Phytochemistry 71 (2010) 1395–1399

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Cytotoxic terpenoids from *Nardophyllum bryoides*

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ARTICLE INFO

Article history:
Received 13 November 2009
Received in revised form 23 February 2010
Available online 20 May 2010

Keywords:
Nardophyllum bryoides
Astereae
Terpenoid
Seco-chiliolidic acid
Seco-*ent*-halimane

ABSTRACT

The investigation of the ethanol extract of fresh aerial parts of the Patagonian shrub *Nardophyllum bryoides* collected in the province of Chubut, Argentina, yielded eleven terpenoids. These include: three seco-*ent*-halimane diterpenoids (**1**–**3**), two *ent*-halimanes (**4**–**5**) and six pentacyclic oleanane and ursane triterpenoids (**6**–**11**). Four of these compounds (**2**, **6**, **8** and **11**) are hitherto unknown, while two others (**1** and **4**) have been previously reported but only as synthetic products. Several of these compounds showed moderate cytotoxicity against a human pancreatic adenocarcinoma cell line while compounds **4** and **5** were active at micromolar concentrations. The main component, seco-chiliolidic acid (**1**), could be isolated from this extract in large amounts, turning *N. bryoides* into a sustainable source of this bioactive compound.

1. Introduction

Plant natural products from abundant species represent an attractive and sustainable source of starting materials for the preparation of new bioactive substances. In this context, we began to investigate the native flora of Argentina in search of readily accessible and abundant natural products which could serve as scaffolds for the preparation of novel structures. Among other local species, we examined a sample of *Nardophyllum bryoides* (Lam.) Cabrera collected in the province of Chubut. A preliminary chromatographic inspection of this extract indicated the presence of a major compound which could fit the abovementioned criteria. After identification of this compound, the rare seco-chiliolidic acid (**1**), and taking into account the lack of chemical information on the genus *Nardophyllum*, we undertook a complete study of the minor terpenoid components of *N. bryoides*.

The genus *Nardophyllum* is native to South America and is widely distributed in the Argentinean and Chilean Patagonia and the Andes (Jakupovic et al., 1986; Bonifacino, 2005). This is a small genus that comprises only six species and is placed in the *Chiliotrichum* group, tribe Astereae (Bonifacino, 2005). From this genus, only *Nardophyllum lanatum* had been previously investigated (Jakupovic et al., 1986; Zdero et al., 1990).

Herein, we report the isolation and structural elucidation of three seco-*ent*-halimane diterpenoids (**1**–**3**), two *ent*-halimanes (**4**–**5**) and six pentacyclic oleanane and ursane triterpenoids (**6**–**11**) from an ethanolic extract of the fresh aerial parts of *N. bryoides*. Four of these compounds (**2**, **6**, **8** and **11**) are new, while two others (**1** and **4**) have been previously reported but only as semi-synthetic products (Jakupovic et al., 1986; Harde and Bohlmann, 1988). Two of these compounds showed cytotoxicity against a human pancreatic adenocarcinoma cell line at micromolar concentrations. To the best of our knowledge, this is the first chemical report for this species, and the first report on the biological activity of seco-chiliolidic acid and derivatives. Furthermore, seco-chiliolidic acid could be isolated from this extract in large amounts, turning *N. bryoides* into a sustainable source of this bioactive compound which will be used as starting material for synthetic transformations.

2. Results and discussion

Fractionation of the ethanolic extract of fresh aerial parts of *N. bryoides* (see Section 4) led to the isolation of five diterpenoids of the *ent*-halimane and seco-*ent*-halimane families, and six pentacyclic triterpenoids belonging to the α and β-amyrin series (Fig. 1).

