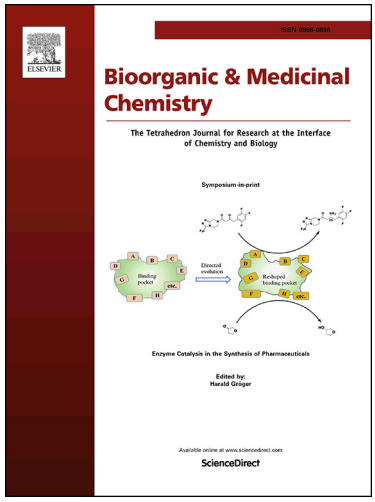

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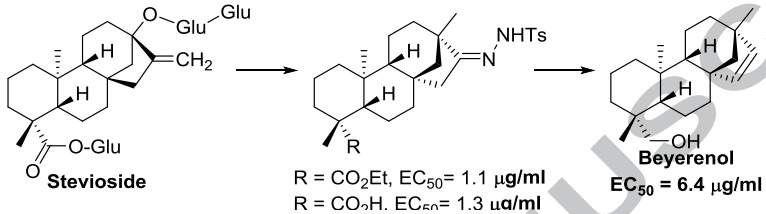

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Jilmar A. Murillo[a], Juan F. Gil[a], Yulieth A. Upegui[b], Adriana M. Restrepo[b], Sara M. Robledo[b], Winston Quiñones[a], Fernando Echeverri[a], Aurelio San Martin[c], Horacio F. Olivo[d], and Gustavo Escobar[a,] *

[a]*QOPN Grupo Química Orgánica de Productos Naturales, Instituto de Química, Universidad de Antioquia*
[b]*PECET, Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia, Calle 70 No. 52-21, Postal Code 0500100, Medellín, Colombia*
[c]*Facultad de Ciencias, Universidad de Magallanes, Punta Arenas, Chile*
[d]*Division of Medicinal and Natural Products Chemistry, The University of Iowa, Iowa City, IA 52242, USA*

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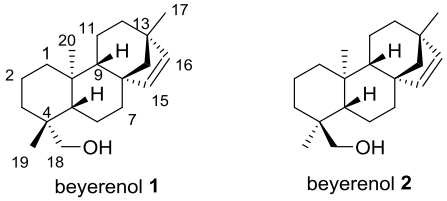
ABSTRACT

We describe the *in vitro* activity of two natural isomeric *ent*-beyerene diterpenes, several derivatives and synthetic intermediates. Beyerenols **1** and **2** showed EC_{50} of 4.6±9.4 and 5.3±9.4 μg/mL against amastigotes of *L. (V) brazilensis*, with SI of 5.1 and 7.7, respectively. Beyerenol **1** was synthesized from stevioside. *In vivo* experiments with bereyenols showed cure in 50% of hamsters infected with *L. (V) brazilensis* topically applied as Cream I (beyerenol **1**, 0.81%, w/w) and Cream III (beyerenol **2**, 1.96%, w/w). These results suggest that beyerenols are potential candidates for cutaneous leishmaniasis chemotherapy by topical application. *In vitro* assays of amastigotes of *L. (V) brazilensis* showed EC_{50} of 1.1±0.1 and 1.3±0.04 μg/mL, with SI of 3.1 and 3.5 for hydrazone intermediates **10** and **11**, respectively.

1. Introduction

Leishmaniasis is an endemic parasitic disease, widely distributed around the world and affecting a large economically disadvantaged population that lives in rural areas of tropical and subtropical countries.[1,2] Available treatments include pentavalent antimonials, miltefosine, pentamidine isothionate and amphotericin B.[3] The use of these products has various difficulties such as the need for parenteral administration (except for miltefosine that is administered orally), very long treatment periods, high doses, serious side effects and high cost. The landscape is complicated by the potential development of resistance to some of these medicines.[4,5] The pharmaceutical companies are not interested to invest in developing new chemical entities to control neither leishmaniasis nor other parasite diseases and it is classified by the World Health Organization as a neglected disease.[6]

In the search for more and better drugs, natural products represent a good alternative to discover new antileishmanial agents.[7] *ent*-Beyerene diterpenoids have been reported in species of Asteraceae and Euphorbiaceae families, among others.[8,9,10,11] In the first family, several species of *Baccharis* have been reported as medicinal plants in Argentina, Bolivia, Chile and Peru, where they are used in traditional medicine for the treatment of wounds and inflammations and their extracts have been studied as antibacterial and anti-inflammatory.[12] Marginal activities have been reported against HIV-1, antimycobacterial and antileukemic.[8,9,13,14,15] There are some reports of active diterpenes against leishmania, with structures as diverse as clerodanes,[16] labdanes,[17] abietanes,[18] and alkaloids,[19] some of them present interesting *in vitro* activities against different species of leishmania, However, the leishmanicidal activity of *ent*-beyerene diterpenoids has not been reported. In the present work, the *in vitro* and *in vivo* leishmanicidal activities of two isomeric compounds isolated from the Chilean plant *Baccharis tola* (Figure 1) and some others obtained by semisynthesis were evaluated. The therapeutic efficacy of natural compounds in experimental cutaneous leishmaniasis (CL) model in hamsters is also reported in the present work.



beyerenol **1** beyerenol **2**

Figure 1. *ent*-Beyer-15-en-18-ol (**1**) and *ent*-beyer-15-en-19-ol (**2**) from *Baccharis tola.*

* Corresponding author. Tel.: +0-000-000-0000; fax: +0-000-000-0000; e-mail: author@university.edu

2. Isolation, synthesis of beyerenol and analogs

Natural products have been a rich source of bioactive molecules for the development of new drugs; however, they have several drawbacks, among them the low concentrations present in the plant and the extensive purification processes. For these reasons, many investigations do not advance until stages that allow to define their potential as a new medicine and thus be able to establish QSAR relationships to optimize their effects. On the other hand, more and better drugs are needed to control neglected parasitic diseases because those that are currently available have many limitations. In this work we analyze the leishmanicidal activity of several natural diterpene molecules, as well as some structural analogues.

