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NONPOLAR CONSTITUENTS OF *Inula grandis* ROOTS. CYTOTOXIC ACTIVITY OF IGALAN

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Inula grandis Schrenk. (syn. *I. macrophylla* Kar. & Kir., Asteraceae) is broadly distributed in Central Asia [1].

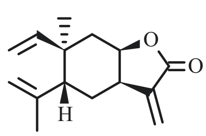
This species is used in folk medicine for tuberculosis, gastrointestinal-tract diseases, and brucellosis and as an anthelmintic [2].

Previously, roots of *I. grandis* growing in Central Asia were shown to produce carbohydrates and sesquiterpene lactones [2]. However, other constituents of the roots, including nonpolar compounds, were not investigated.

Therefore, roots of *I. grandis* that were collected on Chatkal Ridge, Tashkent Oblast (Uzbekistan), in the middle of October 2016 were studied. The constituent composition of the hexane fraction of the EtOH extract of *I. grandis* roots was determined using GC-MS analysis. Table 1 presents the percent contents of identified compounds.

The hexane fraction contained 15 identified compounds accounting for 97.3% of the constituents. Thirteen of the constituents listed in Table 1 were identified for the first time. Of these, thymyl isobutyrate was a monoterpene; kessane, a sesquiterpenoid; the other eleven, sesquiterpenes. The major constituents of roots were the sesquiterpene lactones igalan and alantolactone, which were isolated and described earlier [2]. Their presence in the hexane fraction of the EtOH extract of the roots confirmed that they were isolated pure by column chromatography.

The lactone igalan (**1**) has an elemane-type skeleton with four asymmetric C atoms at C5, C10, and the two C atoms at the fusion of the γ-lactone ring to C6 and C7 so that 16 stereoisomers are possible.



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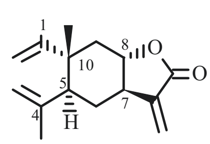

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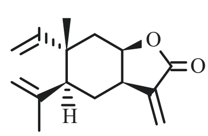

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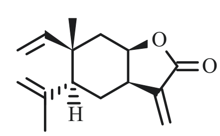

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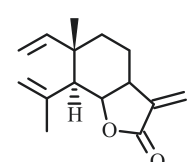

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Previously, PMR spectra of igalan taken on an INM-41H-100 spectrometer at operating frequency 100 MHz did not allow unambiguous assignment of all proton resonances [3]. Therefore, the stereochemistry of igalan was not established.

Later, an elemanolide that was called secoeudesmanolide (**2**, $C_{15}H_{20}O_3$) was isolated as a colorless oil from *Liatris cylindracea* [4]. Two stereoisomeric elemanolides **1** and **3** were isolated as a mixture from the plant *Spilanthes leiocarpa* [5]. Compound **3** unpurified from **2** was isolated from *L. platylepis* [6, 7]; elemanolides **1** and **4**, from *Stevia polyphylla*.

It was noted that the elemanolide igalan isolated earlier from *I. grandis* may have been the **2**–**3** stereoisomer. However, it was also noted there that existing data were inadequate to assign igalan to one of the aforementioned **2**–**3** stereoisomers.

The two stereoisomers **1** and **3** were obtained as a colorless oil from transformation of the germacrane lactone inunolide, which was isolated from *Rudbeckia laciniata* L. [8]. NMR spectral data established their configurations with stereoisomer **1** being attributed to igalan.

Recently, structure **5** was established based on IR, EI-MS, and PMR and ^{13}C NMR spectra of an elemanolide isolated from *L. spicata* [9] and was also assigned to igalan. However, the structure of lactone **5** corresponded to dehydrosaussurea lactone, which was obtained earlier from the germacranolide costunolide using thermal Cope rearrangement [10].

