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Short communication

Synthesis and antiviral activity of scopadulane-rearranged diterpenes

Miguel A. González[a,*], Lee Agudelo[b], Liliana Betancur-Galvis[b]

[a] Departamento de Química Orgánica, Universidad de Valencia, Dr Moliner 50, E-46100 Burjassot, Valencia, Spain
[b] Group of Investigative Dermatology, Department of Internal Medicine, Universidad de Antioquia, Medellín, Colombia

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ABSTRACT

A new bioactive diterpene skeleton resulting from a backbone rearrangement is described. Activity of the rearranged product and several derivatives against *Herpes Virus Simplex type* 2 is reported.

Scoparia dulcis L. (Scrophulariaceae) is a perennial herb, which grows in tropical and subtropical regions. The roots and leaves of the plant are used as a cure of toothache, blennorrhagia, stomach disorders and diabetes. Several groups of researchers studied the plant and reported the isolation of bioactive diterpenes (Hayashi et al., 1987, 1991, 1993; Ahmed and Jakupovic, 1990). The tetracyclic diterpenoids scopadulcic acids A (**1**) and B (**2**), and dulcinol/scopadulciol (**3**) (Fig. 1) were isolated as the first members of a unique carbon skeleton of diterpenoids which was named "scopadulane" by Hayashi et al. (1987). In recent years, further efforts resulted in the isolation of four new natural metabolites, 4-epi-scopadulcic acid B (**4**), dulcidiol (**5**), iso-dulcinol (**6**) and scopadulcic acid C (**7**) (Fig. 1) (Ahsan et al., 2003; Giang et al., 2006).

During the past decade, Hayashi (2000) proved that these natural products, and some derivatives, exhibit a broad pattern of biological activities, including antiviral, antitumor, inhibition of gastric acid secretion and bone resorption as well as potentiation of known antiviral drugs.

The systematic study of phytochemicals from plants of the genus *Calceolaria* (Scrophulariaceae) has resulted in the isolation of many new diterpenes, including thyrsiflorane-type diterpenes having a carbon framework identical to that of scopadulcic acids. For example, thyrsiflorin A (**8**), thyrsiflorin B (**9**) and thyrsiflorin C (**10**) (Fig. 1) were identified as constituents of *Calceolaria thyrsiflora* (Chamy et al., 1991).

The unique carbon skeleton and interesting biological activities of scopadulcic acids together with the absence of reports on the biological activity of the thyrsiflorins prompted us to carry out the synthesis and biological evaluation of several thyrsiflorins (Arnó et al., 2000; Betancur-Galvis et al., 2001) as well as several scopadulcic acid analogues (Arnó et al., 2003). Several of these compounds showed moderate in vitro antiviral activity against herpes simplex virus type 2.

During our synthetic studies towards the preparation of thyrsiflorin C (**10**) (Arnó et al., 2000), we obtained an unexpected product resulting from a p-toluenesulfonic acid-catalyzed rearrangement. This product was tentatively assigned the chemical structure **11** (Fig. 2) on the basis of NMR data. The new compound has been named as "scopadurosane" as it is the result of mixing the scopadulane and the rosane (Connolly and Hill, 1991) carbon skeletons.

In this paper, we report the optimized synthesis of the molecule **11** and several derivatives in order to carry out a biological study of this new carbon framework.

1. Chemistry

Compound **11** was obtained during our studies for the synthesis of thyrsiflorin C (**10**) (Scheme 1) (Arnó et al., 2000). Our attempts to optimize the isomerization of the double bond in enone **12** to give ketal **13** led us to increase the amount of p-toluenesulfonic acid (PTSA). With a PTSA concentration of 0.04 M in refluxing benzene the reaction followed a different reaction pathway leading to the formation of a new product **14** which was identified, after deketalization, as the rearranged ketone **11**. We observed that this new

* Corresponding author. Tel.: +34 96 354 3880; fax: +34 96 354 4328.
E-mail address: miguel.a.gonzalez@uv.es (M.A. González).

