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Short communication

Synthesis and biological evaluation of (+)-labdadienedial, derivatives and precursors from (+)-sclareolide

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ABSTRACT

Labdadienedial and a series of C15,C16-functionalized derivatives were synthesized from commercial (+)-sclareolide and evaluated for their cytotoxic, antimycotic, and antiviral activities. Their precursors were similarly evaluated.

1. Introduction

Zingiberaceae is one of the major tropical plant families. Embers and extracts of the Zingiberaceae plants are famous for their use as spices and as medicinal herbs [1]. Rhizomes of several species are also used as insect repellents. Many compounds with novel structures and a large number of biologically active compounds have been identified from these plants. Among the bioactive compounds isolated from the Zingiberaceous plants, the labdane diterpenoids have been reported to have a broad spectrum of interesting biological activities [2,3]. In particular, (*E*)-labda-8(17),12-diene-15,16-dial (labdadienedial, Fig. 1, R = CHO) has been found as a major constituent in Zingiberaceous plants of the genus Alpinia [4], Hedychium [5], Curcuma [6], and Aframomum [7]. Both enantiomers of labdadienedial (Fig. 1, R = CHO) have been isolated, although to the best of our knowledge, the levorotatory enantiomer has been reported only once in 1980 [4b]. This labdane-type diterpene has shown cytotoxic [5a,8], antifungal [6b,8], mosquitocidal [6b], antiplasmodial [7d], antimicrobial [7e], antiallergic and inhibition of nitric oxide production activities [9]. It also inhibits human platelet aggregation and human 5-lipoxygenase [10]. Additionally, recent studies have demonstrated that labdadienedial (Fig. 1, R = CHO) possesses certain hepatoprotective activity [5c].

These features make labdadienedial (Fig. 1, R = CHO) an interesting bioactive molecule, which has led to two patent applications for several uses including antiparasitic and antifungal [11], and as flavor enhancer [12]. Also, it was patented in 1988 as an antitumor agent [13]. (+)-Labdadienedial (Fig. 1, R = CHO) has been synthesized from commercially available (−)-sclareol [14], and a chemoenzymatically obtained chiral decalin-type precursor [15]. Other labdanes having the same structural characteristics (1,4-enedial or 3-formyl-3-butenal) have been obtained from readily available (+)-larixol [16]. However, the bioactivities of accessible derivatives of labdadienedial (Fig. 1, R = CHO) have not yet been reported to date.

Continuing our research program on the synthesis of bioactive terpenoids [17], we were interested in synthesizing labdadienedial (Fig. 1, R = CHO) and several easily obtainable derivatives (Fig. 1) from commercially available (+)-sclareolide. Our intention was then to study the biological properties of the synthesized molecules.

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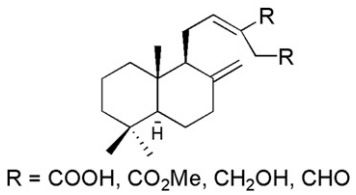


Fig. 1. Chemical structures of some tested labdanes.

In this paper, we describe the syntheses of labdadienedial (Fig. 1, R = CHO) and several derivatives from commercially available (+)-sclareolide (Scheme 1) [18], and present the results of a preliminary evaluation of their cytotoxic, antimycotic and antiviral activities. In this study, an oxygenated moiety (such as methyl ester, alcohol, acid or aldehyde) was introduced into the lipophilic labdane skeleton. In this context, simple and sequential modifications were performed on the double methyl ester **4**. Compound **4** and three derivatives (**5**–**7**) with different functional groups at C15 and C16 were tested together with the synthetic precursors **1**–**3**. All the compounds were easily obtained in good yield by standard or reported chemical procedures. Our synthetic route provides an efficient alternative to prepare bioactive (+)-labdadienedial (**6**) (Scheme 1).