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TABLE 1. Constituents of the Hexane Fraction of the EtOH Extract of *Inula grandis* Roots

Constituent	RI	RT, min	%	Constituent	RI	RT, min	%
α-Longipinene	1358	11.906	0.03	Thymyl isobutyrate	1482	13.634	0.40
(–)-Modhephene	1388	12.324	0.07	β-Eudesmene	1490	13.738	0.26
β-Elemene	1397	12.441	0.26	α-Selinene	1498	13.849	0.19
Petaziten	1403	12.527	0.10	β-Bisabolene	1506	13.966	0.18
(±)-β-Isocomene	1415	12.699	0.05	Kessane	1534	14.365	0.86
β-Caryophyllene	1425	12.841	0.11	Igalan	1836	19.395	42.19
α-Bergamotene	1437	13.007	0.06	Alantolactone	1870	20.256	52.43
(+)-Epi-β-santalene	1450	13.179	0.11	Total			97.30

TABLE 2. Chemical Shifts in PMR and ^{13}C NMR Spectra of Igalan and HMBC Experimental Data (400 MHz, $CDCl_3$, δ, ppm, J/Hz)

C atom	δ_C	δ_H	HMBC (C)
1	147.47	5.71 (dd, J = 17.5, 10.8)	5, 9, 10, 14
2	111.16	4.89 (dd, J = 17.5, 0.9)	1
		4.93 (dd, J = 10.8, 0.9)	
3	113.14	4.68 (br.s)	4, 5, 15
		4.90 (m)	5, 10, 15
4	145.89	–	
5	46.22	1.99 (dd, J = 16.0, 3.6)	3, 4, 6, 14, 15, 10
6	26.69	1.95 (m)	10, 8
		2.17 (ddd, J = 13.6, 12.6, 6.2)	7, 5, 8, 4, 11
7	39.33	3.27 (br.s)	
8	76.00	4.79 (ddd, J = 10.7, 7.7, 5.9)	11, 12
9	42.93	1.29 (dd, J = 13.1, 10.7)	8, 10, 14, 1
		1.93 (dd, J = 13.1, 5.9)	14, 7, 5, 8, 5, 1
10	39.77	–	
11	137.45	–	
12	170.61	–	
13	120.49	5.55 (d, J = 3.5)	12, 7
		6.34 (d, J = 3.5)	11, 12, 7
14	16.57	1.04 (s)	5, 9, 10, 1
15	24.94	1.72 (s)	5, 3, 4

Lactone **1** isolated by us was confirmed to correspond to igalan using PMR and ^{13}C NMR spectral data and HMBC and HSQC experiments (Table 2).

An analysis of high-resolution NMR spectral data for igalan lactone isolated by us from *I. grandis* roots, for the elemanolide stereoisomers isolated from various plants, and for those prepared by chemical transformation [4–10] showed practically complete agreement with stereoisomer **1** reported before [8].

However, they differed in aggregate state according to the literature. Igalan was a crystalline compound; stereoisomer **1**, an oil [8].

Single crystals of **1** were grown from hexane to establish unambiguously the molecular structure from an X-ray crystal structure analysis.

Single crystals of **1** were transparent elongated prisms that crystallized in the monoclinic system, space group $P2_1$, $Z = 2$, $a = 6.491(2)$, $b = 15.560(2)$, $c = 7.282(2)$ Å, $\beta = 114.64(3)°$, $\rho_{calcd} = 1.159$ g/cm^3.

Figure 1 shows the molecular structure of the lactone igalan (**1**). It can be seen that the six-membered elemane ring was *cis*-fused to the lactone ring with the 7,8αH, C10α-Me, and C5β-H oriented axially and the C5α-isopropylene and C10β-ethylene, equatorially.

Thus, the X-ray crystal structure analysis established the molecular structure of igalan as (5*R*,10*S*)-elema-1,3,11(13)-trien-5β,7,8α-8,12-olide, which corresponded to stereoisomer **1**.