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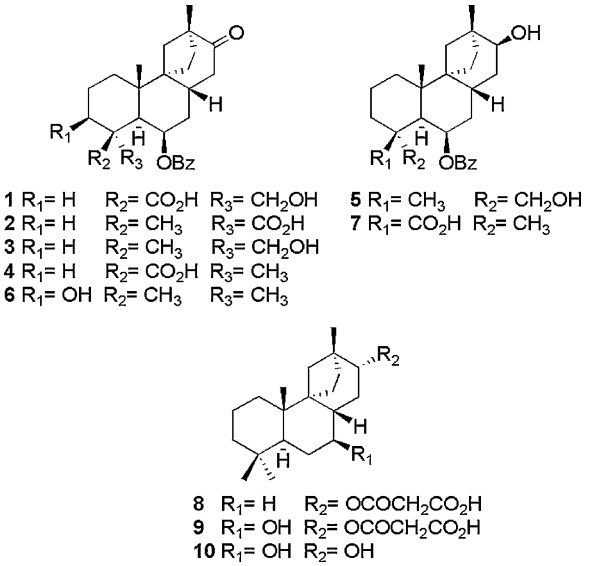


Fig. 1. Chemical structures of **1**–**10**.

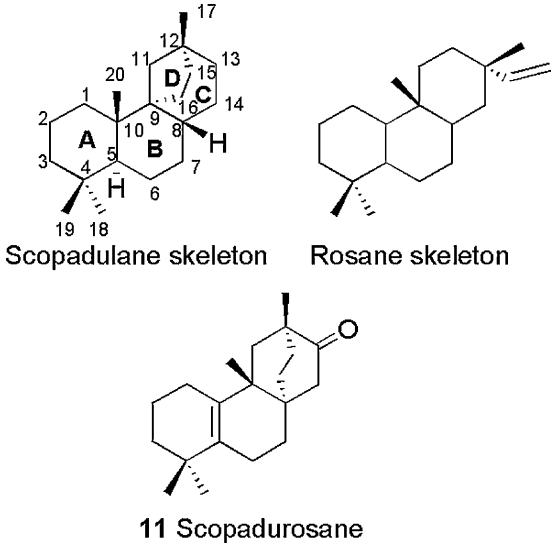
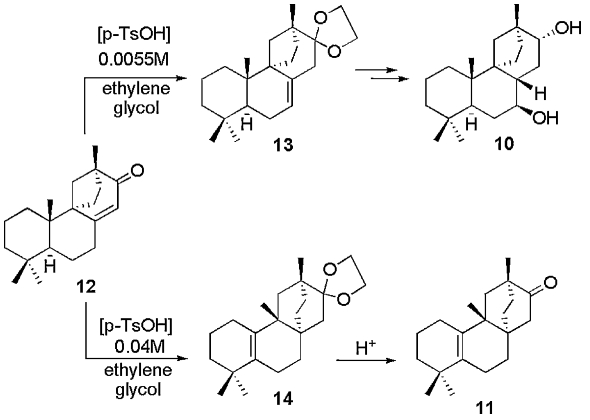
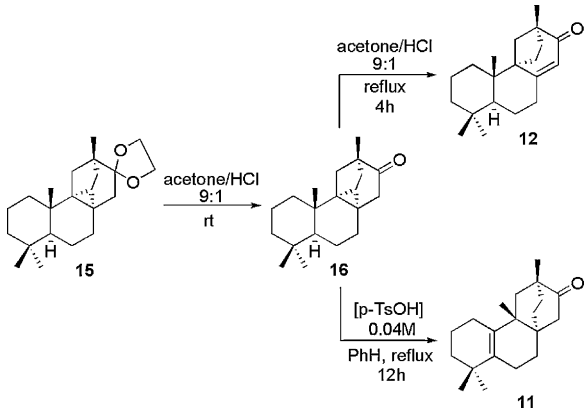


Fig. 2. Chemical structure of **11**.



