Notes

A Glycosyl Analogue of Diacylglycerol and Other Antiinflammatory Constituents from *Inula viscosa*

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Some extracts from *Inula viscosa* were examined for acute antiinflammatory activity in vivo. Three flavonoids: rhamnocitrin (**1**), 7-*O*-methylaromadendrin (**3**), and 3-*O*-acetylpadmatin (**4**); a sesquiterpene lactone, inuviscolide (**2**); a sesquiterpene acid, ilicic acid (**5**); and a digalactosyl-diacylglycerol, inugalactolipid A (**6**), were isolated from the CH_2Cl_2 extract, identified by spectroscopic methods, and characterized as the topical antiinflammatory principles of this species. All these compounds proved to be effective against 12-*O*-tetradecanoylphorbol-13-acetate-induced ear edema in mice, although lacking activity against arachidonic acid-induced edema. In addition, compounds **5** and, markedly, **6** showed notable effects on a multiple-dose murine chronic dermatitis model. This is the first attempt to establish a rationale concerning the documented use of the plant on various skin diseases.

Inula viscosa (L.) Aiton [*Dittrichia viscosa* (L.) Greuter] (Asteraceae) is a perennial herbaceous plant that profusely colonizes sub-nitrophile and sub-saline soils in abandoned and plowed fields in the Mediterranean region. It presents simple alternate leaves, covered with glands secreting a sticky substance, and bright yellow flowers that bloom between August and November. This species is used topically in folk medicine as an anti-scabies, antiinflammatory, and wound-healing agent.[1] Previous studies have demonstrated the antiinflammatory effect of the aqueous extract[2] and the gastric antiulcerogenic activity of the flavonoids from the ether extract.[3] The aim of the present work was the isolation, identification, and pharmacological study, on acute and chronic models, of the compounds that can be considered responsible for the plant's antiinflammatory effect on the skin. The principles found had formerly been observed along with certain sesquiterpenes and flavonoids in the plant, but a novel antiinflammatory compound belonging to the rather unexplored group of plant glycodiacylglycerols is also included.

The CH_2Cl_2-soluble fraction of the MeOH extract of the plant induced a 74% reduction in the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute mouse ear edema, whereas the hexane, EtOAc, and H_2O extracts showed weak activity (20−40% inhibition). The same bioassay was used to discriminate the fractions obtained from the CH_2Cl_2 extract by chromatography on Si gel. The most active of these were fractions 2 (86% edema reduction) and 8 (67% edema reduction). Following the steps succinctly described in the Experimental Section, six compounds (**1**−**6**) were isolated. Compound **1** was identified by UV analysis with standard reagents[4] and ^{1}H NMR spectroscopy as the flavonol rhamnocitrin (3,5,4′-trihydroxy-7-methoxyflavone). According to the ^{1}H NMR data, compound **3** showed the same pattern of hydroxyl substitution as **1**, being two *trans*-coupled doublets between 4.5 and 5.0 ppm indicative of a dihydroflavonol structure. Compound **4** was also a dihydroflavonol, although it possessed an *ortho*-dihydroxyl substitution at ring B and an acetyl substitution on the 3-hydroxyl group, which was perceived by the higher-frequency shift (ca. 0.7 ppm) for protons 2 and 3. On the basis of these results and of those reported previously,[5] **3** was identified as 7-methylaromadendrin (3,5,4′-trihydroxy-7-methoxyflavanone) and **4** as 3-acetylpadmatin (3-acetoxy-5,3′,4′-trihydroxy-7-methoxyflavanone).

Compounds **2** and **5** showed the unequivocal ^{13}C NMR spectral characteristics of sesquiterpenoid compounds containing two saturated rings. They also shared the presence of the very frequent 2-propenoyl substitution on carbon 7 and a *gem*-α-hydroxy-*β*-methyl function demonstrated by the presence of the signals at 80.46 and 24.07 ppm, for **2**, and 71.38 and 23.66 ppm, for **5**, corresponding to carbons 4 and 15, respectively. However, **2** and **5** differed in that **2** showed a double pair of *exo*-methylene protons in the ^{1}H NMR spectra (*δ* 5.56 and 6.23 ppm, 4.97 and 5.11 ppm), while **5** had a single one (*δ* 5.69 and 6.52 ppm). Moreover, **2** was a lactone, for its IR absorbance peak at 1760 cm^{-1} and the ^{13}C NMR signals at 168.48 ppm (carboxy-ester) and 82.33 ppm (*γ* lactone alcoholic carbon). Comparison of all these results with those reported in the literature made it possible to identify compound **2** as inuviscolide [4α-hydroxyguaia-11(13),10(14)-dien-1*β*,5α,7α,8*β*H-12,8-olide)[6,7] and compound **5** as ilicic acid [4α-hydroxyeudesm-11(13)-en-5α,7αH-12-oic acid].[8−10]