2. Results and discussion

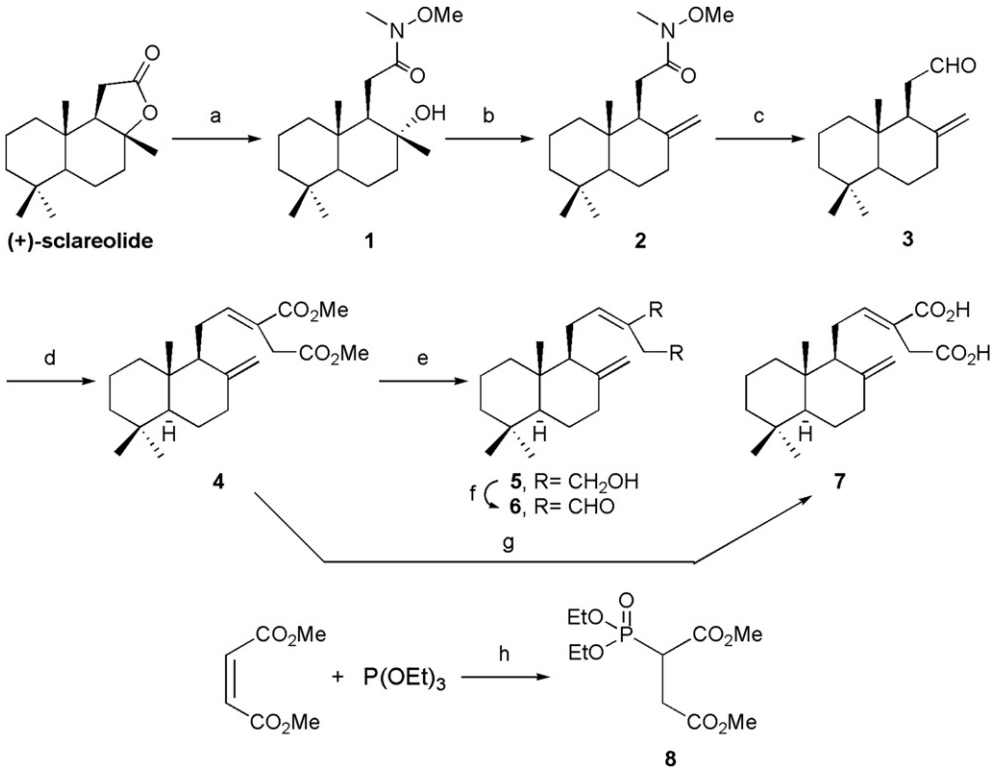
2.1. Chemistry

The syntheses began with the preparation of aldehyde **3** from commercially available (+)-sclareolide following the procedure of de la Torre and co-workers [19a] incorporating the modification reported by Boukouvalas and co-workers (Scheme 1)[19b]. Thus, (+)-sclareolide was reacted with the dimethylaluminium amide derived from *N*-methoxy-*N*-methylamine yielding the desired Weinreb's amide **1** in 85% yield (Scheme 1). The tertiary alcohol of amide **1** was dehydrated in the presence of $SOCl_2$/pyridine at −78 °C to give the *exo*-isomer **2** in 90% yield. Finally, compound **2** was treated with DIBAL-H at −78 °C to give the desired aldehyde **3** in 92% yield.

With the aldehyde **3** in hand, our next objective was to accomplish the introduction of the key unsaturated 1,4-dialdehyde moiety. To this end, we decided to attempt a standard olefination reaction such as the Wittig–Horner reaction. Thus, we prepared the phosphonate ester **8** by hydrophosphinylation following similar reported conditions [20], from dimethyl maleate and triethyl phosphite in 76% yield. The reaction of the anion of phosphonate **8**, generated with NaH in THF, with the aldehyde **3** gave the desired dimethyl succinate **4** in 65% yield. This olefinic product resulted to be by analysis of ^{1}H NMR data, a 3:2 mixture of stereoisomers (*E*/*Z*), which were inseparable by flash chromatography. While we did not try ourselves, this ratio could be improved using a simple phosphorous ylide to exclusively obtain the *E* isomer, as it has been reported for similar ring systems [16]. Then, we carried out the functional group interconversions necessary to obtain three derivatives. Thus, reduction of **4** with $LiAlH_4$ in dry tetrahydrofuran at room temperature gave diol **5** in 93% yield. Oxidation with Dess–Martin periodinane [21] of **5** afforded aldehyde **6** in 55% yield. Finally, ester **4** was saponified with KOH in aqueous methanol to give dicarboxylic acid **7** in 92% yield.

2.2. Biological evaluation

All of compounds **1**–**7** (Scheme 1) were tested for cytotoxic, antimycotic and antiviral activity. Firstly, the compounds (Scheme 1) were tested *in vitro* for potential antitumor and cytotoxic activities to



Scheme 1. *Reagents and conditions*: a) MeONHMe·HCl, $AlMe_3$, DCM, 0 °C to rt, 85%; b) $SOCl_2$/py, DCM, −78 °C, 90%; c) DIBAL-H, Et_2O, −78 °C to −20 °C, 92%; d)NaH, **8**, THF, 0 °C, 65%; e) $LiAlH_4$, THF, 93%; f) Dess–Martin periodinane, DCM, 55%; g) KOH, MeOH, H_2O, 92%; h) AcOH, 100 °C, 76%.

determine the concentration of the compound that induces 50% growth inhibition (IC_{50}) of the human cervix epitheloid carcinoma HeLa tumor cell line and the Vero non-tumor cell line (Table 1). All of the compounds produced a dose-dependent inhibition on the growth of the HeLa tumor cell line and Vero cell line with R^2 (coefficient of linear regression) > 0.85 (data not shown). The compound which showed the highest cytotoxic activity on the HeLa tumor line was γ-bicyclohomofarnesal (**3**) with an IC_{50} value of 2.6 ± 0.2 μg/mL, however, its cytotoxicity in the non-tumor Vero cell line was 10.7 ± 2.4 μg/mL. This compound has shown comparable cytotoxic activities against murine tumor cell lines (P388, IC_{50} = 0.16 μg/mL; B16, IC_{50} = 30.8 μg/mL) and a human tumor cell line (SNU-1, IC_{50} = 3.9 μg/mL)[14b]. In addition, γ-bicyclohomofarnesal (**3**) exhibited the highest selectivity index (SI) with a value of 4.1. According to its SI values, homodrimane **3** is the most selective and potent compound tested, in respect to its cytotoxic properties in the tumor HeLa cell line in comparison to the non-cancerous Vero cell line. However, compound that showed the lowest cytotoxicity against non-cancerous cells (Vero cell line) was the amide **1** with an IC_{50} value of 82.0 ± 8.1 μg/mL. By contrast, the labdane-based compounds **4**–**7** were more cytotoxic against Vero cells than against HeLa cells with the exception of compound **4**. With regard to these results, it is interesting to note that the labdane diester **4** and (+)-labdadienedial **6** were the most cytotoxic against HeLa cells with IC_{50} values of 14.5 ± 1.9 μg/mL and 19.5 ± 1.9 μg/mL, respectively. The cytotoxic activity of (+)-labdadienedial **6** against HeLa cells is comparable with previous bioactivity reports of this compound against human epidermoid carcinoma KB cells (ED_{50} = 40.0 μg/mL)[8], murine lymphocytic leukemia P388 cells, murine melanoma B16 cells, and human gastric adenocarcinoma SNU-1 cells (IC_{50} = 4.1, 41.2 and 12.0 μg/mL, respectively)[14b].

