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Trilobolide and related sesquiterpene lactones from *Laser trilobum* possessing immunobiological properties

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ABSTRACT

Three new and five known sesquiterpene lactones were isolated from the roots of *Laser trilobum* (L.) Borkh. Chemical identity of the known compounds and structural analysis of the new ones were determined by HR MS and NMR spectroscopy. The two new sesquiterpene lactones: 2-acetoxytrilobolide and 2-hydroxy-10-deacetyltrilobolide belong to the guaianolide type, and the third one, eudeslaserolide, to the biogenetically related eudesmanolide type. Both types, together with their biogenetic precursor of germacranolide type (laserolide) are present in *L. trilobum*, as well as in the related *Laserpitium* species. Purposefully selected set of these native sesquiterpene lactones was tested for specific immunobiological properties. The obtained results demonstrate that trilobolide and its acetoxy analog are strong activators of cytokine secretion. On the contrary, the other *L. trilobum* and *Laserpitium siler* constituents are only very mild activators, or even inhibitors of the cytokine and nitric oxide production.

1. Introduction

Laser trilobum (L.) Borkh. became one of the preferred species for investigation during our previous systematic study [1–3] concerning chemotaxonomic significance of sesquiterpene lactones serving as phytochemical markers for detecting phylogenetic

in the roots of *L. trilobum*, allowed not only the full determination of its structure [2,3], but also testing and assessing its cytotoxic properties in human and animal malignant cells [6]. Later, it was investigated together with other sesquiterpene lactones also for its insect deterrent activity [7] expecting chemoecological implication [8], recently upgraded in [9].

Trilobolide (**1**) is the substantial and characteristic sesquiterpene lactone constituent of guaianolide type isolated from *L. trilobum* [1–4]. Its chemical structure is closely related to the

to rapid elevation of intracellular calcium [11–15]. It has already been shown that thapsigargin interferes also with production of nitric oxide (NO) [16]. Since the biosynthesis of NO is under tight control of cytokines, we extended our investigation to the possible effects of thapsigargin (**3**) and its closely related structural analog trilobolide (**1**) on secretion of interferon-γ (IFN-γ) and production of NO [17].

Close structural relation between the trilobolide (**1**) and thapsigargin (**3**), as it is shown in Fig. 1, together with the taxonomic relation between their plant sources *L. trilobum* (L.) Borkh. and *T. garganica* L. (both belonging to the family Apiaceae) indicate also remarkable similarity in their biological

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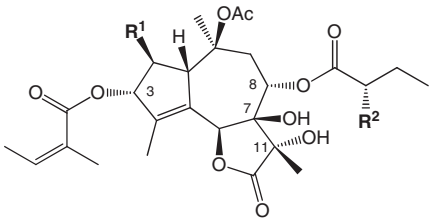
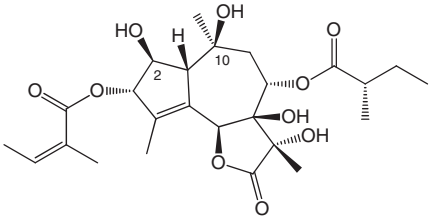


1. trilobolide: **R**^{1} = H; **R**^{2} = CH_3
2. 2-acetoxytrilobolide: **R**^{1} = $OCOCH_3$; **R**^{2} = CH_3
3. thapsigargin: **R**^{1} = O-octanoyl; **R**^{2} = H



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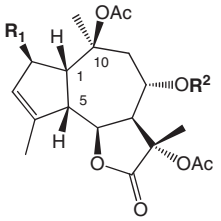
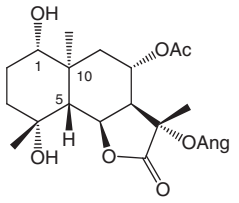
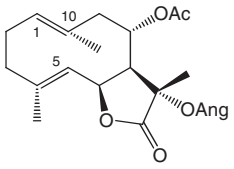


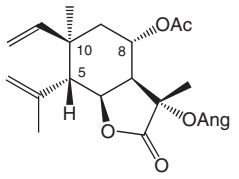
5. archangelolide: **R**^{1} = Ang; **R**^{2} = Mebut
6. 2-deangeloylarchangelolide: **R**^{1} = H; **R**^{2} = Mebut
7. acetylisomontanolide: **R**^{1} = H; **R**^{2} =Ang



8. eudeslaserolide



9. laserolide



10. isolaserolide

Note: **Ac** = acetyl, **Ang** = angelyl (see substituent at C-3 of trilobolide (**1**)) and **Mebut** = methylbutyryl (see subst. at C-8 of **1** or **4**)

Fig. 1. Chemical structures of trilobolide (**1**) and related constituents of *L. trilobum* compared with the thapsigargin (**4**).

activity and pharmacological properties [16]. Moreover, *T. garganica* represents important medicinal plant utilized for medical treatment since ancient times, and thus became a matter of intensive phytochemical and pharmacological study [11,18]. In contrary, *L. trilobum* (syn. *Siler trilobum*) is a common perennial herbaceous mountain plant (known as gladich), although, with fruits occasionally used (e.g. in Turkey) as a spice or condiment [19] with significant antimicrobial effect on a series of pathogen bacteria [20] endangering food- or feed-stuffs. Its German name Rosskümmel might indicate a similar sense. Nevertheless, it remained less attractive for a proper phytotherapeutic study.

Our recent interest in trilobolide (**1**) and structurally related sesquiterpene lactones has been reopened and encouraged by discovering their remarkable immunobiological properties [16–18]. The significant immunostimulatory activity of trilobolide (**1**) led us to submit appropriate patent application [21]. The invention relates to the use of trilobolide (**1**) for stimulation of the immune system, particularly for stimulation of the IFN-γ secretion, and to the possible use of this activity in therapy. It relates also to methods of the trilobolide (**1**) separation from roots, or even more reasonably, from seeds of gladich (*L. trilobum*), and alternatively also from two additional plant sources, laserworts: (*Laserpitium siler* L.) or (*Laserpitium archangelica* Wulf. in Jacq.) [22].