Compound **6** showed five very well differentiated groups of ^{13}C NMR peaks: a large number between 10 and 40 ppm (alkyl chain), 13 signals between 60 and 75 ppm (saccharide methines and methylenes), two signals near 100 and 105 ppm (anomeric sugar carbons), six signals between 125 and

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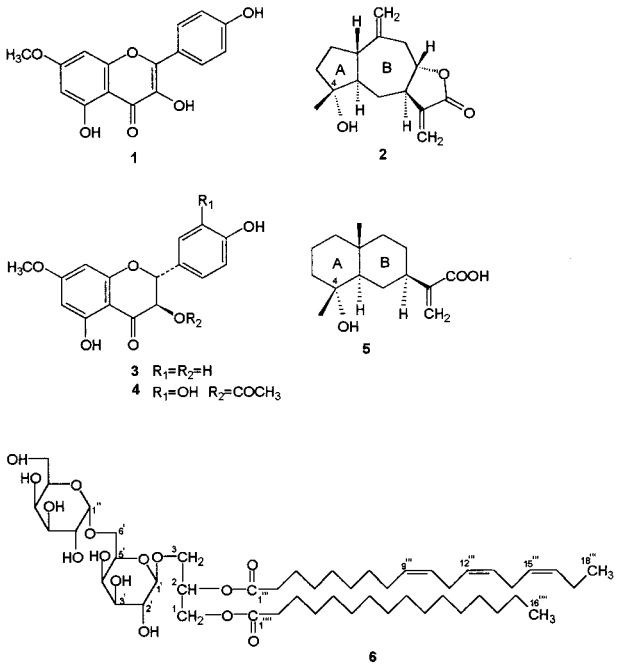
135 ppm (olefinic methines), and two signals near 175 ppm (acid carbonyl). A DEPT experiment indicated that the signals at 63.99 and 68.74 ppm belonged to oxymethylenes from a short-chain polyol like that of glycerol. As no aromatic ^{1}H NMR signals appeared, ^{13}C NMR signals between 125 and 135 ppm can be attributable to isolated double bonds, probably in a fatty acid residue, correlating with the presence of the ^{1}H multiplet centered at 5.29 ppm. A huge multiplet near 1.30 ppm corresponded to aliphatic methylene protons, whereas the signals in the 3.4−5.0 ppm region accounted for the presence of a disaccharide moiety. This disaccharide was identified as α-D-galactosyl(1→6)-β-D-galactose by the full coincidence of its NMR signals with those of the many lipid digalactosyl derivatives found in nature.[11−14] After alkaline hydrolysis, two fatty acid methyl esters were identified as methyl palmitate (hexadecanoate) and methyl linolenate (9*Z*,12*Z*,15*Z*-octadecatrienoate) by gas chromatography analysis with authentic samples. Because the splitting of the ^{13}C NMR signals for the two carbonyl carbons of acid residues is 0.35 ppm, the saturated one (palmitoyl) should be at *sn*-1 position; otherwise, these signals would have been closer or even overlapped.[15] For this reason, the structure of 1-*O*-hexadecanoyl-2-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-3-*O*-[α-D-galactopyranosyl (1″→ 6′)-*O*-β-D-galactopyranosyl] glycerol was attributed to compound **6**, which will be now termed inugalactolipid A. This substance had previously been reported as one of the galactolipids of the roots from the Chinese toxic plant *Arisaema amurense* Nakai (Araceae).[13]



The results on antiinflammatory activity are shown in Tables1−3. It should be noted that the amount of isolated compounds **1**−**4** was not enough to perform repeated treatments against chronic dermatitis or for oral administration against carrageenan-induced edema.

On the edema induced by a single application of TPA, all the compounds tested were fairly active (70−90% inhibition) except inugalactolipid A, which did not reach 50% inhibition. Inuviscolide seemed to act equipotently with respect to indomethacin. In contrast, none of the products significantly restricted the edema induced by arachidonic acid (AA).