In a continuation of our studies, all of the compounds were evaluated for anti- *Candida*, anti- *Aspergillus* and anti-dermatophytes activity. The compounds did not show activity, except (+)-labdadienedial (**6**), against *Candida parapsilopsis*, *Candida. krusei*, *Candida. tropicalis* and *Candida. albicans* at concentrations lower than 100.0 μg/mL (data not shown), (+)-Labdadienedial (**6**) has shown previously antifungal activity against several *Candida* species (*C. albicans* at 1.0 μg/mL, *C. krusei*, and *C. parapsilosis* at 25.0 μg/mL [6b]; *C. albicans* at 25.0 μg/mL, *C. guilliermondii*, and *C. tropicalis* at 6.25 μg/mL, and *C. utilis* at 12.5 μg/mL [8]). In this study, (+)-labdadienedial (**6**) showed activity against *C. parapsilopsis* and *C. krusei* at 6.25 μg/mL, *C. tropicalis* and *C. albicans* at 12.5 μg/mL (data not shown). Furthermore, only the compounds **1** and bioactive (+)-labdadienedial (**6**) showed activity against *Aspergillus fumigatus* with MIC values of 50.0 ± 34.2 μg/mL and 79.4 ± 25.8 μg/mL, respectively (Table 2). There was a lack of activity against *A. flavus* and *A. terreus* (data not shown). As can be seen in Table 2, different functional groups at C15 and C16 (compounds 4, 5, and **7**) to the aldehyde group in (+)-labdadienedial (**6**) did not improve anti- *A. fumigatus* activity. Amphotericin B (AMB), itraconazole (ITZ) and terbinafine (TERB) were used as positive controls for the *Candida*, *Aspergillus* and dermatophytes species, where the MIC values for the three reference drugs were within the established values for the standard protocols.

During our studies we found that (+)-labdadienedial (**6**) was active against *Fusarium oxysporum* with a MIC value of 19.8 ± 6.4 μg/mL. This result is promising when considering the currently limited options for treatment of this fungus due to its relative resistance to most antifungal agents [22]. In addition, we studied the activity of compounds **1**–**7** against *Trichophyton rubrum* and *T. mentagrophytes*, which are the most common etiologic agents of the superficial fungal infection known as dermatophytosis [23]. As a result, all compounds showed activity against *T. rubrum* and *T. mentagrophytes* with the exception of compounds **4**–**5** and **7** (Table 2). (+)-Labdadienedial (**6**) showed the highest activity against *T. rubrum* and *T. mentagrophytes* (MIC = 0.62 ± 0.2 μg/mL and 1.24 ± 0.4 μg/mL, respectively). Again, we confirmed that the oxygenated derivatives of (+)-labdadienedial (**6**) containing either a methyl diester, a dialcohol or a diacid did not improve the overall activity, in this case against *Trichophyton* sp.

The antiviral activity of all synthesized compounds (**1**–**7**) was studied against herpes simplex virus type 1 (HSV-1) and determined using a modified end-point titration technique (EPPT)[25]. Only (+)-labdadienedial (**6**) reduced the HSV-1 replication with values below 100 μg/mL (data not shown). According to Vlietinck et al. [24], only the compounds with reduction factor (R_f) of the viral titer over 1×10^3 (R_f: ratio of the virus titer in the absence over virus titer in the presence of the tested compound) show relevant antiviral activity. (+)-Labdadienedial (**6**) was found to be moderately active against HSV-1 over infected confluent monolayers of Vero cells with R_f value of 1×10^2 at a concentration of 12.5 μg/mL. However, this concentration is very similar to the IC_{50} on Vero cells.

Table 1
Cytotoxic activity of labdane-based compounds, derivatives and precursors **1**–**7** determined by the MTT technique expressed as IC_{50} (μg/mL).[a]

Compound	Cell lines[b]		
	Vero	HeLa	SI[d]
	IC_{50}[c]	IC_{50}	
1	82.0 ± 8.1	66.8 ± 11.7	1.23
2	31.5 ± 3.6	11.7 ± 2.1	2.69
3	10.7 ± 2.4	2.6 ± 0.2	4.11
4[e]	33.7 ± 1.0	14.5 ± 1.9	2.32
5[e]	38.0 ± 6.5	91.0 ± 7.5	0.42
6[f]	13.1 ± 3.9	19.5 ± 1.9	0.67
7[e]	39.0 ± 5.4	84.5 ± 3.3	0.46
Vincristine	1.1 ± 0.2	0.05 ± 0.01	2.2

[a] Concentration of compounds which induces 50% growth inhibition in 48 h.
[b] HeLa, human cervix epitheloid carcinoma ATCC CCL-2; Vero, *Cercopithecus aethiops* African green monkey kidney ATCC CCL-81.
[c] IC_{50} values are expressed as the mean ± S.E.M. of at least four dilutions by quadruplicate.
[d] SI, selectivity index is defined as VERO IC_{50} over HeLa IC_{50}.
[e] 3:2 mixture of *E*/*Z* isomers.
[f] 9:1 mixture of *E*/*Z* isomers.

Table 2
Antifungal activity of labdane-based compounds, derivatives and precursors **1**–**7** expressed as GM-MIC values in μg/mL[a].