Scheme 1. Unexpected synthesis of scopadurosane **11**.



Scheme 2. Synthesis of scopadurosane **11** from cyclopropane **16**.

ketone **11** could be obtained directly from enone **12**, using the same acid concentration, without the presence of ethylene glycol.

Scopadurosane **11** was initially obtained as colorless oil but after several crystallizations attempts the compound crystallized in MeOH–H_2O at −30 °C giving white crystals (m.p. 87–89 °C). The molecular formula $C_{20}H_{30}O$ was established by HRMS ($[M]^+$, m/z 286.2299, calcd 286.2297). The IR spectrum showed absorption peaks at 2924 and 1721 cm^{-1} (carbonyl group). The ^1H and ^{13}C NMR signals of **11** were assigned by different 2D NMR, NOE difference experiments and comparison with reported data (González and Zaragozá, 2005). The ^1H NMR spectrum indicated the presence of four tertiary methyl groups (δ 0.99, 0.98, 0.94, 0.89), two of which are geminal, and one methylene group (δ 2.21, dd, J = 18.3, 3.0 and δ 2.00, d, J = 18.3) next to a carbonyl group (C-13). The ^{13}C NMR and DEPT spectra indicated the presence of 20 carbons which were assigned to seven quaternary carbons, of which one is the carbonyl group and two are olefinic (tetrasubstituted olefin), nine methylene carbons and the absence of methine carbons. NOE correlations in the NOE difference spectrum of **11** irradiating the methyl group at C-12 established the β-axial orientation of the methyl group at C-9.

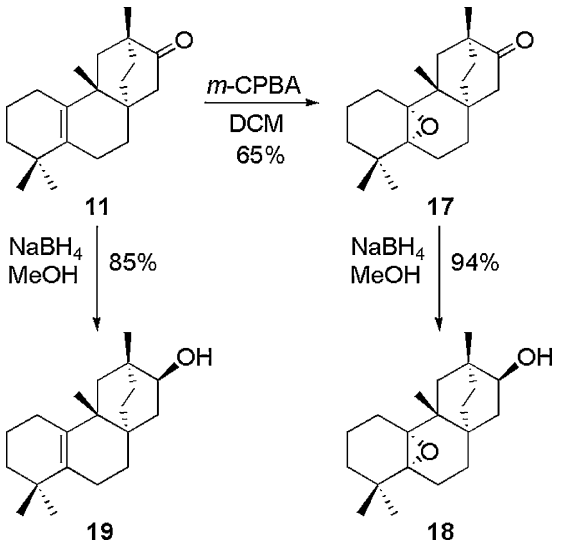
Now, we have also prepared rearranged ketone **11** from cyclopropane **16** (Scheme 2). We have also confirmed that other acidic conditions as refluxing 9:1 acetone/HCl and long reaction time leads to the same result, thus, confirming that is the thermodynamically more stable product.

2. Antiviral evaluation

With the rearranged ketone **11** in hand, we have synthesized three derivatives for their biological evaluation (Scheme 3). Thus, we firstly did the epoxidation of the double bond present in **11** to give compound **17**, which was subsequently reduced to afford alcohol **18**. Scopadurosane **11** was also directly reduced to alcohol **19**.

The antiviral activities of scopadurosanes **11**, and **17**–**19** were evaluated against a common sexually transmitted pathogen, herpes simplex virus type 2 (HSV-2). These compounds were investigated at different concentrations depending on cytotoxicity. The antiviral and cytotoxic activities of the compounds and the positive control acyclovir against HSV-2 were determined initially using a modified end-point titration technique (EPPT) (Vlietinck et al., 1995) and are shown in Table 1.