On the edema induced by subcutaneous administration of carrageenan, ilicic acid produced a moderate but significant inhibition of the mouse-paw swelling, with an effect sustained even after 5 h, whereas inugalactolipid A lacked appreciable activity. A much larger reduction in both ear swelling and neutrophil infiltration was produced by application of the same compounds on the skin in the TPA multiple-dose model: similar percentages were obtained for the inhibition of neutrophil infiltration measured as myeloperoxidase (MPO) levels, while inugalactolipid A exhibited an effect higher than that of ilicic acid by nearly 20%.

Table 1. Antiedematous Effects of the Products Assayed on Ear Acute Models

	TPA[a]		AA[b]	
	ΔW[c]	I.R.[d]	ΔT[e]	I.R.[d]
control	14.0 ± 1.3		151.5 ± 13.9	
rhamnocitrin (**1**)	3.1 ± 0.7**	78	142.3 ± 10.1	6
inuviscolide (**2**)	1.4 ± 0.4**	90	176.9 ± 9.5	−16
7-OMe-aromadendrin (**3**)	3.1 ± 1.2**	78	146.4 ± 8.8	3
3-OAc-padmatin (**4**)	2.4 ± 1.5**	83	153.5 ± 5.4	−1
ilicic acid (**5**)	3.9 ± 1.0**	72	124.3 ± 7.9	18
inugalactolipid A (**6**)	7.3 ± 1.5**	48	144.2 ± 6.5	5
indomethacin	1.2 ± 0.4**	91	nt[f]	
phenidone	nt[f]		74.2 ± 7.2**	51

[a] 12-*O*-tetradecanoylphorbol-13-acetate. [b] Arachidonic acid. [c] Increase in ear weight (mg ± SEM). [d] Inhibition ratio. [e] Increase in ear thickness (μm ± SEM). * $p < 0.05$; ** $p < 0.01$ with respect to the control group (Dunnet's *t*-test). [f] nt, not tested.

Taking all these in vivo results together it can be suggested that the topical antiinflammatory activity of the test compounds lies in the interaction with processes other than arachidonate catabolism, especially with that mediated by 5-lipoxygenase (5-LO), because at the dose used, the AA-ear edema test is selective for inhibitors of this enzyme.[16] In contrast, as many groups of potential antiinflammatory agents such as H_1-histamine antagonists, corticoids, and inhibitors of phospholipase A_2 (PLA_2), cyclooxygenase (COX), or 5-LO have been reported to be effective against TPA-ear edema,[17] several different options are open when it comes to searching for the mechanism of the compounds **1**−**5** active in this model.

The effect of the flavonoids in the TPA test was somewhat milder than that obtained for inuviscolide. As the most active flavonoid proved to be 3-acetylpadmatin, we found that free phenolic hydroxyl groups at 3′,4′ combined with an esterified secondary alcohol at carbon 3 are favorable features, although the differences between the effects of compounds **1**, **3**, and **4** are, in fact, small. It should be noted that this is the first report on the antiinflammatory activity of these dihydroflavonols.

On the other hand, the structure of ilicic acid contains the fairly typical 2-propenoyl function that is repetitive in the side chain of many sesquiterpenoid derivatives from Asteraceae. Inuviscolide differs from ilicic acid in that it contains a γ-lactone over a guaiane nucleus with two *exo*-methylene groups, although the two compounds still have in common some other chemical features: an α-hydroxy-β-methyl substitution at carbon 4, an α-*exo*-methylene function with respect to the carboxyl group, the trans configuration of the A/B rings, and the absence of cyclic unsaturations, which are so frequent in other terpenoids from this plant. The sesquiterpene lactones constitute a group to which a strong reactivity and, therefore, interactions with many biological systems,[18] including the expression of COX-2, one of the most salient pro-inflammatory enzymes,[19] is attributed, when *exo*-methylene and vicinal carbonyl groups occur.

Few studies on the antiinflammatory activity of glycolipids have been reported. One of the points requiring more

Table 2. Effects of the Products Administered Orally against Carrageenan Paw Edema

	ΔV^a			I.R.[b]		
	1 h	3 h	5 h	1 h	3 h	5 h
control	9.3 ± 0.9	13.3 ± 1.0	13.8 ± 1.8			
ilicic acid (**5**)	8.8 ± 0.7	9.0 ± 0.7**	9.7 ± 0.8**	5	32	30
inugalactolipid A (**6**)	10.8 ± 0.9	12.0 ± 0.7*	13.1 ± 0.7	−16	10	5
phenylbutazone	4.3 ± 0.2**	5.6 ± 0.2**	7.9 ± 0.4**	54	58	43

[a] Increase in paw volume (mL × 10^{-2} ± SEM) 1, 3, and 5 h after carrageenan injection. [b] Inhibition ratio; * $p < 0.05$; ** $p < 0.01$ with respect to the control group (Dunnet's *t*-test).