Comp.	GM-MIC[a]			
	Aspergillus fumigatus (ATCC 204305)	*Fusarium oxysporum* (ATCC 48112)	*Trichophyton rubrum* (ATCC 28188)	*Trichophyton mentagrophytes* (ATCC 24198)
1	50.0 ± 34.2	>100	50.0 ± 34.2	39.7 ± 12.9
2	>100	>100	50.0 ± 0.0	19.8 ± 19.6
3	>100	>100	31.5 ± 12.9	31.5 ± 12.9
4[b]	>100	>100	>100	>100
5[b]	>100	>100	>100	>100
6[c]	79.4 ± 25.8	19.8 ± 6.4	0.62 ± 0.20	1.24 ± 0.40
7[b]	>100	>100	>100	>100
TERB	–[d]	–	<0.078 ± 0.0	0.03125 ± 0.0
AMB	2.0 ± 0.0	–	–	–
ITZ	0.17 ± 0.09	–	–	–

[a] Geometric means of minimal inhibitory concentration of tested compounds in μg/mL.
[b] 3:2 mixture of *E*/*Z* isomers.
[c] 9:1 mixture *E*/*Z* isomers.
[d] Not evaluated.

3. Conclusions

In conclusion, we have prepared several labdanes and intermediates and tested then for their cytotoxic, antifungal and antiviral activities *in vitro*. In general, the cytotoxic and antifungal activity of bioactive (+)-labdadienedial (**6**) has been confirmed. We have also demonstrated the antiviral activity of (+)-labdadienedial (**6**) for the first time. Also, we have demonstrated the potent antifungal activity of (+)-labdadienedial (**6**) against two fungal species (*Trichophyton* sp.) not previously studied with this molecule. In general, derivatization of the parent bioactive molecule (+)-labdadienedial (**6**) with oxygenated moieties such as dimethyl diester, dialcohol and diacid did not improve the activity, despite we handled 3:2 mixtures of *E*/*Z* isomers of these compounds. Therefore, these results will encourage us to synthesize additional labdane-related compounds possessing the key pharmacophore 3-formyl-3-butenal (1,4-enedial) moiety, as well as undertaking synergistic studies between antifungal drugs and new labdadienedial-based compounds with the aim of obtaining more effective antifungal treatments.

4. Experimental

4.1. Chemistry

Optical rotations were determined using a 5-cm path-length cell, using dichloromethane as solvent (concentration expressed in g/100 mL). $[\alpha]_D$-values are given in 10^{-1} deg cm^2 g^{-1}. NMR spectra were recorded on a 300 MHz spectrometer with tetramethylsilane as an internal standard. All spectra were recorded in $CDCl_3$ as solvent unless otherwise described. Complete assignments of ^{13}C NMR multiplicities were made on the basis of DEPT experiments. *J* values are given in Hz. Mass spectra (MS) were run by electron impact (EI) at 70 eV. Reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F-254 in 0.25 mm-thick plates. Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a 10% sulfuric acid solution and heating on a hotplate. Purifications were performed by flash chromatography on Merck silica gel (230–400 mesh). All non-aqueous reactions were carried out in an argon atmosphere in oven-dried glassware. Commercial reagent grade solvents and chemicals were used as received unless otherwise noted. Combined organic extracts were washed with brine, dried over anhydrous sodium or magnesium sulfate, filtered and concentrated under reduced pressure. All compounds prepared in this work exhibit spectroscopic data in agreement with the proposed structures.

4.1.1. (1S,2S,4aS,8aS)-N-Methoxy-N-methyl 1-(2-hydroxy-2,5,5,8a-tetramethyldecahydronaphthalenyl)-acetamide (Weinreb's amide, **1**)

Prepared according to a reported procedure [19] with few modifications. To a stirred suspension of *N*,*O*-dimethylhydroxylamine hydrochloride (98%, 8.0 g, 80 mmol) in dry DCM (160 mL) at 0 °C was added Me_3Al (2 M in toluene, 42 mL, 84 mmol) dropwise over 15 min. The mixture was warmed to room temperature and stirred for 3 h until a clear solution was obtained. A solution of (+)-sclareolide (97%, 10.3 g, 40 mmol) in dry DCM (80 mL) was added. The reaction mixture was stirred for 3 h, and after cooling to 0 °C, 60 mL of 10% aqueous H_2SO_4 was added slowly and carefully with the evolution of a large amount of gas. The resulting reaction mixture was allowed to warm to room temperature and the organic layer was separated. The aqueous phase was extracted with DCM (40 mL × 4) and ethyl acetate (40 mL × 1). The combined organic layers were dried, filtered and concentrated under reduced pressure. The residue was purified by chromatography using hexane–ethyl acetate (2:3 to 1:4) as the eluent, to give amide **1** (10.5 g, 85%) as an amorphous solid, whose optical rotation, MS, ^1H NMR and ^{13}C NMR data were in excellent agreement with the reported data [19].

4.1.2. (1S,4aS,8aS)-N-Methoxy-N-methyl 1-(5,5,8a-trimethyl-2-methylenedecahydronaphthalenyl)-acetamide (**2**)

Prepared according to a reported procedure [19] with few modifications. To a stirred solution of Weinreb amide **1** (8.44 g, 27.1 mmol) in dry DCM (120 mL) at room temperature was added dry pyridine (4.5 mL, 2.0 equiv.). After cooling to −78 °C, a solution of thionyl chloride (9.9 mL, 135 mmol, 5 equiv.) in dry DCM (50 mL) and dry pyridine (18 mL, 8.2 equiv.) was added dropwise over 30 min. The reaction mixture was stirred for 1 h at the same temperature before quenching with saturated aqueous $NaHCO_3$ (170 mL). The reaction mixture was allowed to warm to room temperature and the organic layer was separated. The aqueous phase was extracted with DCM (60 mL × 3) and ethyl acetate (60 mL × 3). The combined organic layers were washed with 5% aqueous HCl (40 mL × 3) and brine, and then dried, filtered, and concentrated under reduced pressure to give the crude olefin. This crude was purified by chromatography eluting with hexane–ethyl acetate (1:1) to give olefin **2** (4.95 g, 90%) as an amorphous solid, whose optical rotation, MS, ^1H NMR and ^{13}C NMR data were in excellent agreement with the reported data [19].