Plant sources of various sesquiterpene lactones are common in nature [23] and have long ago been studied in our laboratory as plant representatives of the family Apiaceae (Umbelliferae) [1–5,22]. For the present pharmacology study *L. trilobum* was selected as a source for its high content of trilobolide (**1**), and for containing a whole range of other biogenetically and thus also structurally related minor sesquiterpene lactones [3,4]. Originally, these substances were isolated from the roots of plants growing wild in their

natural localities. Collecting roots, however, appeared for this perennial plant rather destructive. Moreover, almost all localities belong at present to protected natural territories. This led us to resolve the situation by the plant cultivation in suitable field conditions, aiming to raise sufficient production of *L. trilobum* for its harvesting, processing and suggested use [21]. We verified that *L. trilobum* contains trilobolide (**1**) not only in roots, but also in other organs, particularly in seeds [21]. This allows collecting seeds (or whole umbels) from the umbelliformed mature inflorescence of the plant, avoiding in that way its unwanted destruction. We present in this paper, however, results obtained by processing our stored material (extracts, fractions, separated compounds) from the root supplies gained in our previous, at that time unrestricted collections [3]. The aim of this work is to show, that the Temperate Palearctic *L. trilobum* can serve in the phytotherapy analogously as the Mediterranean Palearctic *T. garganica*, known for its use in the traditional medicine.

2. Experimental section

2.1. General methods

Purity of compounds determined for biological assays were analyzed by reversed-phase HPLC as described in Section 2.7. Structural identity of compounds was determined by the following characteristics. Melting points were determined on the Koffler block (Boetius) without correction. Optical rotations were measured using the polarimeter Rudolph Research Analytical Autopol IV. Infrared spectra were recorded on Bruker IPS-88 instrument. NMR spectra were measured on Bruker AVANCE 600 instrument (^{1}H at 600.13 MHz; ^{13}C at 150.9 MHz) in CD_3OD or d6-DMSO at 300 K. Homonuclear 2D-H,H-COSY and 2D-H,H-ROESY spectra were used for structural determination of proton signals, and heteronuclear 2D-H,C-HSQC or 2D-H,C-HMBC spectra, in combination with ^{13}C APT spectra, were used for assigning all carbon signals. Chemical shifts were referred to the solvent signal using relations: δ(H) 3.31 and δ(C) 49.0 ppm in CD_3OD and δ(H) 2.50 and δ(C) 39.7 ppm in d6-DMSO. Mass spectra, including HR-MS, were recorded on LTQ Orbitrap XL (Thermo Fisher Scientific) spectrometer.

2.2. Chemicals

The majority of sesquiterpene lactones, i.e., **1**, **5**–**7**, **9** and **10** were obtained in our earlier investigation [3]. They were purified (see Section 2.7) from our original stored samples and identified by MS and NMR spectroscopy. Thapsigargin (**3**) was purchased from Sigma-Aldrich Company.

2.3. Plant material

Roots of *L. trilobum* were collected in August 1982 in the Malá Farta mountain region, close to the village Timorádza, Czechoslovakia (presently Slovak Republic), identified by botanist Dr. Jan Toman [5]. A voucher specimen is deposited in our Institute in Prague. This stored material was compared with the presently produced plant material in the experimental station of SEVA-Flora s.r.o. in Valtice (Czech Republic) from seeds supplied by the Botanical garden of Masaryk University in Brno (Czech Republic). Voucher specimens and living plants are available in the concerned institutions. The presently used name: *Laser trilobum* (L.) Borkh., is according to the Euro + Med Plantbase of the Flora Europea known also under still unresolved synonyms: *Siler trilobum* (L.) Crantz, or *Laserpitium trilobum* L.

2.4. Animals and cells

Female Wistar rats weighing 175–185 g were purchased from Velaz (Prague, CR). They were kept in transparent plastic cages placed in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France) under controlled 12/12 h light/dark cycle (lights on 06.00 a.m.), temperature (22 ± 2 °C), and relative humidity (50 ± 10%) conditions. Standard pelleted diet and water were provided ad libitum. The animal welfare and all experimental procedures have been approved by the Institution Animal Ethics Committee (no. 13/2006).

Rats were sacrificed by cervical dislocation. The resident peritoneal cells were collected by a lavage using 16 ml of sterile saline. They were washed twice in sterile saline, and the number of live and dead cells was counted under a microscope using trypan blue. The suspension contained 96% of viable cells. The flow cytometry analysis showed that the cell population was composed of 56.5% ± 4.4% of macrophages (mean ± SEM), 12.0% ± 3.0% of granulocytes, 10.0% ± 2.9% of lymphocytes and 7.1% ± 1.7% of natural killer cells. The pooled peritoneal cells were finally re-suspended in complete culture medium RPMI-1640, containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma-Aldrich, St. Louis, MO, USA). They were seeded into 96-well round-bottom microplates (Costar, Cambridge, MA) in final 100-μl volumes, 2×10^6 cells/ml. The plates were maintained at 37 °C, 5% CO_2 in a humidified Heraeus incubator. All experimental variants were run in duplicate.

2.5. Biological assays

2.5.1. Nitric oxide assay

The cells were cultured 24 h in the presence of test compounds. The concentration of nitrites in supernatants of cells was taken as a measure of NO production [24]. It was detected in individual, cell-free samples (50 μl) incubated 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% (1-naphthyl)ethylenediamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to μM nitrite.

2.5.2. Cytokine assays

After an incubation period (24 h), supernatants of cells were analyzed for cytokine secretion. Concentrations of individual cytokines were determined by ELISA kits (R&D Systems, Abingdon, UK), following the manufacturer instructions.

2.5.3. Cell viability assay

Viability of cells was determined using a colorimetric assay based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche Diagnostics,

Mannheim, Germany). The cells were cultured as described above. After the 24-h culture, the WST-1 was added and the cells were kept in the Heraeus incubator at 37 °C for an additional 3 h. Optical density at 450/690 nm was evaluated. The percentage cytotoxicity of test samples was related to the control samples and to the samples with 100% dead cells evoked by 1% Triton, according to the formula: [(exp. value − control value) / (Triton value − control value)] × 100. All control and experimental variants were run in quadruplicate.