The ^{1}H NMR spectrum of compound **1**, the major secondary metabolite in the extract, displayed the typical signals of a monosubstituted furan ring at 6.41, 7.42 and 7.47 ppm (Table 1). The ^{13}C NMR spectroscopic data indicated the presence of a lactone

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doi:10.1016/j.phytochem.2010.04.019

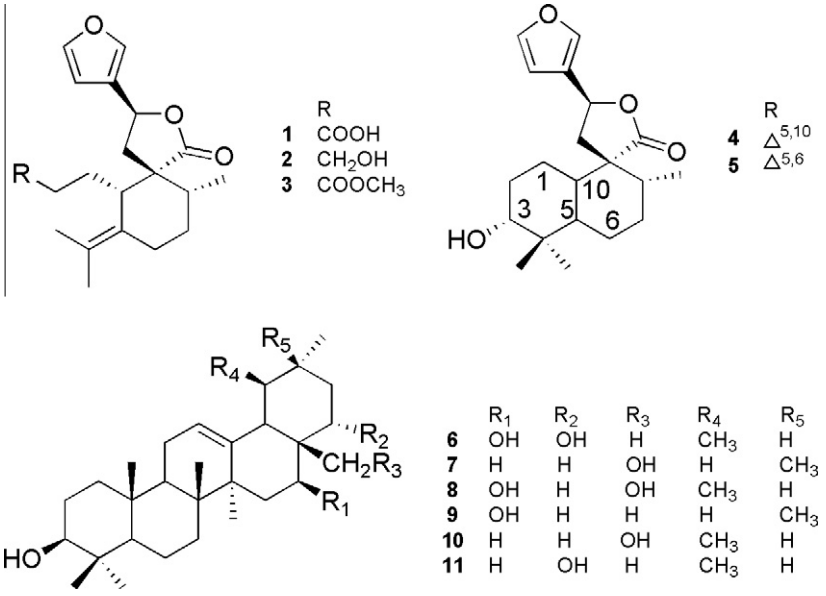

Fig. 1. Structure of compounds **1**–**11**.

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compounds **1**–**3** in $CDCl_3$, δ in ppm.

	1		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	26.0	2.12 *m* 2.04 *m*	27.2	1.82 *m* 1.74 *m*	26.1	2.12 *m* 2.02 *m*
2	32.7	2.22 *m*	32.0	1.50 *m* 1.38 *m*	32.6	2.23 *m* 2.16 *m*
3	179.6	–	63.1	3.61 *m*	173.8	–
4	128.1	–	129.1	–	128.6[a]	–
5	128.3	–	126.7	–	128.0[a]	–
6	21.6	2.43 *br t* (5.6) 2.17 *m*	21.2	2.44 *m* 2.17 *m*	21.4	2.44 *m* 2.17 *m*
7	29.6	1.72 *m* 1.51 *m*	29.8	1.69 *m* 1.51 *m*	29.6	1.70 *m* 1.51 *m*
8	36.4	1.72 *s*	36.3	1.74 *s*	36.3	1.71 *s*
9	52.1	–	52.2	–	52.1	–
10	41.6	2.92 *dd* (11.6, 3.1)	42.2	2.88 *dd* (11.0, 3.4)	41.5	2.92 *dd* (11.9, 3.0)
11	45.2	2.46 *dd* (13.0, 6.0) 2.02 *m*	45.1	2.50 *dd* (12.9, 6.0) 2.01 *dd* (12.9, 11.0)	45.1	2.45 *m* 2.00 *m*
12	70.7	5.37 *dd* (10.5, 6.0)	70.5	5.34 *dd* (10.3, 6.0)	70.4	5.37 *dd* (10.6, 5.9)
13	124.4	–	124.3	–	124.2	–
14	108.6	6.41 *dd* (1.7, 0.6)	108.6	6.40 *dd* (1.8, 0.9)	108.4	6.41 *dd* (1.8, 0.9)
15	144.0	7.42 *t* (1.7)	143.6	7.42 *t* (1.8)	143.4	7.42 *t* (1.7)
16	140.2	7.47 *br t* (0.6)	139.8	7.47 *br s*	139.6	7.47 *m*
17	16.0	1.33 *d* (6.7)	15.9	1.34 *d* (6.8)	15.8	1.34 *d* (6.7)
18	20.9	1.71 *d* (1.8)	20.8	1.73 *d* (2.0)	20.6[b]	1.69 *d* (2.0)
19	20.6	1.73 *d* (1.0)	20.6	1.74 *d* (1.0)	20.5[b]	1.73 *d* (1.2)
20	177.4	–	177.5	–	177.3	–
OMe	–	–	–	–	51.6	3.65 *s*