4.1.3. γ-Bicyclohomofarnesal (**3**)

Prepared according to a reported procedure [19] with few modifications. To a suspension of amide-olefin **2** (3.01 g, 10.27 mmol) in dry diethyl ether (120 mL) at −78 °C was added DIBAL-H (1 M in toluene, 20 mL) under argon atmosphere. The resulting clear solution was stirred at −78 °C for 90 min and at −20 °C for 30 min before quenching with 10% aqueous HCl (120 mL). The resulting mixture was allowed to warm to room temperature and the organic layer was separated. The aqueous phase was extracted with diethyl ether (25 mL × 4). The combined organic layers were washed with brine, dried, filtered and concentrated under reduced pressure. The resulting residue was purified by chromatography eluting with hexane–ethyl acetate (9:1) to give 2.2 g (92%) of pure aldehyde **3** as a colorless oil, whose optical rotation, MS, ^1H NMR and ^{13}C NMR data were in excellent agreement with the reported data [19].

4.1.4. Dimethyl (E)-labda-8(17),12-diene-15,16-dioate (**4**)

A suspension of sodium hydride (60%, 213 mg, 5.33 mmol) in dry THF (2.8 mL) at 0 °C was treated with dimethyl 2-(diethoxyphosphoryl)succinate **8** (see synthesis below, 1.35 g, 4.77 mmol) dropwise. After 20 min, the ice bath was removed and the solution was stirred for 30 min at room temperature before addition of the aldehyde **3** (0.93 g, 3.98 mmol) in THF (4 mL). After being stirred for 75 min, the reaction mixture was quenched with saturated aqueous NH_4Cl (2 mL), diluted with diethyl ether (45 mL), and washed with brine (10 mL). The resulting organic layer was dried, filtered and concentrated under reduce pressure. The residue was purified by chromatography eluting with hexane–ethyl acetate (9.5:0.5) to give 937 mg (65%) of succinate **4** as a colorless oil having a 3:2 mixture of *E*/*Z* isomers by analysis of ^1H NMR data: $[\alpha]_D^{20}$ + 13.7 (*c* 6.1); *E* isomer: ^1H NMR (300 MHz) δ 6.81 (1H, t, $J = 6.0$), 4.75 (1H, br s), 4.33 (1H, br s), 3.63 (3H, s), 3.61 (3H, s), 3.29 (2H, s), 0.80 (3H, s), 0.74 (3H, s), 0.64 (3H, s); ^{13}C NMR (75 MHz) δ_C 170.9 (s), 167.0 (s), 147.8 (s), 147.1 (d), 124.5 (s), 107.6 (t), 56.1 (d), 55.1 (d), 51.7 (q), 51.6 (q), 41.8 (t), 39.3 (s), 39.2 (t), 38.9 (t), 37.6 (t), 33.4 (q), 33.3 (s), 32.1 (t), 23.8 (t), 21.5 (q), 19.1 (t), 14.1 (q); HRMS (EI) *m*/*z* 362.2422 $[M]^+$, calcd for $C_{22}H_{34}O_4$: 362.2457.

4.1.5. (E)-Labda-8(17),12-diene-15,16-diol (**5**)

To a solution of dimethyl succinate **4** (860 mg, 2.37 mmol) in diethyl ether (15 mL) at 0 °C, $LiAlH_4$ (180 mg, 4.75 mmol) was added in portions. After being stirred for 10 min, the ice bath was removed. The mixture was stirred for 4 h at room temperature, cooled to 0 °C and quenched by adding 2 mL of H_2O dropwise. The resulting mixture was diluted with ethyl acetate (70 mL), and washed with 5% aqueous HCl (15 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried, filtered and concentrated under reduced pressure to give an oily residue, which was purified by chromatography eluting with hexane–ethyl acetate (3:7) to give 683 mg (93%) of alcohol **5** as a colorless oil having a 3:2 mixture of *E*/*Z* isomers by analysis of ^{1}H NMR data: $[\alpha]_D^{20}$ + 21.4 (*c* 8.3); *E* isomer: ^{1}H NMR (300 MHz) δ 5.40 (1H, t, J = 6.3), 4.76 (1H, br s), 4.39 (1H, br s), 4.32 (2H, s), 3.60 (2H, m), 0.83 (3H, s), 0.76 (3H, s), 0.65 (3H, s); ^{13}C NMR (75 MHz) δ_C 148.2 (s), 135.3 (s), 131.6 (d), 107.3 (t), 68.1 (t), 61.0 (t), 60.0 (t), 56.9 (d), 55.2 (d), 41.9 (t), 39.3 (s), 38.9 (t), 37.9 (t), 33.5 (q), 33.4 (s), 24.0 (t), 22.1 (t), 21.6 (q), 19.2 (t), 14.2 (q); HRMS (EI) *m*/*z* 306.2584 $[M]^+$, calcd for $C_{20}H_{34}O_2$: 306.2559.