2.5.4. Lipopolysaccharide (LPS) contamination assay

The samples were sterile filtered using non-pyrogenic 0.22 μm filters (Costar). The Limulus Amebocyte Lysate assay (Kinetic-QCL; Cambrex Bio Science, Walkersville, MD) was used in order to exclude possible contamination with LPS. The samples were found to be devoid of any traces of LPS.

2.6. Statistical analysis

Analysis of variance (ANOVA) and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

2.7. Separation and purification

Compounds **1**, **5**–**7**, **9** and **10** were purified by flash chromatography on short silica gel columns using solvents: petroleum ether–ethyl acetate (2:1), as reported in [21]. All purified compounds were subsequently inspected by HPLC analysis (on reverse phase Separon SGX C-18) performed in a gradient mode with combining solvents (55–100% water in methanol), as reported in detail in [21]. Their chemical identity was inspected by MS and NMR spectrometry.

Trilobolide (**1**) was separated from stock extracts obtained originally during the investigation reported in reference [3], corroborating consequently the high chemical stability of the compound. Extract (60 g) containing trilobolide (**1**) was chromatographed on a column of silica gel (deactivated by 12% of water). Elution was performed with a mixture of petroleum ether and ethyl acetate (with a gradient from 1:0 to 1:1), yielding 20 fractions combined according to the content of identical substances (detected by TLC monitoring). From the trilobolide containing fractions, the first portion was isolated by crystallization (from MeOH–EtOAc), obtained in the purity of 98%. From the mother liquors, a next batch of trilobolide by repeated chromatography and crystallization was obtained. A sufficiently large amount of trilobolide (11 g) was prepared by repeating this procedure. The main portion of it was reserved for chemical transformations, intended for the following structure–activity relationship study [25].

The AcOEt soluble portion of the above mentioned stock extract contained according to HPLC analysis 10 major substances; among them the fifth one was trilobolide (**1**). This portion (3.8 g) was fractionated (in two repetitions) by preparative RP-HPLC, using column (26 × 600 mm) packed with Separon SGX C-18, performed in gradient mode with combination of solvents (55–100% water in methanol) for 360 min, at a flow rate of 5 ml/min. Besides trilobolide (**1**), three new sesquiterpene lactones were obtained: 2-acetoxytrilobolide (**2**, 190 mg), 2-hydroxy-10-deacetyltrilobolide (**4**, 12 mg) and eudeslaserolide (**8**, 60 mg). Chemical identity of the new separated compounds, compared with the trilobolide (**1**), is described below.

*2.7.1. Trilobolide (**1**)*

White crystals, m.p.190–192 °C; $[\alpha]_D^{20}$ −66.3 (c 0.74 in MeOH; the former data misquoted in [1,21] is revised). IR (KBr) [cm^{-1}]: 3455, 3480 (hydroxyls), 1785 (γ-lactone), 1725, 1250 (acetate), 1712 (ester conjugated with double bond), 1652 (double bond). TOF-MS ESI$^+$: m/z (%): 545 (100) $[M + Na]^+$, 485 (10), 463 (11), 385 (27), 363 (8), 261 (18), 233 (4). Composition $C_{27}H_{38}O_{10}$ (M = 522) determined by HR-MS: 545.23571 [M + Na], for $C_{27}H_{38}O_{10}Na$ calculated 545.23572. Full structural information based on ^1H and ^{13}C NMR spectra is summarized in Table 1.

*2.7.2. 2-Acetoxytrilobolide (**2**)*

White powder; $[\alpha]_D^{20}$ −40.8 (c 0.38 in MeOH). IR (KBr) [cm^{-1}]: 3456 (hydroxyls), 1780 (γ-lactone), 1726, 1235 (acetate), 1708 (ester conjugated with double bond), 1646 (double bond). TOF-MS ESI$^+$: m/z (%): 603 (100) $[M + Na]^+$, 519 (6), 421 (13), 399 (2), 333 (2). Composition $C_{29}H_{40}O_{12}$ (M = 580) determined by HR-MS: 603.24123 [M + Na], for $C_{29}H_{40}O_{12}Na$ calculated 603.24120. Full structural information based on ^1H and ^{13}C NMR spectra is summarized in Table 1.

*2.7.3. 2-Hydroxy-10-deacetyltrilobolide (**4**)*

White powder; $[\alpha]_D^{20}$ −18.0 (c 0.14 in MeOH). IR (KBr) [cm^{-1}]: 3462 (hydroxyls), 1776 (γ-lactone), 1730, 1236 (acetate), 1717 (ester conjugated with double bond), 1647 (double bond). TOF-MS ESI$^+$: m/z (%): 519 (100) $[M + Na]^+$, 447 (5), 435 (13), 408 (2), 360 (4). Composition $C_{25}H_{36}O_{10}$ (M = 496) determined by HR-MS: 519.22001 [M + Na], for $C_{25}H_{36}O_{10}Na$ calculated 519.22007. Full structural information based on ^1H and ^{13}C NMR spectra is summarized in Table 1.

*2.7.4. Eudeslaserolide (**8**)*

White powder; $[\alpha]_D^{20}$ −42.9 (c 0.18 in MeOH). IR (KBr) [cm^{-1}]: 3434 (hydroxyls), 1776 (γ-lactone), 1737, 1244 (acetate), 1719 (ester conjugated with double bond), 1637 (double bond). TOF-MS ESI$^+$: m/z (%): 447 (100) $[M + Na]^+$, 387 (4), 325 (4), 265 (2), 247 (2). Composition $C_{22}H_{32}O_8$ (M = 424) determined by HR-MS: 447.19883 [M + Na], for $C_{22}H_{32}O_8Na$ calculated 447.19894. Full structural information based on ^1H and ^{13}C NMR spectra is summarized in Table 1.