Diterpenes are a large group of natural molecules with pharmacological activities and very diverse chemical structures. In this group are the *ent*-beyerenes, compounds which have not been reported to possess antiparasitic activity. *ent*-Beyer-15-en-18-ol (**1**) and *ent*-beyer-15-en-19-ol (**2**) were isolated from the *B. tola* plant as previously reported.[20] Four derivatives were prepared from the major diastereomer, beyerenol **1**, Figure 2. Beyerenol **1** was hydrogenated in the presence of Pd/C in ethyl acetate to furnish beyerol **3**. Beyerenol **1** was oxidized with Jones reagent to give beyerenoic acid **4**, and this acid was oxidized with *m*-CPBA to obtain the oxirane beyeroic acid **5** as a single isomer. Finally, beyerenol **1** was oxidized with *m*-CPBA in dichloromethane to obtain oxirane **6**. These simple derivatives (**3** – **6**) obtained from the natural product were valuable to obtain initial relationships between structure and antiparasitic activity.

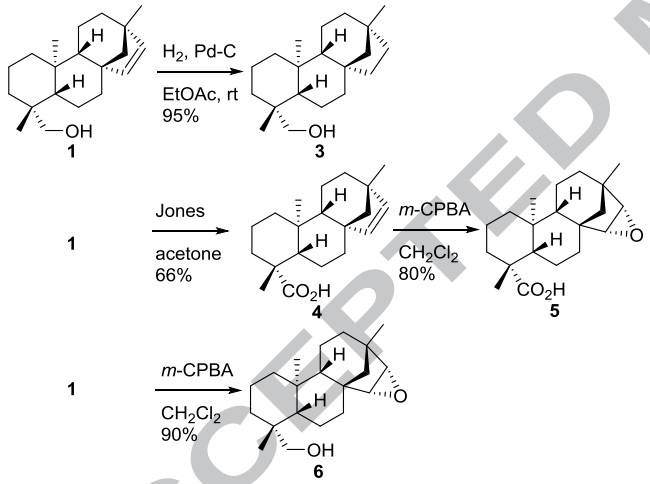


Figure 2. Some derivatives from *ent*-beyer-15-en-18-ol (**1**).

The promising leishmanicidal activity of beyerenol **1**, its low concentration in the Chilean plant *B. tola*[16] and its extremely difficult isolation, forced the search for a source that would allow us to generate more material. Stevioside, a natural sweetener widely used in the food industry emerged as an ideal candidate, given its accessibility, low cost and structural similarity to the molecule of interest. [21,22] To our knowledge, this is the first report of a synthesis of beyerenol **1**.

Isosteviol (**8**), generated by acid hydrolysis of stevioside (**7**),[23] was initially esterified to ethyl ester **9** and then transformed into the corresponding tosylhydrazone **11**, which underwent a Bamford-Stevens reaction employing basic conditions and microwave irradiation to ethyl beyerenoate **12**, Figure 3.[24] This ethyl beyerenoate **12** was reduced with $LiAlH_4$ to furnish the desired beyerenol **1** in 52% overall yield. The same synthetic sequence was carried out with isosteviol, without the need to prepare the ethyl ester, and the overall yield was 38%. Through this synthetic route and the use of low cost reagents, it was possible to obtain several grams of the compound of interest.

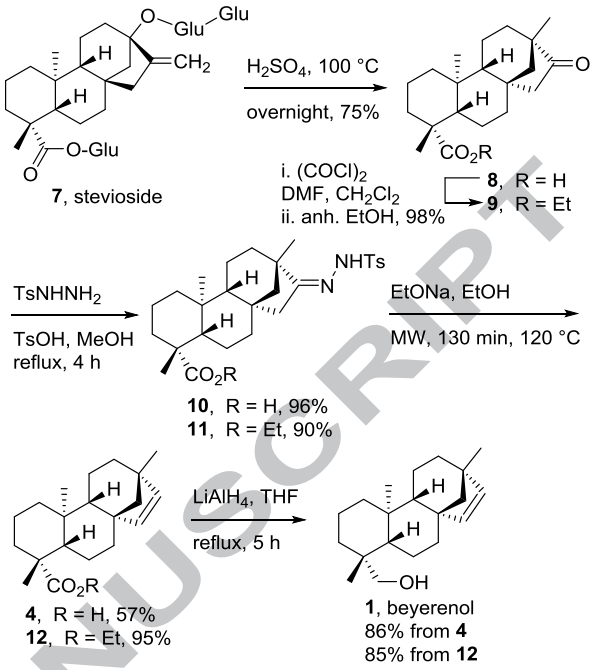


Figure 3. Synthesis of beyerenol **1** from stevioside.

Some simple modifications were made to the cyclopentanone ring of isosteviol ethyl ester **9** following literature procedures, Figure 4. The ketone function of isosteviol **9** was converted to the corresponding oxime **13** on treatment with hydroxylamine and potassium acetate in 96% yield.[25] Oxime **13** underwent a Beckman fragmentation reaction when heated with tosyl chloride in DMF providing nitrile **14** in 87% yield.[26] Isosteviol ethyl ester **9** was reduced with sodium borohydride to give alcohol **15** as a single isomer in 96% yield.[25] The synthesis of isosteviol **8** and its ethyl ester **9** were prepared according to the method reported in the literature as well as for compounds **13**, **14** and **15**.

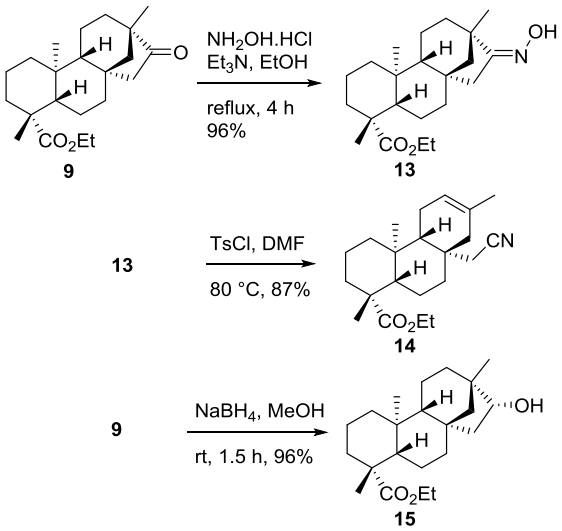


Figure 4. Synthesis of isosteviol ester derivatives **13** – **15**.