TABLE 3. Cytotoxic Activity of Lactone Igalan (Cell Growth Inhibition, %)

Sample	HeLa		HEp-2		Hepatocytes	
	10 μg/mL	1 μg/mL	10 μg/mL	1 μg/mL	10 μg/mL	1 μg/mL
Igalan	95.1 ± 4.1	49.0 ± 2.2	73.8 ± 2.5	8.5 ± 0.2	70.5 ± 3.4	10.8 ± 0.3
Control – cisplatin	74.1 ± 2.9	22.8 ± 1.1	51.6 ± 2.3	35.9 ± 0.8	59.8 ± 2.0	15.5 ± 0.1

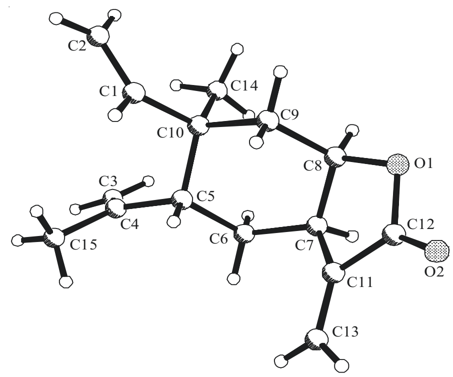

Fig. 1. Molecular structure of igalan (**1**).

Sesquiterpene lactones are known to posses pronounced antitumor activity. Several of them are used as antiblastoma preparations [11].

The cytotoxic activity of sesquiterpene lactone **5** from *L. spicata* against a human hepatic cancer cell line (HepG2) was reported as 3.83 ± 0.043 μg/mL [9]. The effect was comparable with that of the used antitumor drug doxorubicin (3.73 ± 0.036) μg/mL.

Therefore, the cytostatic activity of **1** was studied *in vitro* in HeLa cervical epithelial carcinoma, HEp-2 laryngeal adenocarcinoma, and normal (healthy) liver (hepatocyte) cell cultures. Table 3 presents the results.

Thus, the lactone igalan at a concentration of 10 μg/mL turned out to be highly active for laryngeal adenocarcinoma (73.8%), cervical epithelial carcinoma (95.1%), and liver cells (70.5% cell-growth inhibition). Under these same conditions, the antitumor drug cisplatin, which was used as a reference standard, had weaker cytotoxic activity than **1**.

Reducing the compound concentration by ten times had various effects on cell-growth inhibition. Igalan remained cytotoxic only for HeLa cells (49.0%) with weak action on healthy hepatocytes (10.8%). Cisplatin at a concentration of 1 μg/mL displayed nonselective activity and caused the death of an average of 15.5–35% of all cells. The IC_{50} value of **1** on cervical epithelial carcinoma was ~1 μg/mL.

Thus, the lactone igalan at low concentrations was highly cytotoxic for HeLa carcinoma culture and marginally toxic for healthy cells *in vitro*.

IR spectra were recorded on a PerkinElmer system 2000 FTIR spectrometer (KBr pellets). NMR spectra were taken in $CDCl_3$ with TMS internal standard (0 ppm) on a Varian MR-400 spectrometer at operating frequency 400 MHz.

TLC used Silufol UV 254 chromatography plates and glass plates with a thin layer of silica gel. Spots were detected by I_2 vapor and vanillin solution (1%) in conc. H_2SO_4. Melting points of isolated compounds were determined in glass capillaries on an Electrothermal MEL-TEMP® apparatus (Equipment, USA).

GC-MS Analysis. Milled *I. grandis* roots (100 g) were extracted (3×) with EtOH. The EtOH extract was condensed, mixed with silica gel in a 1:1 ratio, and dried. The dried solid was placed onto a column and eluted with hexane. The hexane extract was condensed and analyzed by GC-MS on an Agilent 7890A GC gas chromatograph with an Agilent 5975C inert MSD quadrupole mass spectrometer as the detector. Mixture constituents were separated over an HP-5ms quartz capillary column (30 m × 0.25 mm, 0.25 μm) using temperature program 50°C(2 min), 10°C/min to 200°C (6 min), 15°C/min to 290°C (15 min). The injected sample volume was 1 mL (hexane, benzene) at mobile-phase flow rate 1.3 mL/min. Constituents were identified by comparing mass spectral characteristics with data in W8N05ST.L and NIST08 electronic libraries and retention indices of compounds determined from the ratio to retention times of an *n*-alkane mixture.