3. Results and discussion

3.1. Chemical identification and structural relations

Our investigation of the immune response induced by trilobolide (**1**) [16,17,21] has been extended also to other related guaianolides **5**–**7** (Fig. 1) isolated either from *L. trilobum* [3] or from the related species *Laserpitium siler* [22]. Two of them are new guaianolides **2** and **4** (Fig. 1) added to the set of compounds selected for the bioassay. Another new compound is eudesmanolide **8**, tested together with two previously identified known lactones: germacranolide **9** and its rearranged analog **10**. Lactones **5**–**10** possess similar substituents in the same positions (C-8 and C-11), but slightly different from the lactones **1**–**4**, which have even more

Table 1
Proton and carbon-13 NMR data of compounds **1**, **2**, **4** and **8**.

Proton	**1** (CD_3OD)	**2** (CD_3OD)	**4** (CD_3OD)	**8** (d6-DMSO)	Carbon	**1** (CD_3OD)	**2** (CD_3OD)	**4** (CD_3OD)	**8** (d6-DMSO)
1	4.42 m	4.36 m	3.46 m	3.13 dt (11.6; 5.0; 4.0)	1	52.53	58.83	63.63	77.05
2a	1.665 ddd (14.6; 5.8; 4.8)	5.48 dd (4.1; 2.9)	4.31 dd (4.3; 3.4)	1.70 m (13.6; 12.5; 11.6; 3.6)	2	33.28	85.61	78.98	26.64
2b	2.51 dt (14.6; 8.6; 8.6)	–	–	1.33 m (12.5; 4.0; 3.6)	3	81.15	79.51	88.08	40.00
3a	5.60 m	5.665 m	5.59 m	1.51 dt (13.6; 3.6; 3.6)	4	134.50	133.23	133.90	69.27
3b	–	–	–	1.46 td (13.6; 13.6; 4.0)	5	142.80	140.94	139.74	49.32
5	–	–	–	1.34 d (11.3)	6	78.70	78.01	78.87	75.73
6	5.71 m	5.675 m	5.67 m	5.18 dd (11.3; 9.2)	7	79.64	79.37	79.61	44.22
7	–	–	–	3.26 dd (9.2; 4.5)	8	67.63	67.34	67.69	67.88
8	5.595 t (3.8; 3.8)	5.59 t (3.8; 3.7)	5.53 t (3.8; 3.6)	5.06 dt (6.4; 4.5; 4.1)	9	39.77	39.43	45.99	41.12
9a	2.20 dd (14.8; 3.8)	2.28 dd (14.6; 3.8)	1.81 dd (14.8; 3.8)	1.63 dd (15.1; 6.4)	10	87.00	85.87	74.06	38.84
9b	3.02 bdd (14.8; 3.8)	2.98 ddd (14.6; 3.7; ~0.5)	2.42 ddd (14.8; 3.6; 0.6)	1.74 dd (15.1; 4.1)	11	79.47	79.48	79.41	78.34
13	1.34 s	1.35 s	1.34 s	1.51 s	12	178.37	178.15	178.44	173.66
14	1.35 bs	1.41 bs	1.14 s	1.01 s	13	16.04	15.86	15.91	20.28
15	1.88 m	1.85 m	1.82 m	1.18 s	14	22.35	23.49	25.11	15.72
OAng	6.16 qq (7.2; 1.5)	6.17 qq (7.2; 1.5)	6.18 qq (7.2; 1.5)	6.20 qq (7.2; 1.5)	15	13.16	12.96	13.17	32.18
	2.00 dq (7.2; 1.5)	1.98 dq (7.2; 1.5)	2.01 dq (7.2; 1.5)	1.91 dq (7.2; 1.5)	OAng	169.17	168.70	169.31	166.13
	1.905 p (1.5)	1.91 p (1.5)	1.93 p (1.5)	1.84 p (1.5)		129.12	128.76	129.02	126.87
OMebu	2.33 m	2.33 m	2.32 m	–		139.21	139.53	139.42	139.43
	1.71 m; 1.45 m	1.70 m; 1.44 m	1.70 m; 1.44 m	–		15.86	16.03	16.07	15.75
	0.92 t (7.4)	0.92 t (7.5)	0.91 t (7.2)	–		20.85	20.71	20.83	20.15
	1.14 d (7.2)	1.14 d (7.0)	1.13 d (7.0)	–	OMebu	176.33	176.28	176.47	–
OAc	1.96 s	2.06 s	–	1.98 s		42.71	42.66	42.74	–
		1.88 s	–			27.27	27.24	27.29	–
1-OH	–	–	–	4.51 d (5.0)		12.02	12.01	12.03	–
4-OH	–	–	–	4.34 s		16.74	16.74	16.75	–
					OAc	172.03	171.96	–	169.87
						22.50	22.58	–	21.33
						–	171.72	–	–
						–	21.07	–	–

substituents in various other positions. Especially the additional hydroxyl in position C-7, forming with the C-11 hydroxyl a characteristic vicinal diol grouping (*trans* glycol) at the lactone ring, rare among natural sesquiterpene lactones [4,23].

The identities of earlier reported major compounds: trilobolide (**1**), archangelolide (**5**), 2-deangeloylarchangelolide (**6**), acetylisomontanolide (**7**), laserolide (**9**) and isolaserolide (**10**) were confirmed comparing their NMR data with the data reported earlier [2,3,26]. The structures of new minor constituents **2**, **4** and **8** (Fig. 1) were elucidated by analysis of their IR, mass, and NMR spectra (for ^1H and ^{13}C NMR data, see Table 1). The ^1H and ^{13}C 1D spectra together with ^1H, ^1H–COSY and ^1H, ^{13}C–HMQC spectra were used for complete structure assignment of carbon and proton signals (Table 1). Characteristic NMR data of compounds **2** and **4** correspond with the data of trilobolide (**1**) [3], thapsigargin (**3**) and their analogs [11,27], as are summarized in [26].