3. Materials and methods

3.1 *In vitro* cytotoxicity in human U937 macrophages

Human promonocytic cells U937 (ATCC CRL-1593.2 previously cultured in RPMI-1640 (Invitrogen) enriched with

10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Sigma) were dispensed into each well of a 96-well culture plate at a concentration of 10^4 cells/100 µL in enriched RPMI-1640. Then, 100 µL of each compounds at one of six concentrations tested (200, 50, 12.5, 3.1, 0.8 and 0.2 µg/ml) and cells were incubated at 37 °C with 5% CO_2. After 72 hours the cytotoxic effect was determined by adding 20 µL/well of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide solution (0.5 mg/mL) (Sigma) and incubating at 37 °C for 3 hours. The reaction was stopped with 100 µL/well of isopropanol (50%) and sodium dodecyl sulfate (10%) and the production of formazan was quantified by reading at 570 nm in a spectrofluorometer (Varioskan Flash Multimode Reader, Thermo Scientific, Waltham, MA, USA). The color intensity (absorbance) was registered as O.D.[27,28] Cells exposed to amphotericin B (Sigma) were used as control for viability (negative control). Non-specific absorbance was corrected by subtracting absorbance (O.D.) of the blank. Determinations were done by triplicate in at least two independent experiments. The results are expressed as the half maximal lethal concentration (LC_{50}) calculated by Probit method.[29]

3.2 *In vitro* antileishmanial activity in intracellular amastigotes of *L. (V) braziliensis*

Human macrophages (U-937 cells, 10^5/ml RPMI-1640) in stationary phase were induced to macrophages with phorbol myristate acetate (0.1 ng/mL) (Sigma). After 72 h, cells were infected with promastigotes of *L. (V) braziliensis* (HMOM/COL/88/UA301-EGFP) in a proportion of 15:1 (parasites: cell). Plates were incubated at 34 °C, 5% CO_2 and 3 h after, non-internalized promastigotes were removed by washing twice with phosphate buffer (PBS). Infected cells were incubated again and 24 h later the infected cells were exposed to 100 µL/well of each compound at one of six concentrations tested (100, 25, 6.2, 1.6 and 0.4 µg/mL) and incubated at 37 °C with 5% CO_2. After 72 h cells were removed using trypsin/EDTA solution and washed twice with PBS by centrifuging 10 min at 1100 rpm, 4°C and analyzed in a flow cytometer (Cytomics FC 500MPL, Brea, CA) by reading at 488 nm excitation, 525 nm emissions over an argon laser and counting 10,000 events. The percentage of infected cells was calculated by dot plot analysis according to positive events for green fluorescence and the parasite load was calculated by histogram analysis according to the mean fluorescence intensity. Infected cells incubated in RPMI-1640 medium were used as control of infection (negative control) and infected cells exposed to amphotericin B were used as control of antileishmanial activity (positive control).[30,31] Results are reported as the half maximal effective concentration (EC_{50}) calculated by Probit analysis.[23]

3.3 *In vivo* therapeutic response of beyerenols (1 and 2) isolated from *B. tola*

The effectiveness of beyerenols **1** and **2** to cure cutaneous leishmaniasis (CL) was tested in the hamster model for CL.[32] Golden hamsters (*Mesocricetus auratus*), both sexes, six weeks-old and 140 g average of weight, were infected by intradermal injection of $10x10^7$ stationary phase promastigotes of *L. (V) braziliensis* (HMOM/COL/88/UA301-EGFP) in the dorsum. After developing the ulcer, hamsters were randomly distributed in three experimental groups (n = 6). Two groups were treated topically with 40 mg/day during 20 days of a cream formulation containing beyerenol **1** at 0.81% (Cream I) or 1.96% (Cream II) and beyerenol **2** at 2.60% (Cream III), concentrations of these compounds were prepared at convenience. An additional group of hamsters was treated with meglumine antimoniate (200 µg intralesion injection, twice per week, during 4 weeks).

Hamsters were monitored every two weeks for lesion size and appearance and weight. Clinical condition was monitored daily. The effectiveness of each treatment was assessed comparing the lesion sizes prior to and after treatments, as identified as *cure* (healing of 100% area and complete disappearance of the lesion); *improvement* (reducing the size of the lesion in >10% of the area); *failure* (increasing the size of the lesion); *relapse* (reactivation of lesion after cure). Animal condition was followed during three months after treatment and then sacrificed humanely in a CO_2 chamber. After necropsy, histopathology analysis was done in organs and tissues when affected. The evaluation of the toxicity associated to the administration of the treatment was made with the monitoring of the weight and the physical condition, as well as the study of the parameters paramedical Alanine aminotransferase (ALT), Cretinina and Blood Urea Nitrogen (BUN) pre-treatment and 45 days after treatment.

3.4 Ethical aspects

This project was approved by the Ethical Committee for Animal Experimentation at the Universidad de Antioquia (Act No. 88, March 27, 2014).

3.5 Statistical analyses

Cytotoxicity was determined according to mortality (%) in each condition (compound and concentration) using Equation 1, where the O.D of control corresponds to 100% viability.

% Inhibition of viability = 100 – [(O.D Exposed cells) / (O.D unexposed cells) × 100] [1]

The % of mortality was used to calculate the half maximal lethal concentration (LC_{50}) using the Probit analysis[23] and then, the cytotoxicity of each compound was graded according to the LC_{50} values as high (LC_{50} < 100 µg/ml), moderate (LC_{50} > 100 to < 200 µg/ml) and low (LC_{50} > 200 µg/ml). Anti-leishmanial activity was determined according to the infection (amount of parasites in infected cells) obtained for each experimental condition according to dotplot analysis by infected cells and histograms by mean fluorescence intensity (MFI) in fluorescent parasites.[24,25] The parasite inhibition was calculated by equation 2, where the MFI in control was 100% of parasites.