[a,b] Assignments may be interchanged.

carbonyl and two further oxygen bearing carbons. Additionally, the resonances of three methyl groups could be observed, one as a doublet and the other two corresponding to olefinic methyl groups at 1.71 and 1.73 ppm. A molecular formula $C_{20}H_{26}NaO_5$ obtained by HRMS (*m*/*z*: 369.1690 $[M + Na]^+$), indicated the presence of three rings in the structure, while a complete set of 2D NMR spectra established that **1** was a member of the 3,4-seco-*ent*-halimane family. Compound **1** was finally identified as seco-chiliolidic acid, previously reported by Jakupovic et al. (1986) from *Chiliotrichium rosmarinifolium* and *Nardophyllum lanatum*, but with incomplete NMR spectroscopic data. Full ^{1}H and ^{13}C NMR data obtained by detailed 2D experiments are listed in Table 1. The NMR spectra of **1**–**3** were quite similar. The difference between compounds **1** and **2** was the presence of a 2H-multiplet at 3.61 ppm in the ^{1}H NMR spectrum of **2**, suggesting the presence of a primary hydroxyl group instead of the carboxylic acid. This assumption was confirmed by the presence of an oxidized methylene carbon at δ 63.1 in the ^{13}C NMR spectrum of **2**. The molecular formula deduced from the NMR data and MS was in agreement with the proposed structure. Thus, compound **2** was identified as the previously unreported seco-chiliolide alcohol. On the other hand, compound **3** was readily identified by NMR and MS data as the methyl ester of seco-chiliolidic acid, which was previously obtained as a semi-synthetic derivative of **1** (Jakupovic et al., 1986), and is reported herein for

the first time as a natural product. A complete set of 2D NMR analyses allowed a detailed and complete assignment of all signals for this group of compounds.

Compounds **4** and **5** belonged to the *ent*-halimane class, and were identified as 3α-hydroxy-5,10-didehydrochiliolide and 3α-hydroxy-5,6-didehydrochiliolide, respectively. Compound **5** had been previously isolated from *Chiliotrichium rosmarinifolium* and *Nardophyllum lanatum*; however, compound **4** was obtained as an intermediate in the synthesis of 3α-hydroxy-5β,10β-epoxychiliolide and is reported here for the first time as a natural product (Jakupovic et al., 1986; Harde and Bohlmann, 1988).

Based on their NMR spectra, compounds **6**–**11** were clearly characterized as pentacyclic triterpenoids. On the basis of its HRMS compound **6** had the molecular formula $C_{30}H_{50}O_3$ (*m*/*z*: 481.3671 $[M + Na]^+$) which indicated six degrees of unsaturation. The ^1H NMR spectrum showed eight methyl signals, six as singlets at δ 0.79, 0.96, 1.00, 1.03, 1.06 and 1.14 and two as doublets at δ 0.76 (*J* = 6.3 Hz) and 1.00 (*J* = 6.3 Hz) (see Table 2). The presence of an olefinic proton was evident from the triplet at δ 5.20 (*J* = 3.6 Hz), while a double doublet at 3.22 (*J* = 11.2, 5.1 Hz) indicated the presence of a C-3 hydroxyl group. These facts strongly suggested the structure of a pentacyclic triterpene with an ursane skeleton. The ^{13}C NMR spectroscopic data showed typical signals for the A, B and C rings of an α-amyrin derivative, and the presence of two additional oxidized carbons at 68.5 and 81.8 ppm. Both of them showed HMBC correlations with a methyl singlet at δ 1.06 (assigned to C-28) indicating that the additional hydroxyls were probably located in the C and D rings (C-16 and C-22). The resonance at δC 68.5, δH: 4.55 (*dd J* = 11.2, 5.1 Hz) was assigned to C-16 due to COSY and HMBC correlations with the double doublet at 1.30 ppm (*J* = 13.0, 5.1 Hz, H-15). The assignment of H-15 in turn was confirmed by the HMBC correlations with the C-27 methyl; C-16 also displayed an HMBC correlation with a multiplet at δ 1.45 (H-18). The remaining signal at δC 81.8, δH: 3.52 (*dd J* = 12.2, 4.2 Hz) was attributed to C-22 due to the COSY and HMBC correlations with the protons at δ 1.65 and δ 1.77 (H-21), and the HMBC correlation with the signal at δ 1.45 (H-18). The relative stereochemistry at C-16 and C-22 was determined by the correlations observed in a phase-sensitive NOESY experiment. NOE correlations between H-16, H-27 and H-19, suggested α configurations for these protons, while correlations between H-22, H-18 and H-28, confirmed β configurations for these signals. Thus, compound **6** was identified as urs-12-ene-3β,16β,22α-triol, which, to the best of our knowledge, is a new compound.