Data of the new eudeslaserolide (**8**) correspond with the data of earlier reported analog lasolide from *L. trilobum* [3]. The ^{13}C NMR spectrum of compound **8** in CD_3OD showed 22 carbon signals and (together with ^1H NMR spectrum) allowed determine a presence of acetate and angelate ester groups. Four oxygen atoms of these ester groups and two oxygen atoms of lactone group compared with formula $C_{22}H_{32}O_8$ indicated that remaining oxygen atoms are probably present in two hydroxy groups. It was confirmed by new measurement of **8** in DMSO, where we have observed tertiary OH group as singlet at 4.34 ppm and secondary OH group as doublet at 4.51 ppm with $J = 5.0$ Hz. The detailed analysis of 2D-H,H–COSY, 2D-H,C–HSQC and 2D-H,C–HMBC spectra allowed us to determine the skeleton as eudesman-12,6-olide with OAc in position 8, OAng in position 11 and OH groups in positions 1 and 4. From a biogenetic relation between compound **8** and laserolide (**9**) we can expect the same relative configuration at

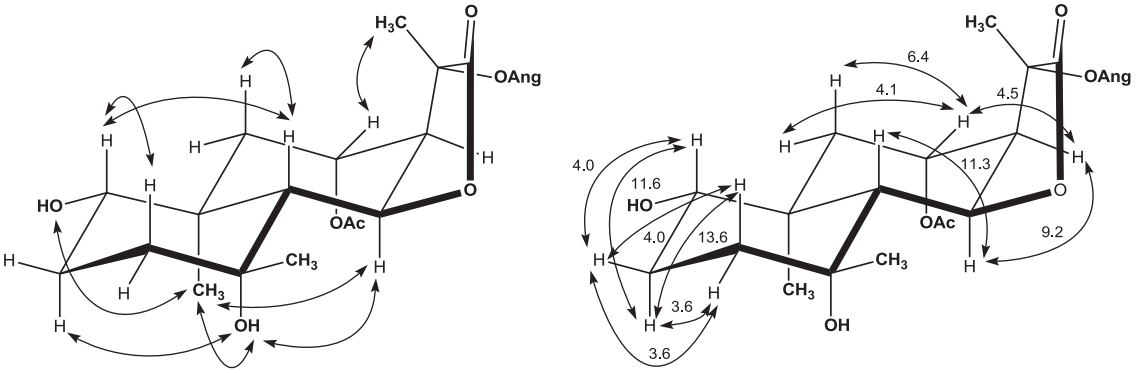


Fig. 2. Eudelaserolide (**8**) with selected observed NOE contacts (left) and vicinal *J*(H,H) (right).

positions 6, 7 and 8. Combining the observed NOE contacts and vicinal *J*(H,H) we could determine the relative configurations in positions 1, 4, 5, 10 and 11 in relation to those at positions 6, 7 and 8. Selected NOEs and *J*(H,H) in the geometry optimized conformation of compound **8** (molecular mechanics MM2) are shown in Fig. 2. The ^{1}H and ^{13}C NMR data are summarized in Table 1. Eudeslaserolide (**8**) was thus identified as 4-hydroxy-8-acetoxy derivative of lasolide or isolasolide [3].

Biogenetic relation of all test compounds, although selected from a single plant source, is not quite apparent. The basic skeletons of compounds **1**–**10** seem to be different (belonging to four different skeletal types), nevertheless, their biogenetic origin is effectively closely related. Their relationship is dependent on a series of biosynthetic steps successively assumed by various authors since early sixties, as reviewed by Fischer et al. [23] based on a summarizing proposal compiled by Herz [28] from many discussions going on at that time. Thereafter, a lot of experimental evidence (including chemical or photochemical biomimetic transformations) have been performed and published, for proving the formerly proposed biogenetic relationships, as reported by Adio [29]. Lactones collected from our plant source belong really to the four biogenetically related structural types [4]. The most abundant are guaianolides (see e.g. structures **1**–**7**) formed by 1,5-cyclisation from the basic germacranolide type, represented here by laserolide (**9**). From the same precursor it is derived also the eudesmanolide type, represented by eudeslaserolide (**8**) formed by 5,10-cyclisation, and finally elemanolide type, represented by isolaserolide (**10**) shaped by Cope rearrangement forming the 5,10-bond with simultaneous opening the former 2,3-bond. Structure differences of the four basic types, increased even more by varied additional substituents, actually indicate also a presumption of various biological properties.

3.2. Immunobiological properties

For primary evaluation of immunobiological activities of lactones **1**–**10** (0.01–25.0 μM), the effects on NO production by rat peritoneal cells were investigated (Fig. 3). Confirming our previous findings [16,17], the NO biosynthesis was stimulated by trilobolide (**1**) and thapsigargin (**3**). In this paper, we demonstrate for the first time, that also the new sesquiterpene lactone, 2-acetoxytrilobolide (**2**), possesses strong ability to induce NO production. The effects of all these compounds are characterized by a bell-shaped dose–response curve. The most plausible explanation of this phenomenon is the enhanced cytotoxicity at higher concentrations of the compounds. As described previously [16,17], both, trilobolide (**1**) and thapsigargin (**3**) become cytotoxic at concentrations of 4–10 μM. The same is true for 2-acetoxytrilobolide (**2**) (data not shown).

On the other hand, another newly isolated and structurally closely related 2-hydroxy-10-deacetyltrilobolide (**4**) as well as other structurally related guaianolides (**5**–**7**) or biogenetically related germacranolide-derived lactones (**8**–**10**) did not show any immunostimulatory activity. These results indicate that stimulation of NO production is induced by guaianolide type of sesquiterpene lactones. The biological activity is related to the presence of 7,11-dihydroxy-guaianolide skeleton with extended acyl substituents at positions 3 and 8, and with fixed acetyl substituent at position 10. Similar structural conditions have been reported for determining the appropriate pharmacophore model resulting from the receptor mapping of the thapsigargin