Most of the compounds reduced moderately the virus replication, without significant cytotoxicity, except for the parent structure **11** which showed no activity. The examination of this preliminary assay indicates that the presence of an hydroxyl group at C-13 provides this skeleton with antiviral activity. More accurate values of anti-HSV-2 activity for compounds **17**–**19** were obtained



Scheme 3. Synthesis of scopadurosanes **17**–**19**.

by the standard plaque reduction assay (Table 1). As a result, the new derivative **19** displayed the highest inhibitory value improving those previously reported by us for other scopadulane-type diterpenes (Betancur-Galvis et al., 2001; Arnó et al., 2003).

In summary, as we described earlier (Betancur-Galvis et al., 2001; Arnó et al., 2003), these results confirm the importance of polar groups at C-13 in scopadulane-based skeletons for anti-HSV-2 activity. The antiviral activity found in some new scopadurosanes improves the activities reported by us for scopadulane-type diterpenes; however, their effect on other viruses as well as the mechanism of action remains unknown, and needs further investigation.

3. Experimental

3.1. General experimental details

General details as reported previously (Arnó et al., 2003). All compounds prepared in this work exhibit spectroscopic data in agreement with the proposed structures.

3.2. Preparation and data for compounds *11*, *16* and *17*–*19*

3.2.1. Compound *11*

A solution of ketal **15** (Arnó et al., 2000) (200 mg, 0.606 mmol) in a 9:1 mixture of acetone/concentrated HCl was stirred at rt for 30 min. Then, it was diluted with diethyl ether, washed with 10% sodium bicarbonate, brine and concentrated. The residue was chromatographed on silica eluting with 9:1 hexane–ethyl acetate to give 168 mg (96%) of known cyclopropane **16** as white solid. The spectroscopic data were in agreement with the reported data (Arnó et al., 2000).

A solution of cyclopropane **16** (100 mg, 0.35 mmol) and p-toluenesulfonic acid (200 mg, 1.05 mmol) in benzene (26 mL) was heated at reflux for 12 h. Then, it was diluted with diethyl ether, washed with 10% sodium bicarbonate, brine and concentrated. The residue was chromatographed on silica eluting with 95:5 hexane–ethyl acetate to give 70 mg of ketone **11** as colorless oil. The spectroscopic data were in agreement with the reported data (Arnó et al., 2000).

3.2.2. Compound *17*

To a stirred solution of rearranged ketone **11** (10.4 mg, 0.036 mmol) in DCM (0.5 mL) at 0 °C, *m*-CPBA (85%; 15 mg, 0.087 mmol) was added in one portion. After being stirred for 3 h, the reaction mixture was diluted with diethyl ether. The organic phase was washed with saturated aqueous Na_2SO_4, 10% $NaHCO_3$ and brine. Workup as usual of the resulting organic extract gave a white, glassy solid which was purified by flash chromatography with 9:1 hexane–ethyl acetate to afford pure epoxide **17** (7.0 mg, 65%) as a colorless oil which solidified upon standing: $[\alpha]_D^{20}$ – 122.9 (c 0.7); IR (NaCl) 2932, 2865, 1721 cm^{-1}; ^1H NMR (300 MHz) δ 2.14 (1H, dd, J = 18.3, 2.8, H-14β), 1.06, 1.00, 0.98 and 0.86 (3H each, each s, H-17, H-18, H-19 and H-20); ^{13}C NMR (75 MHz) δ_C 217.65 (s), 69.65 (s), 68.09 (s), 46.22 (t), 45.81 (t), 43.87 (s), 38.37 (t), 38.29 (s), 35.79 (s), 33.09 (s), 31.12 (t), 27.57 (q), 27.45 (t), 26.30 (t), 26.14 (t), 24.51 (q), 21.13 (t), 20.12 (q), 19.91 (q), 17.23 (t); MS (EI) m/z 302 (M^+, 30), 287 (50), 233 (100); HRMS $C_{20}H_{30}O_2$ requires 302.2246, found 302.2256.