The spectroscopic data for compound **7** were in agreement with those reported for the oleanane longispinogenin (Tori et al., 1976). Compounds **8** and **6** had the same molecular formula ($C_{30}H_{50}O_2$, *m*/*z*: 481.3671 $[M + Na]^+$). Although **8** had also an ursane skeleton, only one methyl doublet (Me-29) could be observed in the ^1H NMR spectrum, since the Me-30 doublet multiplicity was masked due to strong coupling to H-20 which had exactly the same chemical shift, a fact that was evident in the DEPT–HSQC spectrum. As

Table 2
1H and ^{13}C NMR spectral data of compounds **6**, **8** and **11** in $CDCl_3$ in ppm.

	6		**8**		**11**	
	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
1	39.0	1.67 *m* 1.02 *m*	38.7	1.65 *m* 1.01 *m*	39.2	1.65 *m* 1.01 *m*
2	27.5	1.62 *m*	27.3	1.62 *m*	27.5	1.62 *m*
3	79.3	3.22 *dd* (11.2, 5.1)	79.0	3.23 *dd* (11.3, 5.0)	79.2	3.23 *dd* (11.3, 4.8)
4	39.0	–	38.8	–	38.9	–
5	55.4	0.72 *m*	55.2	0.74 *m*	55.5	0.74 *dd* (11.9, 1.4)
6	18.5	1.57 *m* 1.42 *m*	18.2	1.57 *m* 1.43 *m*	18.6	1.56 *m* 1.41 *m*
7	33.0	1.57 *m* 1.39 *m*	32.7	1.58 *m* 1.41 *m*	32.9	1.56 *m* 1.36 *m*
8	40.3	–	46.5	-	40.5	–
9	47.1	1.49 *m*	46.9	1.49 *m*	47.7	1.53 *m*
10	37.0	–	36.8	-	37.1	–
11	23.7	1.95 *m* 1.93 *m*	23.3	1.91 *m*	23.6	1.93 *m* 1.53 *m*
12	126.1	5.20 *t* (3.6)	125.4	5.13 *t* (3.5)	125.4	5.14 *t* (3.6)
13	137.3	–	136.8	–	138.9	–
14	43.1	–	43.6	–	42.7	–
15	36.3	1.75 *m* 1.30 *dd* (13.0, 5.2)	36.4	1.94 *m* 1.50 *m*	26.3	1.75 *m* 1.04 *m*
16	68.5	4.55 *dd* (11.2, 5.1)	68.7	4.33 *dd* (11.8, 4.3)	21.0	1.72 *m* 1.34 *m*
17	42.1	–	41.3	–	39.3	–
18	60.4	1.45 *m*	56.2	1.36 *m*	58.8	1.33 *m*
19	39.4	1.38 *m*	39.6	1.31 *m*	39.4	1.37 *m*
20	38.1	1.1 *m* 0.99 *m*	39.5	0.96 *bs*	37.5	1.04 *m*
21	39.8	1.77 *t* (3.8) 1.65 *m*	29.8	1.55 *m* 1.26 *m*	39.3	1.64 *m* 1.38 *m*
22	81.8	3.52 *dd* (12.2, 4.2)	30.4	2.57 *m* 1.13 *m*	79.3	3.35 *dd* (11.3, 4.3)
23	28.3	1.00 *s*	28.0	1.01 *s*	28.5	1.00 *s*
24	15.8	0.79 *s*	15.5	0.79 s	15.9	0.80 *s*
25	15.9	0.96 s	15.6	0.95 *s*	16.0	0.96 *s*
26	17.0	1.03 *s*	16.6	1.05 *s*	17.1	1.02 *s*
27	25.0	1.14 *s*	24.4	1.15 *s*	23.6	1.09 *s*
28	19.0	1.06 *s*	71.4	3.11 *dd* (11.0, 0.7) 4.15 *d* (11.0)	24.8	0.97 *s*
29	17.9	0.76 *d* (6.3)	17.4	0.78 *d* (6.2)	17.7	0.79 *d* (6.2)
30	21.2	1.00 *d* (6.3)	21.2	0.96 *bs*	21.3	0.97 *d* (6.2)