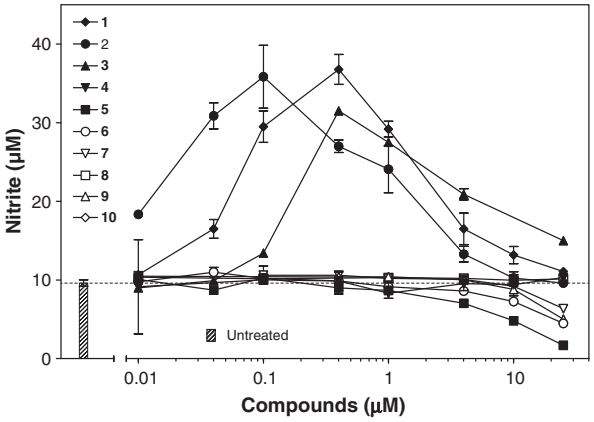


Fig. 3. NO production induced by lactones **1**–**10** at concentration range 0.01–25.0 μM was determined after 24-h culture of rat peritoneal cells. The test compounds are: trilobolide (**1**), 2-acetoxytrilobolide (**2**), thapsigargin (**3**), 2-hydroxy-10-deacyltrilobolide (**4**), archangelolide (**5**), 2-deangeloylarchangelolide (**6**), acetylisomontanolide (**7**), eudeslaserolide (**8**), laserolide (**9**), and isolaserolide (**10**). Each point is the mean ± S.E.M. The data are representative of five independent experiments (n = 3–4, each).

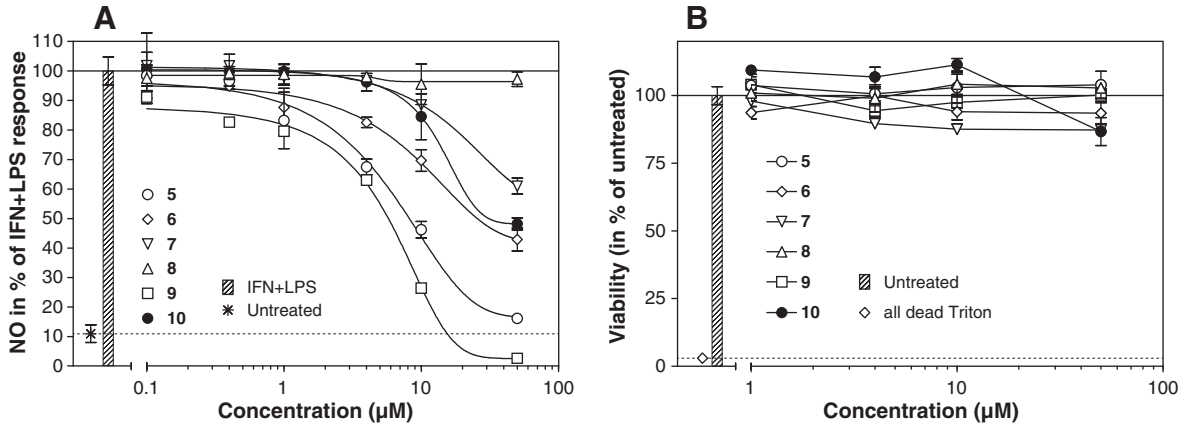


Fig. 4. Dose-dependent inhibition of NO production by lactones **5–10** in IFN-γ + LPS-stimulated rat peritoneal cells macrophages (A) and their viability in the WST-1 assay (B) corresponding with the toxicity of archangelolide (**5**), 2-deangeloylarchangelolide (**6**), acetylisomontanolide (**7**), eudeslaserolide (**8**), laserolide (**9**), and isolaserolide (**10**). Rat peritoneal cells 2×10^6/ml (A) and 1×10^6/ml (B) were cultured 24 h. The effects are expressed as the percent of the NO response after stimulation with IFN-γ + LPS (A) or as the percent of untreated control (B). The points are means ± S.E.M. Data are representative of five (A) and two (B) experiments.

binding site responsible for the SERCA pump influencing [15,30].

Sesquiterpenoids are usually described as agents with inhibitory activity on cell functions, including NO production [31]. Such inhibitory properties were detected (Fig. 4A) in the group of trilobolide-related guaianolides **5–7** and in biogenetically related analogs **8–10** with the −OAc substituents in position C-8 and −OAng in position C-11.

The effect was more pronounced for laserolide (**9**, $IC_{50} = 3.3$ μM), archangelolide (**5**, $IC_{50} = 6.19$ μM) and 2-deangeloylarchangelolide (**6**, $IC_{50} = 20.87$ μM), less for isolaserolide (**10**, $IC_{50} = 40.51$ μM) and acetylisomontanolide (**7**, $IC_{50} = 65.66$ μM), and almost none for eudeslaserolide (**8**, $IC_{50} > 100$ μM), as summarized in Table 2. Compounds **5–10** possess similar ester substituents in their lactone moiety at position C-11, but with the lactone ring anelated to 10, 7 or 6 membered rings, with their gradually decreased conformation flexibility, directly related with the decreasing bio-activity. The inhibitory effect of compounds on NO synthesis was not related to the cell viability. Using WST assay, the toxicity was not detected in the concentration range of test compounds (Fig. 4B).

We further examined the effect of the same representatives of germacranolide, guaianolide, eudesmanolide and elemanolide lactones **5–10** on cytokine secretion (Table 2, Fig. 5).

Highly enhanced levels of four monitored cytokines (IFN-γ, IL-6, IL-1β and VEGF) were induced by LPS (1 μg/ml) and the data are expressed in % of LPS response for individual cytokine. The highest inhibitory activity was observed for laserolide (**9**) with IC_{50} for IFN-γ, IL-6, IL-1β and VEGF: 4.5 μM, 5.2 μM, 19.3 μM and 5.4 μM, respectively. The suppression of cytokine secretion by other lactones of guaianolide type (**5–7**) and elemanolide type (**10**) is more pronounced for IFN-γ and IL-6. Among these lactones, archangelolide (**5**) decreased IFN-γ secretion with $IC_{50} = 24.4$ μM and IL-6 secretion with $IC_{50} = 25.9$ μM. Isoaserolide (**10**), which was less potent in NO production, showed only moderate inhibitory activity $IC_{50} = 61.4$ μM and 64.7 μM for IFN-γ and IL-6, respectively. Except laserolide (**9**), all trilobolide-unrelated 11-O-substituted lactones **5–10** mildly inhibited IL-1β and VEGF secretion. A newly identified eudeslaserolide (**8**), which was ineffective to influence NO production, was also without any effect on cytokine secretion. Taken into account differences in the anelated ring moieties of guaianolides **5–7**, eudesmanolide **8** and germacranolide **9**, it is evident that inhibitory activity is dependent on the lactone-ring anelation to the rigid 6-membered or flexible 10-memgered ring moiety. The highest

Table 2
Inhibition of compounds **5–10** on the NO production and cytokine secretion in the (IFN-γ + LPS)-activated rat peritoneal cells. IC_{50} values (in μM concentrations with 95% limits of confidence) were obtained in the assays displayed in Figs. 3–5.