3.2.3. Compound *18*

To a solution of rearranged ketone-epoxide **17** (4.2 mg, 0.014 mmol) in MeOH (0.55 mL) at 0 °C, $NaBH_4$ (6 mg, 0.158 mmol) was added in one portion. After being stirred for 40 min, the reaction mixture was diluted with diethyl ether. The organic phase was washed with water and brine, dried (Na_2SO_4) and concentrated. The obtained residue was chromatographed with 8:2 hexane–ethyl acetate to give epoxi-alcohol **18** (4.0 mg, 94%) as a colorless oil: $[\alpha]_D^{22}$ – 97.5 (c 0.8); IR (NaCl) 3600–3400, 2933, 2860, 1453 cm^{-1}; ^1H NMR (300 MHz) δ 3.54 (1H, d, J = 9.0, H-13α), 1.19, 1.01, 0.98 and 0.87 (3H each, each s, H-17, H-18, H-19 and H-20); ^{13}C NMR (75 MHz) δ_C 73.90 (d), 69.85 (s), 69.69 (s), 39.62 (t), 39.34 (t), 38.78 (t), 37.28 (s), 34.11 (s), 33.16 (s), 33.00 (s), 32.91 (t), 27.99 (t), 27.90 (q), 26.97 (t), 26.47 (t), 24.62 (q), 24.51 (q), 21.78 (t), 20.60 (q), 17.44 (t); MS (EI) m/z 304 (M^+, 12), 286 (67), 271 (80), 253 (68), 235 (100); HRMS $C_{20}H_{32}O_2$ requires 304.2402, found 304.2407.

3.2.4. Compound *19*

To a stirred solution of rearranged ketone **11** (7.0 mg, 0.024 mmol) in MeOH (0.9 mL) at 0 °C, $NaBH_4$ (10 mg, 0.264 mmol)

Table 1
Cytotoxicity and anti-HSV-2 activity of scopadurosane-type diterpenes on VERO[a] cells determined by the end-point titration technique (EPPT) and the plaque reduction assay.

Compound	CC_{100} (μg/mL)[b]	Antiviral activity (μg/mL)[c]	Antiviral activity (EC_{50}) (μg/mL)[d]
11[e]	35	N.A.	N.A.
17	>50	25	7.8 ± 0.5
18	>50	25	9.1 ± 0.3
19	50	25	2.5 ± 0.7
Acyclovir	>600	6.0	–

[a] VERO, *Cercopithecus aethiops* African green monkey kidney cell line ATCC CCL 81.
[b] Minimal toxic concentration that detached 100% of the cell monolayer.
[c] EPPT results obtained at 48 h, maximal non-toxic dose that showed the highest viral reduction factor. N.A., no activity.
[d] 50% effective concentration based on plaque reduction assay results obtained at 72 h.
[e] Data taken from Betancur-Galvis et al. (2001).

was added in one portion. After being stirred for 30 min, the reaction mixture was diluted with diethyl ether. The organic phase was washed with brine, dried (Na_2SO_4) and concentrated. The obtained residue was chromatographed with 98:2 hexane–ethyl acetate to afford pure alcohol **19** (6.0 mg, 85%) as a colorless oil: $[\alpha]_D^{25}$ – 96.0 (c 0.5); IR (NaCl) 3570–3260, 2945, 2910, 2861, 1457 cm^{-1}; ^1H NMR (300 MHz) δ 3.57 (1H, br d, J = 7.0, H-13α), 1.14, 0.97, 0.94 and 0.83 (3H each, each s, H-17, H-18, H-19 and H-20); ^{13}C NMR (75 MHz) δ_C 135.07 (s), 131.40 (s), 74.22 (d), 41.03 (t), 40.32 (t), 38.77 (t), 38.32 (s), 34.64 (s), 34.05 (s), 33.48 (s), 32.47 (t), 29.51 (t), 29.01 (q), 28.00 (q), 27.59 (t), 26.29 (t), 25.54 (q), 24.81 (q), 21.53 (t), 20.25 (t); MS (EI) m/z 288 (M^+, 16), 273 (50), 255 (100); HRMS $C_{20}H_{32}O$ requires 288.2453, found 288.2449.

