, obtained with low yields [17]. Therefore, high yields should not be expected.

The compounds modified were identified as compound A, 3β-acetoxy-12α-bromo-13-hydroxyursan acid γ-lactone and compound B, 3β-acetoxy-30-bromo-lup-20(29)-en-28-oic acid. In the IR spectrum, for both compounds, it is possible to verify regions of strong absorption corresponding to the functional groups of the lactone, acetate, ether and C-Br stretch. The ^{1}H NMR spectrum of the compound A showed the signal corresponding to the germinal proton to the bromine atom at C-12 (δ_H 4.28), and no signals of double bond were present (δ_H 5.20). In the ^{13}C NMR spectrum of this compound, the C-12 signal that appeared nearly 110 ppm (range of alkenes) became to 52 ppm, proving the absence of the double bond. Moreover, at 91 ppm is possible to identify the presence of a signal corresponding to ether (C-O-C). The mass spectrum reveals the loss of the COO^- group (lactone), bromine, and confirms the presence of ursane scaffold. In the ^{1}H NMR spectrum of the compound B, it is possible to note that the protons of 20,29-double bond are preserved (3.73a ; 3.49b), and the singlets of the three protons of C-30 (1.5-2.0 ppm) was displaced to 4.33 ppm, corresponding to the signal of CH_2-Br. Its

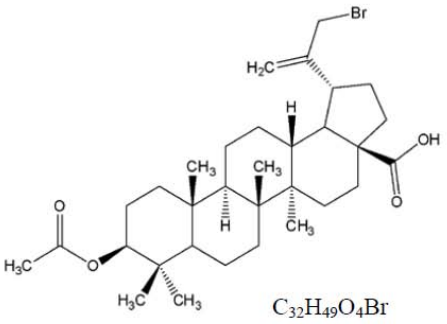
Table 1: Data of compounds identification.

A



3β-acetoxy-12α-bromo-13-hydroxyursan acid γ-lactone

B



3β-acetoxy-30-bromo-lup-20(29)-en-28-oic acid

IR (in KBr) spectra data					
A	2930 (C-H)	1776 (C=O γ-lactone)	1728 (C=O acetate)	1247 (C-O ether)	757 (C-Br)
B	2931 (OH-acid)	2361 (C-H)	1765 (C=O ester)	1728 (C=O acid)	615 (C-Br)

^{1}H-NMR and ^{13}C-NMR spectroscopic data (400 MHz; $CDCl_3$)				
Position	**δ H (A)**	**δ C (A)**	**δ H (B)**	**δ C (B)**
1	1.21 ; 1.64	35	1.55	39.8
2	1.68	28	1.62	25
3	4.52	81	4.46	80.7
4	-	38	-	39
5	0.89	55	1.22	51
6	1.61	16.5	1.77	19
7	1.31 ; 1.57	36	1.40	36
8	-	40	-	40
9	1.81	45	1.36	47
10	-	36.5	-	38
11	2.36	31	1.49	20
12	4.28	52	1.53	26
13	-	91	1.32	28.5
14	-	42	-	41
15	1.24 ; 1.98	29	1.86	31.5
16	2.15	23	1.02	35
17	-	43	-	28
18	1.70	56	1.42	46
19	1.85	39	1.89	42
20	1.17	39.1	-	148
21	1.43 ; 1.62	30	1.68	34
22	1.76	32	1.72	37
23	0.87	28.5	0.88	21
24	0.84	14	0.85	24
25	0.90	16	0.90	16.5
26	1.28	20.5	0.83	15
27	1.51	17	0.82	13
28	-	179	-	178.5
29	1.54	21	3.73a ; 3.49b	80.9
30	0.99	19	4.33	56
31	-	171	-	171
32	2.04	13	2.03	29

GC/MS spectra data	
A	532.49 (M^+-COO^- (1%)); 496.32 (M^+-Br (4%)); 437.31 (26%); 247.11 (30%); 203.13 (56%); 189.11 (100%); 135.05 (34%).
B	501.50 (M^+-Br (10%)); 473.22 (14%); 327.08 (6%); 281.03 (32%); 253.06 (12%); 207.03 (46%); 189.10 (100%).

Abbreviations: GC/MS: Gas Chromatography/Mass Spectrometry; **NMR:** Nuclear Magnetic Resonance; **IR:** Infrared Spectroscopy; **MHz:** Megahertz.

^{13}C NMR spectrum showed the C-29 displaced from 110.0 to 80.9 ppm, and C-30 from nearly 18 ppm to 56 ppm, corresponding to the effect of interaction of C-Br. The mass spectrum reveal the loss of the bromine and makes it clear that it is an acetylated lupane compound (Table 1). Therefore, according to spectroscopic data, the semi-synthesis of triterpenes derivatives was accomplished with success.

In the reactions performed by our group, the pure isolated compounds achieved were UA-bromolactone and BA C-30 modified compound, nevertheless, it is important to highlight that several compounds could be produced in the bromination reactions by different mechanisms [16]. In the UA-bromolactone presented here, probably a severe strain in the molecule due to the non-bonded interaction of axial bromine at C-12 atom and methyl group C-29, lead the addition of bromine occur in the α-side of the double bond [15]. The bromolactonization create a new cycle and change the conformational structure of triterpene skeleton.

The antileishmanial activity is shown in Figure 2. Compounds A and B at 100μM inhibited the viability of 28.66% and 47.41% of promastigotes forms, respectively. The bromination of triterpenes structure showed an increase in the antileishmanial activity, since the scaffolds, UA and BA, were ineffective at 100μM, in the test conditions (Figure 2). Moreover, it was observed that the conformational changes induced by bromolactonization in UA derivative favored the antileishmanial activity.

CONCLUSION

Herein we propose a pathway to the semi-synthesis of 3β-acetoxy-12α-bromo-13-hydroxyursan acid γ-lactone and 3β-acetoxy-30-bromo-lup-20(29)-en-28-oic acid; UA and BA brominated derivatives, respectively. This study brings forth the discussion about the bromolactonization of ursane derivatives and BA 30-bromo derivative semi-synthesis and characterization, and reports for the first time the activity of these derivatives against *L. amazonensis*.

ACKNOWLEDGEMENTS

S.T.C. and A.F.S. performed the reactions and analysis of the compounds; A.P.A.S. and C.B.G.T. carried out the biological assays and helped in manuscript writing; G.G. and S.B.G. coordinated and the study and provide financial support; and S.T.C. and S.B.G. drafted and editing the manuscript. The authors are grateful to the Brazilian agencies FIOCRUZ - RO, CAPES and CNPq for the work financial supported and student's fellowships and to the LRNANO for NMR analysis.

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Cite this article

Cargnin ST, Staudt AF, de Azevedo dos Santos AP, Gosmann G, Garcia Teles CB, et al. (2017) Effective Approach to Semi-Synthesis of Lupane and Ursane Brominated Derivatives and its Effects on Viability of Leishmania amazonensis. Ann Med Chem Res 3(1): 1020.