% Inhibition of infection = 100 – [(MFI Exposed parasites) / (MFI unexposed parasites) × 100] [2]

As above, % of inhibition of infection was used to calculate the half maximum effective concentration (EC_{50}) determined by the Probit analysis,[23] and based on EC_{50} values, the activity was graded as high when EC_{50} <25 µg/ml, moderate with EC_{50} values >25 and <50 µg/ml and low when EC_{50} was >50 µg/ml. The Index of Selectivity was calculated with the equation: IS = LC_{50}/EC_{50}. The *in vivo* efficacy of the compound was expressed as the percentage of healing, improvement or failure, and compared to the efficacy observed in the group treated with meglumine antimoniate. Parametric and non-parametric tests were carried out in order to compare percentages and frequencies. Variances were expressed as standard errors and the statistical significance was determined using the analysis of variance (ANOVA) when comparing between groups.

4. Biological activities

4.1 *In vitro* cytotoxicity and antileishmanial activity of *ent*-beyerene diterpenoids and derivatives

All compounds showed high cytotoxicity in human U937 macrophages with values lower than 100 μg/mL (Table 1). At the same time, *in vitro* tests on amastigotes of *L. (V) braziliensis* showed a high activity for most of the *ent*-beyer-15-en-18-ol derivatives except when the hydroxymethyl in C-18 is oxidized to carboxylic acid or its ester, a situation confirmed by isosteviol and its ester (**8**, **9**). The oxidation of the alkene to an epoxide (**5**, **6**) decreases the activity, however, the presence of nitrogenous groups on the carbons C-15 or C-16 significantly increases its leishmanicidal activity (**10**, **11**, **13**, **14**), although also its cytotoxicity.

In spite of the high cytotoxic activity for U937 cells, the biological activity against *Leishmania* amastigotes was selective, being higher for amastigotes than for the host cells. This selectivity was evidenced by a SI >1. The most selective compounds were compounds **2**, **1**, **15** and **11** with SI of 7.6, 5.1, 3.9 and 3.3, respectively (Table 1).

Table 1. *In vit*ro cytotoxicity and antileishmanial activity of *ent*-beyerene diterpenoids and derivatives.

compd	U937 LC_{50} μg/mL (μM)	Intracellular amastigotes *L. braziliensis* EC_{50} μg/mL (μM)	SI
1	32.9 ± 9.4 (114.2 ± 32.6)	**4.6 ± 0.9** (16.0 ± 3.1)	5.1
2	35.3 ± 3.4 (122.2 ± 11.8)	**5.3 ± 1.7** (18.1 ± 6.0)	7.7
3	8.6 ± 0.3 (29.6 ± 1.0)	**5.3 ± 1.7** (18.1 ± 6.0)	1.6
4	39.2 ± 7.9 (129.5 ± 26.2)	>20 (>66)	>2
5	20.6 ± 0.2 (64.8 ± 0.7)	**13.8 ± 3.5** (43.3 ± 11.0)	1.5
6	35.8 ± 1.8 (117.6 ± 6.0)	>19.2 (>63)	>1.9
8	21.3 ± 4.5 (66.9 ± 14.2)	>6.4 (>20)	>3.3
9	18.0 ± 5.0 (51.9 ± 14.5)	**20.9 ± 5.3** (36.3 ± 9.2)	0.86
10	3.4 ± 0.5 (5.9 ± 0.8)	**1.1 ± 0.1** (2.3 ± 0.2)	3.1
11	4.5 ± 0.2 (8.8±0.4)	**1.3 ± 0.04** (3.0 ± 0.1)	3.5
13	5.7 ± 1.1 (15.7 ± 3.1)	**3.1 ± 0.8** (8.8 ± 2.2)	1.8
14	3.1 ± 0.1 (9.1 ± 0.4)	**5.1 ± 1.2** (14.1 ± 3.3)	0.6
15	10.0 ± 0.9 (28.8 ± 2.6)	**7.0 ± 0.4** (13.6 ± 0.7)	1.4
AmB	50.3 ± 0.1 (54.4 ± 0.1)	0.7 ± 0.2 (0.8 ± 0.2)	71.8

Data represent median Lethal Concentration (LC_{50}) mean value for each compound ± SD evaluated in the cell lines U937 and Effective Concentration 50 (EC_{50}) in μM for each compound ± DS in *L. brazilensis* (intracellular amastigotes). SI: LC_{50}/EC_{50}. AmB: amphotericin B. Note: Numbers in bolt highlight the active compounds.

4.2 Therapeutic response of isomers of beyerenol

The evolution of skin lesions in hamsters with experimental cutaneous leishmaniasis caused by *L. braziliensis* is shown in Figure 5. Three preparations were used: Cream I (beyerenol **1**, 0.81%), Cream II (beyeronol **1**, 1.96%), and Cream III (beyerenol **2**, 2.6%). Treatment consisted in applying Creams for twenty days, with 40 mg/day. The control group of hamsters was treated with intralesion injection of meglumine antimoniate (200 μg), twice per week, 4 weeks. Lines represent the size of lesion in mm^2 during the study. TD0: Before treatment; TD20: end of treatment; PTD30: day 30 after the end of treatment; PTD60: day 60 after treatment ended; PTD90: day 90 after the end of treatment.