3.3. Anti-HSV-2 assay

3.3.1. Cell culture and virus

The cell line was *Cercopithecus aethiops* African green monkey kidney cells (VERO cell line ATCC CCL-81). Cells were grown in MEM supplemented with 10% FBS, 100 units mL^{-1} of penicillin, 100 mg mL^{-1} of streptomycin, 2 mM L-glutamine, 0.07% $NaHCO_3$, 1% non-essential amino acids and vitamin solution. The cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere.

HSV-2 was obtained from the Center for Disease Control (Atlanta, GA). The virus stock was prepared from HSV-2-infected VERO cell cultures. The infected cultures were subjected to three cycles of freezing–thawing and centrifuged at 2000 rpm for 10 min. The supernatant was collected, titrated, and stored at −170 °C in 1-mL aliquots. To titrate the virus suspension, confluent monolayer VERO cells were grown in 96-well flat-bottomed plates, infected with 0.1 mL of serial 10-fold dilutions of the virus suspension in quadruplicate and incubated for 48 h.

All compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma) at 30 °C. Stock solutions of diterpenes were prepared in DMSO and frozen at −70 °C. The concentration of DMSO in biological assays was of 0.05%. Cell controls with DMSO at 0.05% were used.

3.3.2. End-point titration technique (EPTT)

The virus titer was 10^3 (the dilution of the virus required to obtained 50% lytic effect of the culture in each well in 100 μL of viral suspension, $CCID_{50/0.1\,mL}$) using the Spearman–Käber formula. The technique EPTT (Vlietinck et al., 1995), with few modifications was used. Confluent monolayer VERO cells were grown in 96-well flat-bottomed plates. Two-fold dilutions of the compounds in maintenance medium (MM), identical to growth medium except for FBS which was 3%, were added 1 h before viral infection. The treated cells were infected with 0.1 mL of 1 CCID50 of the previously titrated virus suspension and incubated again at 37 °C in humidified 5% CO_2 atmosphere for 48 h.

Controls consisted of cells with serial 10-fold dilutions (from 10 to 10^{-3} CCID50) of HSV-2 in the absence of the compounds, treated non-infected cells and untreated non-infected cells. The antiviral activity is expressed as the maximal non-toxic dose of the test compound needed to obtain maximum reduction of virus titer. The reduction in virus titer was determined as the reduction factor (Rf) of the virus titer, i.e. the ratio of the virus titer in the absence over virus titer in the presence of the compound. The technique of crystal violet was used to stain the plates. The plates were observed through an inverted microscope and the degree of inhibition was determined by comparison with the controls. The CC_{100} was defined as minimal toxic dose that detached 100% of the cell monolayer of VERO cells. Three assays were carried out in triplicate with at least five concentrations of compounds.

3.3.3. Plaque reduction assay

Confluent monolayer VERO cells were grown in 24-well flat-bottomed plates.

Two-fold dilutions of 500 μL of the compounds in MM were added 1 h before viral infection. The treated cells were inoculated with 100 μL of approximately 100 PFU (plaque forming units) of virus; after 1 h 400 μL of MM with 2% carboxymethylcellulose were added and the cells well incubated again at 37 °C in humidified 5% CO_2 atmosphere for 72 h. At least two assays were carried out in duplicate with four concentrations of compound and reproducible results were obtained. The 50% effective concentration (EC_{50}) was defined as the concentration that reduced by 50% plaque forming units. EC_{50} for each compound were obtained from dose–effect curves for linear regression methods and EC_{50} values are expressed as the mean ± S.E.M. of at least four quadruplicate dilutions.

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