Table 3. Antiinflammatory Effects of the Products Assayed on Chronic Dermatitis

	W^a	I.R.[b,c]	MPO[d]	I.R.[b,d]
control	23.4 ± 1.0		1473 ± 110	
ilicic acid (**5**)	15.1 ± 1.6**	78	268 ± 40**	82
inugalactolipid A (**6**)	13.1 ± 0.5**	97	231 ± 40**	84
dexamethasone	13.7 ± 0.6**	92	120 ± 12**	92

[a] Ear weight (mg ± SEM). [b] Inhibition ratio percentage. [c] Swelling inhibition relative to Me_2CO-only control (12.8 ± 0.6 mg). [d] Myeloperoxidase assay; (mOD/biopsy ± SEM, $n = 5$). ** $p < 0.01$ with respect to the control group (Dunnet's *t*-test).

attention to be paid is the fact that inugalactolipid A showed low activity throughout the acute tests but was the most valuable compound on the model of chronic dermatitis induced by TPA. Given that inugalactolipid A does not appear to be able to act immediately against inflammatory stimuli, but, when repeatedly applied, prevented the development of a skin inflammation characterized by edema, epidermal hyperplasia, leukocyte accumulation, and fibrosis,[20−21] this compound may possess lasting actions, possibly related to phospholipid metabolism of or to leukocyte extravasation. These hypotheses are supported, respectively, by recent work on the inhibitory activity of plant glycolipids, especially digalactolipids, on PLA_2 enzymes of various origins,[22] and the P-selectin receptor binding activity of sulfated cerebrosides.[23] In the latter case, a possible structural analogy might be found among the sulfoquinovosyl derivatives, another class of plant glycolipids to which one of the active principles of the antipsoriatic fern *Polypodium decumanum* (calaguala) belongs.[24]

Experimental Section

General Experimental Procedures. NMR solvents were purchased from Sigma Chemical Co., St. Louis, MO. Analytical grade reagents and solvents for UV spectroscopy were purchased from Merck, Darmstadt (Germany), sodium methoxide, from Aldrich Chemical Co., St. Louis, MO. AA, carrageenan, TPA, hydrogen peroxide, phosphate buffer saline (PBS), *N*,*N*-dimethylformamide, hexadecyltrimethylammonium bromide (HTAB), tetramethylbenzidine (TMB), dexamethasone, indomethacin, phenidone, and phenylbutazone were purchased from Sigma and sodium acetate from Panreac, Barcelona (Spain).

^1H and ^{13}C NMR spectra were recorded on Varian 400 MHz and Bruker 250 MHz spectrometers. UV-vis analysis was performed (MeOH solution) on a Perkin−Elmer Lambda 15 spectrophotometer, and IR spectra (Nujol) on a Perkin−Elmer 843 spectrophotometer.

Sodium methoxide 2.5% (4 mL) in MeOH was added to 20 mg of **6** and left standing for 3 h at room temperature. The mixture was then neutralized with HCl 2N in MeOH and extracted with hexane−MeOH (1:1). After the hexanic phase was dehydrated with Na_2SO_4, it was concentrated to yield a residue of 8 mg. A 4% solution of the lipid residue obtained by alkaline hydrolysis (2 μL) was injected into a 20% DEGS−PS, on Chromosorb W-AW 80/100 mesh, steel column (2 m × 1/8 in.) installed on a KNK 3000-HRGC apparatus (Konik Instruments, Sant Cugat del Vallés, Spain). Oven temperature: 180 °C; injector and FID detector temperature: 280 °C. Nitrogen flow rate: 40 mL/min. A methyl esters mixture from Sigma was run as a standard.