Treatment with Creams I, II and III resulted in notable reduction of the size of skin lesions in hamsters, Figure 5. Three of the six hamsters treated with the Cream I and Cream III showed 100% reduction in the size of the lesion three months after treatment (PTD90), Figure 5a and 5c. However, in the group treated with Cream I the remained three hamsters showed a reduction in the size of their lesion in 10.9% (1-0.8-2-♀), 41.2% (1-0.8-4-♂) and 48.4% (1-0.8-5-♂), Figure 5a. In the group treated with Cream III, only one hamster showed reduction in the size of lesion of 21.2% (2-2.6-1-♀) and in the other two hamsters the treatment had no effect (2-2.6-2-♀ and 2-2.6-6-♂) and therefore, they were considered as a failure (Figure 5c). The increase in the dose did not increase the cure rate. Only two hamsters of the group treated with a higher dose (1.96%) of Cream II did cure while other three hamsters showed a significant reduction of 52.34% (1-1.9-1-♀), 76.64% (1-1.9-3-♂) and 7.84% (1-1.9-6-♂) (Figure 5b). One of cured hamsters that had cured at the end of treatment showed a reactivation of the lesion one month later and was considered as relapse (Figure 5b). Five of the six hamsters treated with the control medicine (meglumine antimoniate) showed complete cure at the end of treatment and remained cured until the end of the study (PTD90), except in one case that had a reactivation of its lesion in the second month of clinical follow-up (PTD60) (Figure 5d). The remained hamster of this group (MA-2-♂) the size of its lesion decreased in 85.5% with respect to the size before treatment. Notably, treatment with Cream I and Cream III showed cure until the end of the treatment, similar to what was observed in the group treated with meglumine antimoniate. On the other hand, in the group treated with a lower dose, Cream I, the cure was observed from day 30 to 90 post-treatment (Figure 5a).

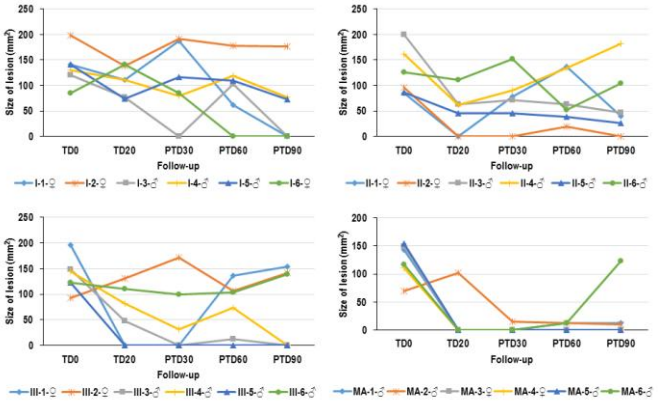


Figure 5. Effect of treatment with Creams I, II, and III *vs* meglumine antimoniate. TD0: Before treatment; TD20: end of treatment; PTD30: day 30 after the end of treatment; PT60: day 60 after treatment ended; PTD90: day 90 after the end of treatment.

A summary of clinical outcome after treatment with beyerenols **1** (Creams I and II) and **2** (Cream III) in comparison to treatment with meglumine antimoniate is shown in Table 2.

Table 2. Effectiveness of treatment with beyerenols **1** and **2**

Treatment/ Compound[a]	Therapeutic effectiveness (%)[a]			
	Cure	Improvement	Failure	Relapse
Cream I [b]	3 (50)	3 (50)	0 (0)	0 (0)
Cream II [b]	1 (16.7)	3 (50)	1 (16.7)	1 (16.7)
Cream III [b]	3 (50)	0 (0)	2 (33.3)	1 (16.7)
Meglumine antimoniate [c]	4 (66.7)	1 (16.7)	0 (0)	1 (16.7)

[a] At 3 months after the end of treatment.
[b] Cream I: beyerenol **1**, 0.81%; Cream II: beyerenol **1**, 1.96%; Cream III: beyerenol **2**, 2.6%.
[c] *n* = 6 animals per group.
[d] Topical cream 40 mg/day for 20 days. [c] Via IL (200 μg) twice / week / 4 weeks

4.3 Toxicity upon treatment

No signs associated with toxicity of the treatments were observed. The weight of animals treated with Creams I, II and III did not differ significantly during the study ($p > 0.05$, ANOVA) (Figure 6). In contrast, animals treated with meglumine antimoniate (MA) experienced small weight loss one month after treatment, without increase during the second month after treatment followed by a decrease at the time the study ended.

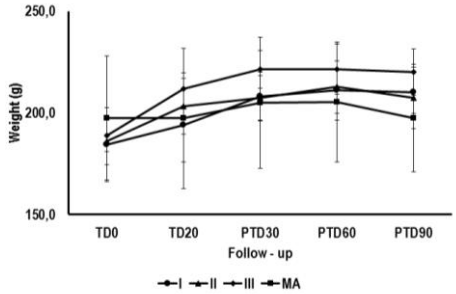


Figure 6. Effect of treatment with Creams I, II and III *vs* meglumine antimoniate in the weight of hamsters with cutaneous leishmaniasis caused by *L. (V) braziliensis.* Lines represent the weight in grams during the study. TD0: Before treatment; TD20: end of treatment; PTD30: day 30 after the end of treatment; PT60: day 60 after treatment ended; PTD90: day 90 after the end of treatment.

Figure 6 shows the evolution of weight in hamsters after treatment with Creams I, II, and III (40 mg/day/20 days). A group of hamsters was treated with intralesion injection of meglumine antimoniate (200 μg/twice per week/4 weeks). The *y*-axis shows the median of weight ± SD of each group of treatment and *x*-axis corresponds to time points of study. TD0: before treatment; TD20: last day of treatment; PTD30: post-treatment days 30; PTD60: 60 days after treatment ended; PTD90: 90 days after the end of treatment.

Chemical analysis of blood samples did not show changes in the normal levels of creatinine and alanine aminotransferase (ALT), blood ureic nitrogen (BUN) or creatinine in any of the animals studied before and after and after treatment (Figure 7). No significant statistical differences in the levels of these biochemical markers were found between animals treated with respect to normal values.

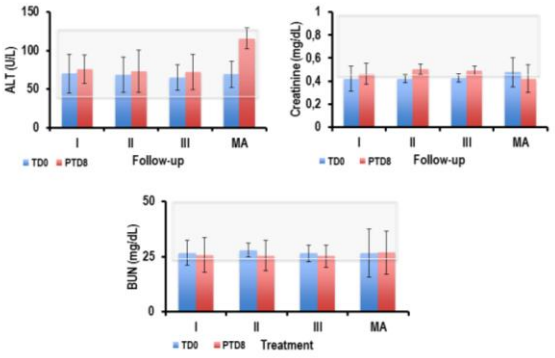


Figure 7. Levels of ALT, creatinine and BUN in serum of hamsters treated with Creams I, II, and III, before and after treatment.