Comp.	NO	IFN-γ	IL-6	IL-1β	VEGF
5	6.19 (4.82–7.95)	24.44 (4.55–131.2)	25.89 (11.05–60.65)	>100	>100
6	20.87 (13.49–32.28)	>100	78.54 (22.40–275.4)	>100	>100
7	65.66 (55.45–77.74)	78.50 (40.08–153.70)	64.42 (40.66–102.0)	>100	>100
8	>100	>100	>100	>100	>100
9	3.30 (1,56–6.98)	4.51 (3.57–5.72)	5.20 (3.29–8.23)	19.93 (11.83–33.57)	5.41 (2.52–11.61)
10	40.51 (30.30–54.16)	61.41 (23.91–157.7)	64.72 (39.83–105.2)	>100	>100

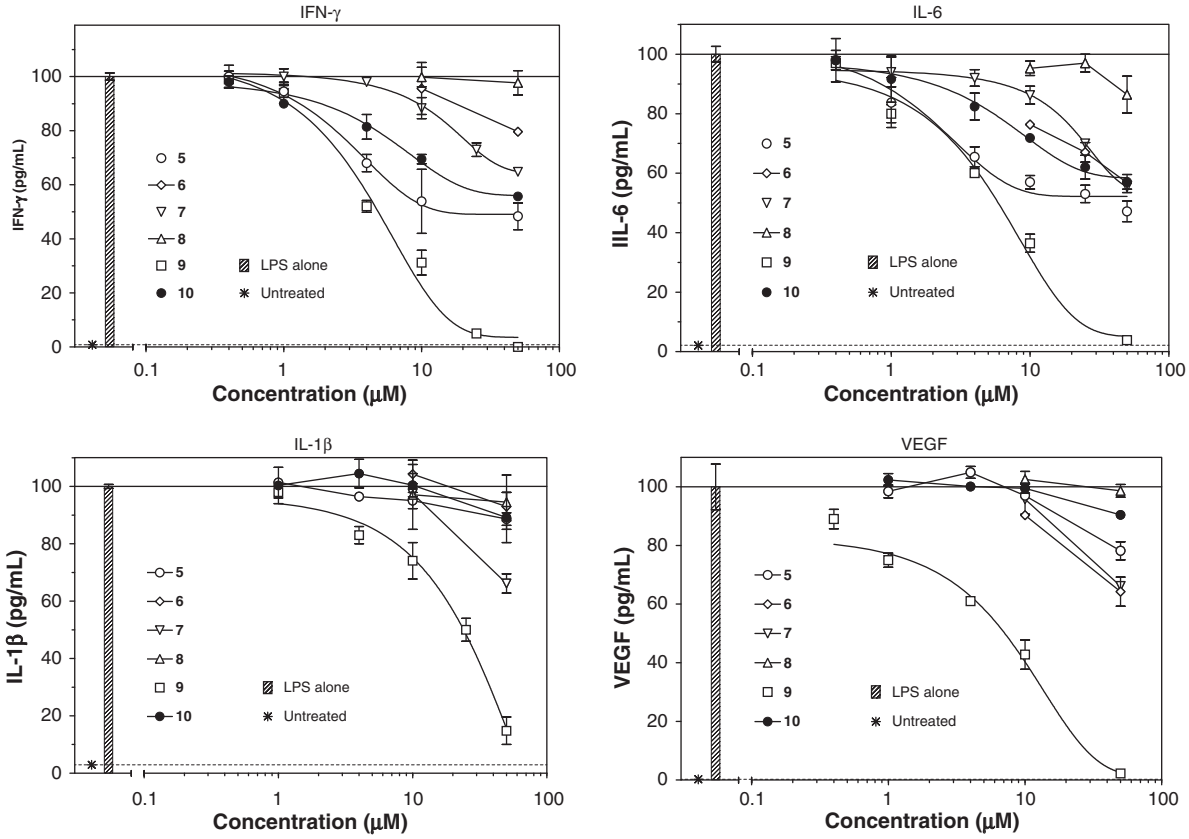


Fig. 5. Evaluation of cytokine (IFN-γ, IL-1β, IL-6, VEGF) secretion induced by sesquiterpene lactones **5**–**10** in IFN-γ + LPS-stimulated rat peritoneal cells. Cells (2×10^6/ml) were cultured in the presence of the test compounds (0.01–50.0 μM): archangelolide (**5**), 2-deangeloylarchangelolide (**6**), acetylisomontanolide (**7**), eudeslaserolide (**8**), laserolide (**9**), and isolaserolide (**10**). Incubation period was 24 h. Cytokines were assayed using ELISA. The data are means ± S.E.M. representing one of two identical experiments.

potential to inhibit cytokine and NO production is possessed by laserolide (**9**), which is effective at IC_{50} of approximately 5 μM. It also inhibits VEGF, a factor known to be associated with the tumor growth. In contrast to many other sesquiterpene lactones, laserolide (**9**) is free of cytocidal effects up to a concentration of 50 μM. This compound appears to be a promising candidate for further research [32].