4.1.6. (E)-Labda-8(17),12-diene-15,16-dial (**6**)

A solution of the diol **5** (350 mg, 1.14 mmol) in DCM (5 mL) was treated with Dess–Martin periodinane (1.20 g, 2.83 mmol)[21], which was added in several portions. After being stirred for 15 h, the mixture was diluted with diethyl ether (10 mL), and saturated aqueous $Na_2S_2O_3$ (10 mL) and $NaHCO_3$ (10 mL) were added. The resulting mixture was stirred vigorously during 30 min and the layers were separated. The aqueous phase was extracted with DCM and the combined organic extracts were washed with brine and concentrated under vacuum. The residue was purified by chromatography eluting with hexane–ethyl acetate (8:2) to give 190 mg (55%) of labdadienedial (**6**) as a slightly yellow oil having a 9:1 mixture of *E*/*Z* isomers by analysis of ^{1}H NMR data: $[\alpha]_D^{20}$ + 22.1 (*c* 2.35); ^{1}H NMR (300 MHz) δ 9.63 (1H, t, J = 1.5), 9.40 (1H, s), 6.77 (1H, t, J = 6.6), 4.86 (1H, d, J = 1.2), 4.37 (1H, d, J = 1.2), 3.43 (1H, AB q, J = 17), 0.89 (3H, s), 0.82 (3H, s), 0.73 (3H, s); ^{13}C NMR (75 MHz) δ_C 197.3 (d), 193.5 (d), 159.9 (d), 148.0 (s), 134.8 (s), 107.8 (t), 56.4 (d), 55.4 (d), 41.9 (t), 39.5 (s), 39.3 (t), 39.2 (t), 37.8 (t), 33.5 (q), 33.5 (s), 24.6 (t), 24.0 (t), 21.7 (q), 19.2 (t), 14.3 (q); HRMS (EI) *m*/*z* 302.2267 $[M]^+$, calcd for $C_{20}H_{30}O_2$: 302.2246. All data were in excellent agreement with that reported for the natural product [4–7]. We also carried out a standard PCC oxidation obtaining a lower yield (40%).

4.1.7. (E)-Labda-8(17),12-diene-15,16-dioic acid (**7**)

A mixture of succinate **4** (200 mg, 0.55 mmol), KOH (85%, 1.5 g, 22 mmol), H_2O (3 mL) and methanol (12 mL) was refluxed for two days. After this time, the reaction mixture was then cooled, poured into aqueous HCl (1.5 M, 30 mL) and extracted three times with ethyl acetate. The organic extract was dried and concentrated under reduced pressure to give the crude acid, which was purified by chromatography eluting with hexane–ethyl acetate (4:6) to give diacid **7** (170 mg, 92%) as a yellowish oil having a 3:2 mixture of *E*/*Z* isomers by analysis of ^{1}H NMR data: $[\alpha]_D^{20}$ + 15.3 (*c* 8.5); *E* isomer: ^{1}H NMR (300 MHz) δ 11.7 (2H, br s), 7.06 (1H, t, J = 6.6), 4.83 (1H, br s), 4.39 (1H, br s), 3.40 (1H, br s), 0.88 (3H, s), 0.82 (3H, s), 0.73 (3H, s); ^{13}C NMR (75 MHz) δ_C 177.0 (s), 172.2 (s), 150.4 (d), 147.9 (s), 123.9 (s), 107.8 (t), 56.2 (d), 55.2 (d), 41.9 (t), 39.4 (s), 39.3 (t), 39.1 (t), 37.7 (t), 33.5 (q), 33.5 (s), 24.3 (t), 24.0 (t), 21.6 (q), 19.2 (t), 14.3 (q); HRMS (EI) *m*/*z* 334.2156 $[M]^+$, calcd for $C_{20}H_{30}O_4$: 334.2144.

4.1.8. Dimethyl 2-(diethoxyphosphoryl)succinate (**8**)

A solution of dimethyl maleate (96%, 6.5 mL, 0.05 mol) and triethylphophite (98%, 10.9 mL, 0.0625 mol) in acetic acid (5.7 mL, 0.1 mol) was refluxed at a bath temperature of 100 °C, under an argon atmosphere, for 24 h. After this time, the mixture was extracted with ethyl acetate (20 mL × 3) and the combined organic extracts were washed with saturated aqueous $NaHCO_3$ (10 mL × 3), dried and concentrated to give the crude phosphonate **8** (76%), which was used without further purification: ^{1}H NMR (300 MHz) δ 4.16 (4H, m), 3.79 (3H, s), 3.70 (3H, s), 3.47 (1H, ddd, J = 24.3, 12.9, 3.6), 3.09 (1H, ddd, J = 18.6, 11.1, 7.5), 2.82 (1H, ddd, J = 17.4, 8.1, 3.6), 1.34 (6H, m); ^{13}C NMR (75 MHz) δ_C 171.3 (s), 168.5 (s), 62.8 (t), 62.7 (t), 52.6 (q), 52.0 (q), 40.9 (d, J = 131.1), 31.1 (t), 16.1 (q), 16.1 (q).

4.2. Biological assays

Stock solutions of compounds were prepared in sulfoxide (DMSO, Sigma) and frozen at −70 °C until required. The concentration of DMSO in biological assays was of 0.05%. Cell controls with DMSO at 0.05% were used.