Data corresponds to the median ± SD of ALT (a), creatinine (b) and BUN (c) in serum of hamsters with cutaneous leishmaniasis before treatment (TD0, white bars) and 8 days after the end of treatment (PTD8, black bars) with Cream I, Cream II, Cream III or meglumine antimoniate (MA). No significant differences were seen between groups ($p > 0.05$). Shaded area corresponds to normal value.

The analysis of the skin, in the hamsters that suffered a clinical cure, shows that the group treated with Cream I topically, presented a more popular inflammatory response with the migration of all cell types evaluated in relation to the other groups of the treatment, the migration of lymphocytes, plasmocytes, and macrophages were a common finding in all the groups. The process of re-epithelialization based on tissue fibrosis occurred only when Cream III and MA were employed. Contrary to what was expected, it was found for the groups with the best therapeutic response (Cream I) and meglumine antimoniate, which was confirmed with the determination of parasitic load by PC (RT-PCR), which in these cases were <500 parasites per milligram of tissue.

In the liver, the degree of alterations was mild to moderate in all cases, common to all groups. There was vacuolar degeneration, pigment (hemosiderin), congestion and infiltration of lymphocytes and macrophages. Hyperplasia only occurred in hamsters treated with MA. Other findings associated with changes due to external stress conditions were presented in the MA group and without treatment it was binucleation.

At the renal level, an association with toxicity exclusive of the group that received MA was presented, confirming that, despite the change in the route of drug administration, there were derived damages, such as turbid degeneration, edema, fibrosis and infiltration of plasma cells and lymphocytes in tissue. Common to all groups was a vacuolar degeneration that was more severe in the group treated with meglumine antimoniate.

4. Conclusions

The *ent*-beyerene diterpenes studied in this work showed high *in vitro* activity against Leishmania in spite of being relatively unfunctionalized substances. In addition, the similar activity of both beyerenol isomers (**1** and **2**) indicates that the orientation of the hydroxymethyl group is not important. However, this functional group is indispensable, since its oxidation produces a derivative that is much less active (6.4 μg/ml vs >20 μg/ml). Other transformations in the molecule, such as the oxidation of

alkene, do not improve activity, although they increase cytotoxicity, especially with nitrogenous groups. The *in vivo* therapeutic efficacy of the formulations tested with the two compounds showed a 50% cure. These results show the need to improve their activity by exploring other formulations that can improve the penetrability of the compound in the skin, as well as changes in the treatment scheme. Additional studies exploring transformations in rings A and B are currently been undertaken.

5. Experimental

5.1 General methods

All commercially available reagents and solvents were obtained from commercial suppliers and used without further purification. Commercial stevia (100%) was purchased from Amazon (Wholesale Healt Connection, WHC, 502 Fredericksburg Rd., San Antonio, TX 78201, USA). The reaction progress was monitored with thin layer chromatography on silica gel TLC aluminum sheets (60F254, Merck, Darmstadt, Germany) and visualized with *p*-anisaldehyde spray reagent on heating at 105 °C. The melting points were determined using a Mel-Temp apparatus (Electrothermal, Staffordshire, UK) and are uncorrected. FTIR spectra were obtained on a Bruker Alpha FTIR spectrometer (Bruker Optic GmbH, Ettlingen, Germany). ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded using Bruker Fourier 300 spectrometer (Bruker Bio-Spin GmbH, Rheinstetten, Germany) operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Samples were dissolved in $CDCl_3$ using TMS as internal standard.

5.1.1 *ent*-beyer-19-ol (3). Beyerenol **1 (**30 mg, 0.10 mmol) and palladium on carbon (Pd/C) (2.0 mg, 10% mol Pd, 0.01 mmol) in EtOAc (5 mL) were stirred under a hydrogen atmosphere at room temperature for 12 hrs. The reaction mixture was filtered through a celite path and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 5:1) yielding beyerol **3** as a white solid (29 mg, 95% yield). Mp 131-132 °C. IR (KBr): 3400, 1050-1005 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 1.0 (3H, s), 1.04 (3H, s), 3.1 (lH, d, *J* = 11.1 Hz), 3.4 (1H, d, *J* = 11.1 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 15.6 (C-20), 17.9 (C-18), 17.8 (C-2), 20.0 (C-7), 20.5 (C-11), 27.2 (C-18), 29.6 (C-4), 33.9 (C-6), 35.3 (C-12), 37.6 (C-15), 39.3 (C-3), 40.0 (C-16), 40.9 (C-1), 49.5 (C-9), 56.8 (C-5), 57.7 (C-14), 72.3 (C-19).

5.1.2 *ent*-beyer-15-en-19-oic acid (4). Beyerenol **1** (60 mg, 0.20 mmol) dissolved in acetone was treated with the Jones reagent (prepared by carefully adding 0.5 mL of concentrated H_2SO_4 to 2.5 mL of a 2M solution of CrO_3 in H_2O cooled at 0 °C). After 2 h the reaction at 0 °C, TLC analysis showed that starting material was consumed; the oxidant was then quenched by the addition of 2-propanol (1 mL). The mixture was diluted with water, and extracted with CH_2Cl_2 (3×15 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 9:1), yielding beyerenoic acid **4** as a white solid (40 mg, 66% yield). Mp 138-141 °C. IR (KBr): 3300-3000 (br, sh), 2650, 1695, 1450, 1270 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 1.0 (3H, s), 1.3 (3H, s), 5.5 (1H, d, *J* = 5.4 Hz), 5.7 (1H, d, *J* = 5.7 Hz), 10.78 (1H, s). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 15.3 (C-20), 16.6 (C-17), 17.7 (C-11), 20.02 (C-2), 22.9 (C-6), 24.9 (C-18), 29.7 (C-10), 33.10 (C-12), 36.7 (C-7), 37.0 (C-3), 38.2 (C-1), 43.7 (C-4), 47.4 (C-13), 49.2 (C-8), 50.0 (C-9), 52.8 (C-5), 61.0 (C-14), 135.0 (C-15), 136.6 (C-16), 185.1 (C-19).