The stimulatory effect of well-known thapsigargin (**3**) and structurally related trilobolides (**1**, **2** and **4**) on NO production leads us to hypothesize that these compounds are potent stimulators of cytokines, especially IFN-γ. Recently we documented [16,17] that thapsigargin (**3**) and trilobolide (**1**) are potent stimulators of IFN-γ secretion in a range of low concentrations (10 nM–10 μM). Here (see Fig. 6), we report for the first time a substantial stimulation of IFN-γ secretion induced by newly isolated 2-acetoxytrilobolide (**2**). The IFN-γ secretion in the presence of compound **2** (at 1 μM and 4 μM) reached the levels triggered by compounds **1** and **3**. On the other hand, another structurally transformed 2-hydroxy-10-deacetyltrilobolide (**4**) did not show any activity.

We have revealed the significant induction of IFN-γ production by several specific guaianolides (Fig. 6). This is a unique property among natural compounds. Nevertheless, it may be presumed that this stimulatory activity is accompanied by secretion of other cytokines. We further investigated secretion of IL-1β and IL-6 cytokines, VEGF and GM-CFS. We observed that thapsigargin (**3**), trilobolide (**1**) and 2-acetoxytrilobolide (**2**) stimulated production of these cytokines. In sharp contrast with the immunostimulatory activity of those three trilobolide-related derivatives was the 2-hydroxy-10-deacetyltrilobolide (**4**). The results did not show cytokine production of the compound **4**. We can conclude, that beyond the 7,11-diol formation, ester substituents in positions C-2 and C-10 (or none in the C-2 of the trilobolide (**1**)) are important for immunostimulatory activity, i.e. cytokine secretion and NO synthesis (see Fig. 3). If they are replaced by hydroxyl groups (as it is in the compound **4**), the ability to secrete cytokines was completely lost.

Acknowledgments

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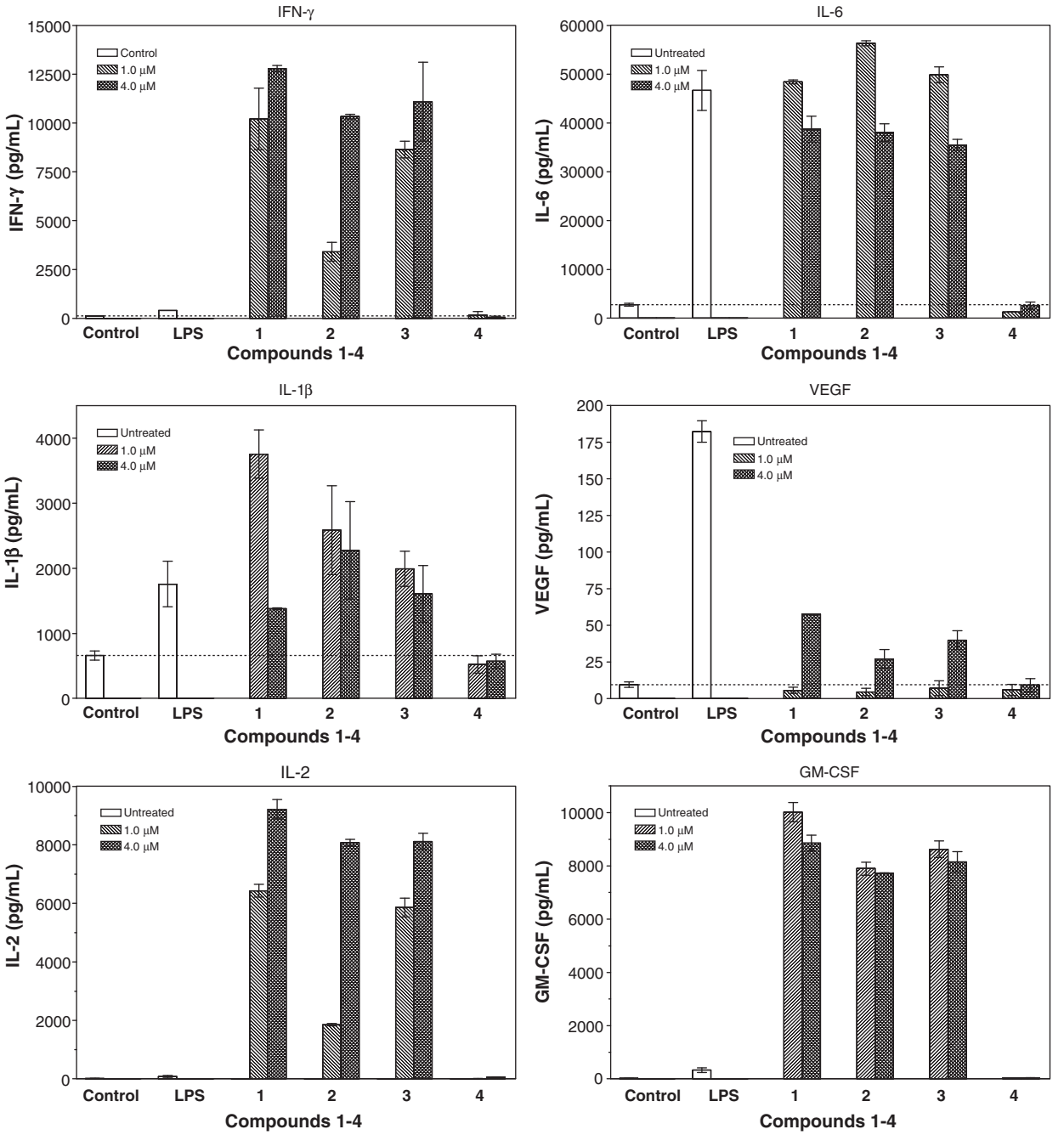

Fig. 6. Evaluation of cytokine secretion (IFN-γ, IL-1β, IL-2, IL-6, VEGF, GM-CSF) induced by sesquiterpene lactones **1**–**4** (1 μM) in LPS-stimulated rat peritoneal cells. Cells (2×10^6/ml) were cultured with the test compounds: trilobolide (**1**), 2-acetoxytrilobolide (**2**), thapsigargin (**3**) and 2-hydroxy-10-deacetyltrilobolide (**4**) in two concentrations (1.0 and 4.0 μM). Incubation period was 24 h. The data are means ± S.E.M. representing two independent experiments.

memory of our late colleague Dr. Z. Smítalová (1953–1988) (see [3,7,22]).

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