Table 3
ID50 values (μM) for the *in vitro* screening against human solid tumor cells.

Cell line	1	2	3	4	5	6	11
Compound							
PANC1	20	25.1	31.6	2.5	0.3	63.1	44.7
LM3	28	20.0	14.1	3	40	25.1	0.8

for compound **6**, a signal at δH: 4.33 (*dd J* = 11.8, 4.3 Hz) clearly established the presence of a C-16 hydroxyl group, while a pair of resonances at δ 3.11 (*dd J* = 11.0, 0.7 Hz) and 4.15 (*d J* = 11.0 Hz) corresponding to a methylene, indicated the presence of an oxidized methyl carbon (δC 71.4). This was attributed to C-28 due to the HMBC correlations with carbons at 30.4 (C-22), 41.3 (C-17) and 68.7 (C-16). A NOESY correlation between H-16 and Me-27 confirmed a β stereochemistry for the C-16 hydroxyl. In this way, compound **8** was identified as the previously unreported urs-12-ene-3β,16β,28-triol.

The spectroscopic data for compounds **9** and **10** were in agreement with reported chemical shifts (Quijano et al., 1998; Siddiqui et al., 1986) for the oleanane maniladiol, and the ursane uvaol, respectively.

Compound **11** had also an ursane skeleton and a molecular formula $C_{30}H_{50}O_2$ (*m/z*: 465.3699 $[M + Na]^+$). The ^{13}C NMR spectrum showed two signals of oxidized carbons at δ 79.2 and 79.3 which were assigned to C-3 and C-22. In particular, H-22 (δ 3.35 *dd* (11.3, 4.3)) showed COSY correlations to both C-21 protons, as well as C-22 showed HMBC correlations with the same signals. On the other hand, a NOESY correlation between H-22 and Me-28 indicated that the hydroxyl group at C-22 was α. Thus, compound **11** was identified as urs-12-ene-3β,22α-diol, which is reported here for the first time.

2.1. Biological activity

The biological activity of seco-chiliolidic acid and chiliolide derivatives had not been previously investigated. The effect of compounds **1**–**6** and **11** on cell growth was assayed on log phase unsynchronized monolayers of two different cell lines: LM3 (murine lung adenocarcinoma cells) and PANC1 (human ductal pancreatic carcinoma). The observed ID50 values (μM, PANC1, LM3) are depicted in Table 3.