4.3. Antifungal assay

The antifungal activity of labdane-based compounds, derivatives and precursors **1**–**7** was evaluated following the standard method proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) [25] for fermentative yeasts [26] and the Clinical and Laboratory Standards Institute M38-A (CLSI M38-A, 2002) protocol for filamentous fungi with modifications. The yeast *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258), *C. tropicalis* (CECT 11901), *C. albicans* (ATCC 10231) and filamentous fungi *A. fumigatus* (ATCC 204305), *A. flavus* (ATCC 204304), *A. terreus* (CDC 317), *F. oxysporum* (ATCC 48112) and the dermatophytes *T. rubrum* (ATCC 28188) and *T. mentagrophytes* (ATCC 24198) were used to evaluate antifungal activity. Briefly, seven serial dilutions of the compounds were dispensed into 96-well flat-bottom microdilution plates in duplicate at final concentrations between 100.0 μg/mL and 2.0 μg/mL. Amphotericine B (Sigma Chemical Co, MO, USA) with *A. fumigatus* and *A. flavus,* itraconazole (Sigma Chemical Co, MO, USA) with *C. krusei* and *C. parapsilosis* and Terbinafine (Recalcine Laboratories, Santiago de Chile, Chile) with dermatophytes were used as positive controls at a range of 0.031–16.0 μg/mL. The inoculum size for microdilution plates were 0.5–2.5 × 10^5 and 0.2–2.5 × 10^5 CFU/mL for yeast and filamentous fungi, respectively. For the AFST-EUCAST method, the Minimal Inhibitory Concentrations (MICs) were determined after 24 h at 35 °C of incubation by spectrophotometric reading at 405 nm and is defined as the lowest concentration that resulted in a 90% reduction of growth. For the CLSI M38-A method, the MICs were determined after 48 h of incubation at 35 °C to *Aspergillus* spp. and at 28 °C to *F. oxysporum.* The activity against the dermatophytes was determined after 6 days of incubation at 28 °C. The MICs were defined as the lowest dilution that resulted in an 80% of inhibition of visible growth. MICs results were expressed as range and geometric mean (GM) of duplicates of each compound tested three different times against each of the fungi species in different assays.

4.4. Antitumor activity and cytotoxicity

The cell lines used were human cervix epitheloid carcinoma cells (HeLa cell line ATCC CCL-2) and *Cercopithecus aethiops* green monkey kidney cells (Vero cell line ATCC CCL-81). HeLa and Vero cells were grown in MEM supplemented with 10% FBS, 100 units/mL of penicillin, 100.0 μg/mL of streptomycin, 20.0 mg/mL of glutamine, 0.14% $NaHCO_3$, and 1% each of nonessential amino acids and vitamin solution. The cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere.

The antitumor activity against HeLa and cytotoxic activity against Vero cells were carried out using an *in vitro* on cell growth and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) colorimetric assay, according to the protocol reported by us [27], which was used with few modifications. HeLa and Vero cell monolayers were trypsinized and washed with culture medium and then plated at 1.5×10^4cells per well for HeLa and 1.25×10^4cells per well for Vero cells in a 96-well flat-bottomed plate. After 24 h of incubation, each diluted compound was added to the appropriate wells and the plates were incubated for further 48 h at 37 °C in a humidified incubator with 5% CO_2. Vincristine was used as positive control. The IC_{50} for each compound were obtained from dose–effect curves for linear regression methods and IC_{50} values are expressed as the mean ± S.E.M. of at least four dilutions by quadruplicate.

4.5. Antiviral assays

Herpes simplex virus type 1 (HSV-1) was obtained from the Center for Disease Control (Atlanta, GA). The virus stock was prepared from HSV-1-infected Vero cell cultures. The antiviral activity against HSV-1 was carried out on Vero cells using an end-point titration technique, according to the protocol reported by us [28], which was used with few modifications. Two-fold dilutions of the compounds and viral suspension (one infection dose; the dilution of the virus required to obtain 50% lytic effect of the culture in each well in 100 mL of viral suspension, $TCDI_{50/0.1\ mL}$) were mixed and incubated for 0.5 h at 37 °C before they were added to confluent monolayer cells and further incubated at 37 °C in humidified 5% CO_2 atmosphere for 36 h. Acyclovir and Heparin sodium salt were used as positive controls.