Isolation of Compounds. Plant material was collected near Torrent (Valencia, Spain), and a specimen was deposited in the Herbarium of the Faculty of Pharmacy, University of Valencia. Dried and powdered aerial parts (2.16 kg) were defatted with hexane and then macerated with MeOH to obtain an extract that was partitioned with CH_2Cl_2 and EtOAc to obtain the corresponding extracts. Another sample (100 g) of dried powder was treated with hot H_2O to obtain an infusion. From the CH_2Cl_2 extract, 12 fractions were obtained over a Si gel column by eluting with $CHCl_3$−MeOH mixtures. From fraction 2 a pure compound (**1**, 29 mg) was obtained by precipitation. The rest of this fraction was chromatographed on Si gel, eluting with hexane−CH_2Cl_2−Me_2CO−MeOH mixtures, to give 13 fractions. Fractions 2−7 were subjected to gel-filtration on Sephadex LH-20 with MeOH to give compound **2** (18.9 mg) and a mixture of two very similar compounds. The latter two substances (**3**, 9.8 mg, and **4**, 7.2 mg) were separated from fractions 2−8 by reversed-phase liquid chromatography. Compounds **5** (162 mg) and **6** (397 mg) were isolated from fraction 8 by gel-filtration on Sephadex LH-20 with MeOH.

Pharmacological Experiments. Animals. Groups of six female Swiss mice weighing 25−30 g were used. All animals were fed a standard diet ad libitum and housed in a temperature-controlled room with a 12 h light/dark schedule.

Carrageenan-Induced Mouse Paw Edema. An edema was induced on the right hind foot of mice by subplantar injection of 0.05 mL of a solution of 3% carrageenan in 0.9% saline (w/v). The volumes of the injected and contralateral paws were measured 1, 3, and 5 h after induction of inflammation using a plethysmometer (Ugo Basile), and the edema was expressed as an increase in paw volume due to carrageenan injection. A reference group was treated with phenylbutazone (100 mg/kg, po). Pure compounds dissolved in EtOH−Tween 80−H_2O (2:2:20 v/v/v) were administered orally at 100 mg/kg (0.50 mL), 1 h before carrageenan injection. A control group received the vehicle only.

TPA-Induced Mouse Ear Edema. An edema was induced on the right ear by topical application of 2.5 μg of TPA dissolved in 20 μL of Me_2CO. The left ear received the vehicle. Pure compounds, dissolved in Me_2CO, were applied topically (0.5 mg/ear) simultaneously with TPA. The mice were killed by cervical dislocation 4 h after TPA application. Circular biopsies of each ear were taken with a leather punch. The edema was expressed as an increase in the ear weight due to TPA application. A reference group was treated with indomethacin (0.5 mg/ear).

AA-Induced Mouse Ear Edema. An edema was induced on the right ear by topical application of 2 mg/ear of AA in 20 μL of Me_2CO. Pure compounds, dissolved in Me_2CO, were applied topically (0.5 mg/ear) 30 min before AA. The thickness of the ears was measured before and 1 h after induction of inflammation using a micrometer (Mitutoyo Series 293). The edema was expressed as an increase in the ear thickness due to AA application. A reference group was treated with phenidone (1 mg/ear).

Mouse Ear Inflammation Induced by Multiple Topical Applications of TPA. A chronic process was induced by topical application of 10 μL of TPA (2.5 μg/ear) on both the inner and outer surface of both ears of each mouse with a

micropipet on alternate days. Test compounds were dissolved in Me_2CO and applied topically (0.5 mg/ear) twice daily for 4 days, in the morning immediately after TPA application, and 6 h later. Dexamethasone was used as the reference drug (0.05 mg/ear). The mice were killed by cervical dislocation 6 h after the last compound application. Circular biopsies of each ear were taken with a leather punch. Ear weight is presented as the mean of 12 determinations. After weighing, biopsies were frozen for future MPO assay.

Myeloperoxidase Assay. Each biopsy was homogenized with 0.75 mL of 0.5% HTAB in 80 mM sodium phosphate buffer (PBS, pH 5.4). After adding a second 0.75 mL aliquot, the sample was centrifuged at 12 000 *g* at 4 °C for 20 min. The supernatant (30 μL) was mixed with 100 μL of 80 mM PBS, 85 μL of 0.22 M PBS, 20 μL of TMB 18.4 mM in 8% aqueous formamide, and 15 μL of H_2O_2 0.017% in a 96-well microtiter plate. After 3 min at 37 °C the reaction was stopped with 30 μL of 1.46 M NaOAc (pH 3.0) and enzyme activity was determined using a Labsystems Multiskan MCC/340 plate reader set at 620 nm. This activity reflects, in a specific mode, the accumulation of neutrophils in the inflamed tissue, which was maximal after 3−4 days of TPA application.

Statistics. Data are expressed as the mean with SEM. One-way ordinary ANOVA and Dunnet's *t*-tests for unpaired data were used for statistical evaluation. Unless otherwise stated, groups were made up of six animals.

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