5.1.3 *ent*-beyer-15,16-epoxy-19-oic acid (5). Beyerenoic acid **4** (30 mg, 0.1 mmol), 3-chloroperbenzoic acid (*m*CPBA, 89 mg, 0,36 mmol), in dichloromethane (5 mL) was stirred at room temperature for 2 h; then a 2M solution of $NaHSO_3$ was added to the reaction mixture and extracted with CH_2Cl_2 (2×15 mL). The combined organic layer was washed with brine solution (30 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 4:1) yielding epoxybeyeroic acid **5** (25 mg, 80% yield) as a white solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 1.0 (3H, s), 1.04 (3H, s), 3.1 (1H, s), 3.4 (1H, s), 11.4 (1H, s). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 14.1 (C-20), 16.0 (C-17), 21.5 (C-2), 22.7 (C-18), 22.72 (C-6), 29.4 (C-12), 29.7 (C-7), 32.0 (C-3), 32.6 (C-1), 37.0 (C-10), 38.4 (C-4), 46.7 (C-14), 50.2 (C-5), 55.9 (C-15), 56.4 (C-16), 60.2 (C-9), 183.6 (C-19).

5.1.4 *ent*-beyer-15,16-epoxy-19-ol (6). Beyerenol **1** (35 mg, 0.12 mmol), 3-chloroperbenzoic acid (*m*-CPBA, 89 mg, 0,36 mmol), and 1 mL of 1N solution of NaOH in dichloromethane (5 mL) was stirred at RT for 2 h; then a 2M solution of $NaHSO_3$ was added to the reaction mixture and extracted with CH_2Cl_2 (2 × 15 mL). The combined organic layer was washed with brine solution (30 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 95:5) yielding epoxyberenol **6** (33 mg, 90% yield) as an amorphous white solid. Mp 101-102 °C. IR (KBr) cm^{-1}: 3450, 2840, 1450, 1380, 1040, ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 1.0 (3H, s), 1.04 (3H, s), 3.1 (lH, d, *J* = 2.7 Hz), 3.1 (1H, d, *J* = 10.8 Hz), 3.4 (1H, d, *J* = 2.7 Hz), 3.5 (2H, s). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 16.2 (C-20), 17.9 (C-17), 18.0 (C-2), 19.3 (C-6), 19.5 (C-11), 21.5 (C-18), 32.8 (C-7), 35.2 (C-12), 35.4 (C-3), 37.4 (C-10), 37.5 (C-4), 38.9 (C-1), 39.0 (C-13), 44.2 (C-8), 46.8 (C-14), 49.0 (C-15), 56.1 (C-9), 56.4 (C-16), 60.3 (C-5), 72.0(C-19).

5.1.5 *ent*-16-*p*-Toluenesulfonyl hydrazide-beyeran-19-oic acid (10). Isosteviol **8** (370 mg, 1.16 mmol), *p*-toluenesulfonyl hydrazide (306 mg, 1.63 mmol), *p*-toluenesulfonic acid monohydrate (20 mg, 0.116 mmol) and methanol anhydrous (10 mL) was stirred at reflux for 8h; water was added to the reaction mixture and extracted with AcOEt (2 × 15 mL). The combined organic layer was washed with brine solution (20 mL), dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 3:1) yielding hydrazone **10** (542 mg, 96% yield) as an amorphous white solid. Mp 243-245 °C. IR (KBr) cm^{-1}: 2942, 2845, 1716, 1384,1162, 1012, ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 1.1 (3H, s), 1.2 (3H, s), 2.4 (3H, s), 7.3 (2H, d, *J* = 7.9 Hz), 7.8 (2H, d, *J* = 8.3 Hz), 10.60 (1H, s). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 13.4 (C-20), 21.5 (C-17), 21.9 (C-27), 29.1 (C-18), 41.1 (C-8), 43.5 (C-8), 44.9 (C-4), 54.0 (C-9), 56.3 (C-5), 127.9 (C-22,26), 129.3 (C-23,25), 135.2 (C-21), 143.8 (C-24), 183.2 (C-19).

5.1.6 Ethyl *ent*-16-*p*-Toluenesulfonyl hydrazide-beyeran-19-oate (11). Isosteviol ethyl ester **9** (550 mg, 1.72 mmol), *p*-toluenesulfonyl hydrazide (610 mg, 3.3 mmol), *p*-toluenesulfonic acid monohydrate (30 mg, 0.174 mmol) and anhydrous ethanol (15 mL) was stirred at reflux for 3 h; water was added to the reaction mixture and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layer was washed with brine solution (30 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 5:1) yielding hydrazone **11** (736 mg, 90% yield) as an amorphous white solid.

Mp 172-174 °C. IR (KBr) cm^{-1}: 2923, 1689, 1452, 1259, 1168, 799, 665. ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.6 (3H, s), 0.9 (3H, s), 1.1 (3H, s), 1.2 (3H, s), 2.3 (3H, s), 4.0 (2H, m), 7.2 (2H, d, *J* = 8.1 Hz), 7.7 (2H, d, *J* = 8.5Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 13.4 (C-20), 14.18 (C-22), 21.6 (C-17), 21.9 (C-29), 28.8 (C-18), 43.3 (C-13), 43.6 (C-9), 43.6 (C-8), 44.8 (C-4), 56.9 (C-5), 60.1 (C-21), 128.0 (C-24,28), 129.3(C-25,27), 135.3 (C-23), 143.8 (C-26), 177.3 (C-19).

5.1.7 Ethyl *ent*-beyer-15-en-19-oate (12). Hydrazone **11** (500 mg, 0.97 mmol) and anhydrous EtOH (8 mL) was added metallic sodium. The resulting mixture was irradiated in a microwave reactor (102 °C, 300 watts, 130 min) then solvent (EtOH) was evaporated and crude was dissolved in CH_2Cl_2 (3 x 20 mL). The organic layers were combined and washed with brine solution (25 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 20:1) yielding compound **12** (303.4 mg, 95% yield) as a yellow oil. IR (KBr) cm^{-1}: 2946, 1723, 1449, 1152, 1026, 749 ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.6 (3H, s), 1.0 (3H, s), 1.2 (3H, s), 4.1 (2H, q, *J* = 7.1 Hz), 5.5 (1H, d, *J* = 5.7 Hz), 5.7 (1H, d, *J* = 5.6 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 13.8 (C-20), 14.2 (C-22), 24.9 (C-17), 29.1 (C-18), 37.7 (C-3), 37.8 (C-10), 38.2 (C-6), 39.3 (C-1), 49.1 (C-4), 43.7 (C-8), 43.8 (C-13), 52.2 (C-9), 57.1 (C-5), 59.9 (C-12), 134.8 (C-16), 136.5 (C-15), 177.6 (C-19).