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References

[1] O. Pancharoen, U. Prawat, P. Tuntiwachwuttikul, Stud. Nat. Prod. Chem. 23 (2000) 797–865.
[2] C. Demetzos, K.S. Dimas, Stud. Nat. Prod. Chem. 25 (2001) 235–292.
[3] I. Chinou, Curr. Med. Chem. 12 (2005) 1295–1317.
[4] (a) P.S. Hema, M.S. Nair, Biochem. Syst. Ecol. 37 (2009) 52–54 (and references cited therein);
(b) H. Itokawa, M. Morita, S. Mihashi, Chem. Pharm. Bull. 28 (1980) 3452–3454.
[5] (a) H. Itokawa, H. Morita, I. Katou, K. Takeya, A.J. Cavalheiro, R.C.B. de Oliveira, M. Ishige, M. Motidome, Planta. Med. 54 (1988) 311–315;
(b) N. Nakatani, H. Kikuzaki, H. Yamaji, K. Yoshio, C. Kitora, K. Okada, W. G. Padolina, Phytochemistry 37 (1994) 1383–1388;
(c) S. Nakamura, Y. Okazaki, K. Ninomiya, T. Morikawa, H. Matsuda, M. Yoshikawa, Chem. Pharm. Bull. 56 (2008) 1704–1709;
(d) N. Chimnoi, S. Pisutjaroenpong, L. Ngiwsara, D. Dechtrirut, D. Chokchaichamnankit, N. Khunnawutmanotham, C. Mahidol, S. Techasakul, Nat. Prod. Res. 22 (2008) 1249–1256.
[6] (a) K. Firman, T. Kinoshita, A. Itai, U. Sankawa, Phytochemistry 27 (1988) 3887–3891;
(b) G.N. Roth, A. Chandra, M.G. Nair, J. Nat. Prod. 61 (1998) 542–545;
(c) F. Abas, N.H. Lajis, K. Shaari, D.A. Israf, J. Stanslas, U.K. Yusuf, S.M. Raof, J. Nat. Prod. 68 (2005) 1090–1093.
[7] (a) S.F. Kimbu, B. Ngadjui, L.B. Sondengam, T.K. Njimi, J.D. Connolly, C. O. Fakunle, J. Nat. Prod. 50 (1987) 230–231;
(b) M. Marlier, G. Le Guellec, G. Lognay, J.-P. Wathelet, M. Severin, Planta. Med. 59 (1993) 455–457;
(c) P.M. Abreu, R.G. Noronha, Flavour Fragr. J. 12 (1997) 79–83;
(d) G. Duker-Eshun, J.W. Jaroszewski, W.A. Asomaning, F. Oppong-Boachie, C. E. Olsen, S.B. Christensen, Planta. Med. 68 (2002) 642–644;
(e) S.J.N. Tatsimo, P. Tane, J. Melissa, B.L. Sondengam, C.O. Okunji, B. M. Schuster, M.M. Iwu, I.A. Khan, Planta. Med. 72 (2006) 132–135.
[8] H. Morita, H. Itokawa, Planta. Med. 54 (1988) 117–120.
[9] T. Morikawa, H. Matsuda, I. Toguchida, K. Ueda, M. Yoshikawa, J. Nat. Prod. 65 (2002) 1468–1474.
[10] M. Abe, Y. Ozawa, Y. Uda, Y. Morimitsu, Y. Nakamura, T. Osawa, Biosci. Biotechnol. Biochem. 70 (2006) 2494–2500.
[11] C.O. Okunji, M.M. Iwu, J.E. Jackson, J.D. Tally, Jr., C. Bacchi, J.F. Ayafor, PCT Int. Appl. WO 2000024411 A2 20000504 (2000).
[12] X. Yang, Z. Jia, R. Li, J. Jordan, PCT Int. Appl. WO 2008138162 A1 20081120 (2008).
[13] H. Itokawa, H. Morita, JP Patent 63162644 A (1988).
[14] (a) M. Jung, S. Lee, B. Yoon, Tetrahedron Lett. 38 (1997) 2871–2874;
(b) M. Jung, I. Ko, S. Lee, S.J. Choi, B.H. Youn, S.K. Kim, Bioorg. Med. Chem. Lett. 8 (1998) 3295–3298.
[15] M. Nozawa, E. Ono, H. Akita, Heterocycles 53 (2000) 1811–1819.
[16] J. Aslaoui, H. Li, C. Morin, Tetrahedron Lett. 46 (2005) 1713–1716.
[17] (a) M.A. González, D. Romero, B. Zapata, L. Betancur-Galvis, Lett. Org. Chem. 6 (2009) 289–292;
(b) M.A. González, D. Pérez-Guaita, J. Correa-Royero, B. Zapata, L. Agudelo, A. Mesa-Arango, L. Betancur-Galvis, Eur. J. Med. Chem. 45 (2010) 811–816.
[18] Known compounds **1**–**3**, **5**, and **6** are identical by comparison of specific rotation and spectral properties. Synthetic precursors **4**, **7**, and reagent **8** also gave satisfactory physical and spectral data.
[19] (a) M.C. De la Torre, I. García, M.A. Sierra, Tetrahedron Lett. 43 (2002) 6351–6353;
(b) J. Boukouvalas, J.X. Wang, O. Marion, B. Ndzi, J. Org. Chem. 71 (2006) 6670–6673.
[20] R.G. Harvey, Tetrahedron 22 (1966) 2561–2573.
[21] D.B. Dess, J.C. Martin, J. Am. Chem. Soc. 113 (1991) 7277–7287.
[22] M. Nucci, E. Anaissie, Clin. Microbiol. Rev. 20 (2007) 695–704.
[23] J. Brasch, U.C. Hipler, Clinical aspects of dermatophyte infections, , In: Human and Animal Relationships, vol. 6. Springer, Berlin, 2008, pp. 263–286.
[24] A.J. Vlietinck, L. Van Hoof, J. Totté, A. Lasure, D. Vanden Berghe, P.C. Rwangabo, J. Mvukiyumwami, J. Ethnopharmacol. 46 (1995) 31–47.
[25] National Committee for Clinical Laboratory Standards Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. M38-A. Approved Standard. National Committee for Clinical Laboratory Standards, Wayne, PA, 2002.
[26] M. Cuenca-Estrella, C.B. Moore, F. Barchiesi, E. Chryssanthou, D.W. Denning, J. P. Donnelly, F. Dromer, B. Dupont, J.H. Rex, M.D. Richardson, B. Sancak, P. E. Verweij, J.L. Rodríguez-Tudela, Clin. Microbiol. Infect. 9 (2003) 467–474.
[27] M. Arnó, L. Betancur-Galvis, M.A. González, J. Sierra, R.J. Zaragozá, Bioorg. Med. Chem. 11 (2003) 3171–3177.
[28] L. Betancur-Galvis, C. Zuluaga, M. Arnó, M.A. González, R.J. Zaragozá, J. Nat. Prod. 64 (2001) 1318–1321.