Isolation of Sesquiterpene Lactones. Sesquiterpene lactone igalan and alantolactone were prepared by the literature method [3] except that the chromatography column was eluted by a mixture of extraction hydrocarbons and EtOAc (9:1). Eluates containing igalan were obtained first; then, a mixture of igalan and alantolactone; and then, alantolactone. Recrystallization of these isolated pure igalan (hexane) and alantolactone (EtOH).

Igalan (1). $C_{15}H_{20}O_3$, mp 79–80°C (hexane), yield 0.56%. IR spectrum (KBr, ν, cm^{-1}): 3501 (free OH), 3434 (bound OH), 3081, 2984, 2921, 2876, 2210, 1863, 1843, 1758 (C=O γ-lactone), 1660 (C=C stretching), 1640, 1445, 1405, 1378, 1324, 1299, 1265, 1235 (C=C bending), 1131, 1107, 1043, 988, 972, 931, 920, 891, 814, 735, 706, 665, 604, 587, 557, 519, 456, 430, 408.

Alantolactone, $C_{15}H_{20}O_2$, mp 75–76°C (EtOH) was identified by comparing IR spectra and the lack of melting-point depression of a mixed sample with an authentic sample; yield 0.68%.

X-ray Crystal Structure Analysis (XSA). Unit-cell constants of the crystals were determined and refined on a CCD Xcalibur Ruby diffractometer (Oxford Diffraction) using Cu Kα-radiation (293 K, graphite monochromator). A three-dimensional dataset of reflections was obtained on the same diffractometer ($\theta \le 75.87°$). Absorption corrections were introduced semi-empirically using the SADABS program [12]. The structure was solved by direct methods using the SHELXS-97 software and refined using the SHELXL-97 program [13]. All nonhydrogen atoms were refined using anisotropic full-matrix least-squares methods (over F^2). Positions of H atoms were found geometrically and refined isotropically with fixed thermal parameters $U_{iso} = nU_{eq}$, where n = 1.5 for methyls and 1.2 for others and U_{eq} is the equivalent isotropic thermal parameter of the corresponding C atom. The final agreement factors (R) for **1** were $R_1 = 0.925$ ($wR_2 = 0.1628$) for 356 reflections [$I > 2\sigma(I)$] and $R_1 = 0.2900$ ($wR_2 = 0.3025$) over all 1732 reflections. Experimental XSA data were deposited in the Cambridge Crystallographic Data Centre (CCDC No. 1891691).

Biological Activity. Passaged HeLa and HEp-2 cells were obtained from the Institute of Cytology, RAS (RF). Hepatocytes were cultured according to the literature [14]. These cell lines were highly sensitive to known antitumor drugs. The cytotoxicity test used concentrations of 1 and 10 μg of compound per 1 mL of growth medium. The reference standard was cisplatin preparation containing *cis*-diamminedichloroplatinum (II). Cells in culture medium were used as a negative control.

Cytotoxic activity was studied using three methods: the MTT assay, counting living cells using trypan blue, and morphology. For this, cells were inoculated into 96-well plates at a concentration of 20,000 cells/mL for passaged cell cultures and 50,000 cells/mL for hepatocytes. Compounds were added before incubation for 24 h at 37°C and 5% CO_2. Then, each well was treated with MTT solution (20 μL, thiazolyl blue tetrazolium bromide) at a concentration of 5 mg/mL (Sigma, USA). The plates were left for 4 h. The growth medium was decanted. The cells were treated with DMSO (50 μL, Sigma, USA). Absorbance at 620 nm was measured after 15 min on an EnSpire 2600 spectrophotometer (PerkinElmer, USA).

Cells were removed by versene solution for counting using trypan blue and were carefully pipetted. Then, a sample (50 μL) of the suspension was mixed with an equal volume of trypan blue solution and transferred to a Goryaev chamber for cell counting.

Morphological analysis used a LeicaDMIL microscope (USA) at 80× magnification.

Results were processed statistically and plotted using Excel software to determine the means and standard errors. Differences were considered statistically significant for $p \le 0.05$.

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