5.1.8 *ent*-beyer-15-en-18-ol (1): Carboxylic acid **12** (300 mg, 0.91 mmol) was dissolved in dry THF (5 mL) and was added an excess of $LiAlH_4$ (105 mg, 2.8 mmol). The reaction mixture was refluxed for 5 h. Then the mixture was filtered through celite and evapored under reduced pressure. The residue was puritied by flash column chromatography on silica gel (hexane/EtOAc, 10:1) obtaining a white solid 221.3 mg in 85% yield. ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 1.0 (3H, s), 1.0 (3H, s), 3.5 (1H, d, *J* = 10.9 Hz), 3.8 (1H, d, *J* = 10.9 Hz), 5.5 (1H, d, *J* = 5.6 Hz), 5.7 (1H, d, *J* = 5.6 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 15.8 (C-20), 19.3 (C-6), 20.2 (C-2), 20.3 (C-11), 25.0 (C-17), 27.0 (C-18), 33.2 (C-12), 35.6 (C-7), 37.2 (C-10), 37.7 (C-3), 38.5 (C-4), 39.2 (C-1), 43.6 (C-13), 49.2 (C-8), 52.9 (C-9), 56.8 (C-5), 61.1 (C-14), 65.5 (C-19), 135.1 (C-16), 136.5 (C-15).

5.1.9 Ethyl *ent*-16-hydroximinobeyeran-19-oate (13). To solution of isosteviol ethyl ester **9** (150 mg, 0.430 mmol) in EtOH (5 mL) was added $NH_2OH.HCl$ (170 mg, 2.4 mmol, 5.5 eq) and $NaHCO_3$ (208 mg, 2.41 mmol, 5.7 eq); the resulting mixture was refluxed for 4 h. The reaction mixture was then concentrated under reduced pressure, and extracted with CH_2Cl_2 (2 x 10 mL) and brine solution. The organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by flash column chromatography on silica gel (hexane/ EtOAc, 6:1) to give compound **13 (**145 mg 96% yield) as an amorphous white solid. Mp 42-44°C; ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 1.1 (3H, s), 1.2 (3H, s), 1.3 (3H, m), 4.1 (2H, m). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 13.3 (C-20), 14.2 (C-22), 18.9 (C-11), 20.4 (C-2), 21.6 (C-6), 22.2 (C-17), 28.9 (C-18), 36.9 (C-12), 38.1 (C-1), 39.4 (C-3), 40.0 (C-7), 40.7 (C-15), 41.0 (C-10), 43.7 (C-4), 44.0 (C-13), 54.8 (C-5), 56.3 (C-14), 57.0 (C-9), 170.7 (C-16), 177.5 (C-19).

5.1.10 Ethyl-15-cyanomethyl-4,10,13-trimethyl-dodecahydrophenanthrene-4-carboxylate (14). To the solution of compound **13** (100 mg, 0.30 mmol) in CH_2Cl_2 (4 mL) was added methanesulfonyl chloride (45 µL, 0.6 mmol, 2.0 equiv) and triethylamine (0.3 mL, 2.15 mmol). The resulting mixture was stirred at 0 °C for 4 h. The reaction mixture was concentrated under reduced pressure and diluted with CH_2Cl_2, and washed with brine solution. The organic layer was dried over Na_2SO_4, filtered and and concentrated under reduced pressure. The reaction crude was dissolved in MeOH/toluene (5:1) and then 4 mL of 2N HCl was added, the resulting mixture was heated to 60 °C and stirred overnight. The reaction was quenched with 10% $NaHCO_3$ solution and the organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 10:1) to give compound **14** (82.4 mg, 87% yield) as a white amorphous solid. ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.7 (3H, s), 1.2 (3H, s), 1.3 (3H, m), 1.7 (3H, s), 2.6 (1H, d), 2.6 (1H, d), 4.2 (2H, m), 5.4 (1H, s). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 13.6 (C-19), 14.2 (C-21), 18.9 (C-2), 19.7 (C-6), 20.1 (C-15), 23.4 (C-17), 28.8 (C-16), 35.2 (C-10), 37.4 (C-8), 37.8 (C-12), 43.7 (C-4), 45.7 (C-1), 60.1 (C-20), 118.7 (C-15), 119.9 (C-14), 130.7 (C-13), 177.3 (C-18).

5.1.11 Ethyl *ent*-16-hydroxbeyeran-19-oate (15). To solution of isosteviol ethyl ester **9** (530 mg, 0.923 mmol) and sodium borohydride (86.4 mg, 2.28 mmol) in ethanol (15 mL) was stirred at room temperature for 3 h. Then the reaction mixture was concentrated under vacuum, diluted with CH_2Cl_2 (2 x 10 mL) and washed with brine solution. The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/ EtOAc, 8:1) to give compound **15** as white solid (509.6 mg, 96% yield). Mp 152-153°C. ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.8 (3H, s), 0.9 (3H, s), 1.2 (3H, s), 1.3 (3H, m), 4.1 (2H, q, *J* = 7.1 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 13.3 (C-20), 14.1 (C-22),19.0 (C-11), 20.3 (C-2), 21.6 (C-6), 25.0 (C-17), 29.0 (C-18), 33.8 (C-12), 38.0 (C-1), 38.1 (C-10), 41.8 (C-3), 42.8 (C-7), 43.7 (C-4), 43.9 (C-13), 54.8 (C-5), 55.2 (C-14), 57.1 (C-9), 60.0 (C-21), 177.4 (C-19).

Supplementary Material

Copies of ^1H and ^{13}C NMR for all compounds are included.

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