3. Conclusions

This work describes the terpenoid profile of *N. bryoides*, with a total of eleven isolated and completely characterized compounds. As a result of this work, four new compounds (a 3,4-seco-*ent*-halimane and three ursane triterpenoids) were identified and described. Two additional compounds were isolated for the first time as natural products, and the complete NMR spectroscopic data of seco-chiliolidic acid (**1**) were reported for the first time. It is interesting to note that 3,4-seco-*ent*-halimane compounds may be chemotaxonomic markers for the *Chiliotrichum* group, based on these and previous findings. However, a more thorough investigation on the chemistry of related species will be necessary to confirm this proposal. Interestingly, in a previous paper, Bohlmann proposed that compounds **1**, **4** and **5** may arise from a common precursor, 3α,5α-dihydroxychiliolide, which was isolated from *Chiliotrichium rosmarinifolium* and *Nardophyllum lanatum* (Jakupovic et al., 1986). However, this compound could not be detected among the minor components of this extract.

The preliminary cytotoxicity results indicated that compounds **4** and **5** were highly active against PANC 1 (pancreatic carcinoma) cell line. On the other hand, ursane tritrpenoid **11** was highly and selectively active towards the murine LM3 cell line. These promising results will have to be completed by a more extensive screening of other solid tumor cell lines as well as mechanistic studies. In the case of compound **1**, although the activity can only be considered as moderate, the results are still important, since **1** is an abundant component of this extract (80 mg of HPLC purified substance from 100 g of fresh plant material) which can be transformed into more bioactive derivatives. These results, together with the fact that *N. bryoides* is a sustainable biological resource, suggests that **1** can be used as a suitable scaffold for structural modifications which will further lead to a structure–activity study, and eventually to large-scale preparation of derivatives with increased bioactivity.

4. Experimental

4.1. General

Optical rotations were measured on a Perkin–Elmer 343 polarimeter, whereas ^1H and ^{13}C NMR spectra were acquired using a Bruker Avance-2 (500 MHz) and AC-200 (200 MHz) spectrometers, with $CDCl_3$ as solvent. Proton chemical shifts were referenced to the residual signal of $CHCl_3$ at δ 7.26 ppm, with ^{13}C NMR spectra referenced to the central peak of $CDCl_3$ at 77.0 ppm. Homonuclear ^1H connectivities were determined by COSY experiments. The edited reverse-detected single quantum heteronuclear correlation (DEPT–HSQC) experiment allowed determination of carbon multiplicities, as well as one-bond proton-carbon connectivities, and the heteronuclear multiple bond correlation (HMBC) experiment allowed the determination of long-range proton-carbon connectivities. The relative stereochemistry was determined by gradient-enhanced NOESY experiments. All 2D NMR experiments were performed using standard pulse sequences. HRESI mass spectra were recorded using a MicrOTOF QII Bruker mass spectrometer. Reversed-phase vacuum flash chromatography was carried out on octadecyl functionalized silica gel (Aldrich Chemical Co.). HPLC separations were performed using HPLC-grade solvents, a Thermo Separations Spectra Series P100 pump, a Thermo Separations Refractomonitor IV RI detector and a Thermo Separations SpectraSeries UV 100 UV detector, HPLC-grade solvents and an YMC RP-18 (5 μm, 20 mm × 250 mm) column. UV detection was performed at 220 nm. Sephadex LH-20 was obtained from Pharmacia Inc., TLC was carried out on Merck Sílicagel 60 F_{254} plates. TLC plates were sprayed with 2% vanillin in concentrated H_2SO_4. All other solvents were distilled prior to use.

4.2. Plant material

Specimens of *N. bryoides* were collected at Escalante Department, Province of Chubut, Argentina in February 2008 (summer). A voucher specimen (HRP6865) was identified by María Elena Arce (Universidad Nacional de la Patagonia San Juan Bosco, Argentina) and was stored at the Herbario Regional Patagónico, Universidad Nacional de la Patagonia San Juan Bosco.

4.3. Extraction and isolation

Ground aerial parts of fresh plant material (100 g) were extracted with EtOH (3 × 1l, 24 h each) at room temperature and evaporated at reduced pressure to yield a syrupy residue 7 g. The latter was partitioned between MeOH: H_2O (9:1) and cyclohexane, with the polar phase (5.8 g) subjected to vacuum flash chromatography on reversed phase-silica gel column (H_2O/MeOH gradient). The fraction eluted with MeOH: H_2O 7:3 (NOT-3, 2.1 g) was then permeated through a Sephadex LH-20 column (2 × 50 cm), using

MeOH as eluant. After TLC comparison, the fractions showing similar TLC patterns were pooled into eight groups (S1–S8). Fraction S5 (1.16 g) was purified by dry column flash chromatography on silica gel using a CH_2Cl_2/EtOAc gradient. The fraction eluted with CH_2Cl_2 (E1, 26 mg) was purified by preparative TLC with CH_2Cl_2: MeOH 95:5 as eluant to yield **3** (10 mg). Separation of E4 (120 mg, eluted with CH_2Cl_2: EtOAc, 6:4) by preparative HPLC with MeOH: H_2O (7:3) as eluant afforded compounds **1** (80 mg), **2** (1.6 mg), **4** (1.1 mg) and **5** (8.0 mg).

Separation of NOT-5 (eluted with MeOH: H_2O 9:1, 226 mg) by preparative HPLC with MeOH: H_2O (95:5) as eluant afforded **6** (2.7 mg) and two impure fractions: NAR-2 (15 mg) and NAR-4 (80 mg). NAR-2 was purified by preparative HPLC using CH_3CN as eluant to yield **7** (1 mg) and **8** (1.2 mg). The same conditions were applied to the separation of NAR-4, which afforded compounds **9** (17 mg), **10** (4 mg) and **11** (1 mg).

4.3.1. Seco-chiliolide alcohol **2**

Oil, $[\alpha]-71.7$ (c 0.06, $CHCl_3)_{25D}$; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESI–MS m/z $[M + Na]^+$ 355.1868 (calc. for $C_{20}H_{28}NaO_4$, 355.1879).

4.3.2. Urs-12-ene-3β,16β,22α-triol **6**

White amorphous powder, $[\alpha]+11.8$ (c 0.135, $CHCl_3)_{25D}$; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; ESI–MS m/z $[M + Na]^+$ 481.3671 (calc. for $C_{30}H_{50}NaO_3$, 481.3652).

4.3.3. Urs-12-ene-3β,16β,28-triol **8**

White amorphous powder; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; ESI–MS m/z $[M + Na]^+$ 481.3671 (calc. for $C_{30}H_{50}NaO_3$, 481.3652).

4.3.4. Urs-12-ene-3β,22α-diol **11**

White amorphous powder; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; ESI–MS m/z $[M + Na]^+$ 465.3699 (calc. for $C_{30}H_{50}NaO_2$, 465.3703).

4.4. Biological activity

The effect of the different compounds on cell growth was assayed on log phase unsynchronized monolayers of two different cell lines: LM3 (murine lung adenocarcinoma cells) (Urtreger et al., 1997) and PANC1 (human ductal pancreatic carcinoma).

Briefly, 2×10^3 cells/well from each cell line in complete medium were seeded in 96 multiwell plates. After 24 h, cells received 10 nM, 100 nM, 1 μM, 10 μM, 100 μM and 1 mM of compound or vehicle (0.1% DMSO) for 3 days. Cell viability was assessed by reduction of the tetrazolium salt MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] to the formazan product in viable cells (Cell Titer 96 TM, Promega Corp.) as calculated by the 492/620 nm absorbance ratio. The ID50 (Inhibitory Dosis) was calculated from the log-phase of each growth curve.

Acknowledgements

This research was supported by grants from CONICET (PEI 6478), UBA (X 260 Programación 2004–2007), UNPSJB (PI 734) and ANPCyT (PICT (2003) 14321). We thank Mr. José Gallardo and Lic. Gernot Eskuche (UMYMFOR–CONICET) for recording some of the NMR spectra. M. Sánchez and G. Siless thank CONICET for postdoctoral and doctoral fellowships respectively. M. Mazzuca thanks Bioq. M. Barría for helping in the collection and preparation of plant material.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.04.019.

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