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STRUCTURE AND IN VITRO ANTIVIRAL ACTIVITY OF SESQUITERPENE GLYCOSIDES FROM *CALENDULA ARVENSIS*

NUNZIATINA DE TOMMASI, COSIMO PIZZA,*

Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli, via Domenico Montesano, 49, I-80131 Napoli, Italy

CINZIA CONTI, NICOLA ORSI,

Istituto di Microbiologia, Università degli Studi "La Sapienza" Facoltà di Medicina, P. le Aldo Moro 5, I-00185 Roma, Italy

MARIA LUISA STEIN

Dipartimento di Studi Farmaceutici, Università degli Studi "La Sapienza," P. le Aldo Moro 5, 00185 Roma, Italy

ABSTRACT.—Previous research on the aerial parts of *Calendula arvensis* led to the isolation of the epicubebol glycoside **1** and of the sesquiterpene glycosides **2–5** based on the alloaromadendrane skeleton. A further investigation has revealed two new glycosides, **6** and **7**, derived from the same sesquiterpene, the structures of which were elucidated by spectral studies.

Furthermore a series of antiviral tests has been performed on glycosides **1–7** by examining their ability to interfere with rhinovirus 1B and vesicular stomatitis virus infection in vitro. Only glycoside **1** slightly reduced rhinovirus multiplication. All the compounds were able to inhibit vesicular stomatitis virus infection, **1** and **2** being the most effective.

In the course of a systematic search for natural compounds isolated from plant extracts with possible antiviral activity, research has been carried out on glycosides from *Calendula arvensis* L. (Compositae). This herbaceous plant was used in Italian folk medicine as an anti-inflammatory, anticancer, antipyretic remedy, and in a recent pharmacological study the extracts of aerial parts of *C. arvensis* have been demonstrated to possess an anti-inflammatory activity (3).

In previous studies we reported the isolation and structure determination of four triterpenoid saponins from the MeOH extract (4) as well as of the epicubebol glycoside **1** (1) and of four alloaromadendrol glycosides **2–5** from the $CHCl_3$ extract (2).

We now report a reexamination of the $CHCl_3$ extract of aerial parts of *C. arvensis* which yielded, in addition to compounds **1–5**, the two new alloaromadendrol glycosides **6** and **7**.

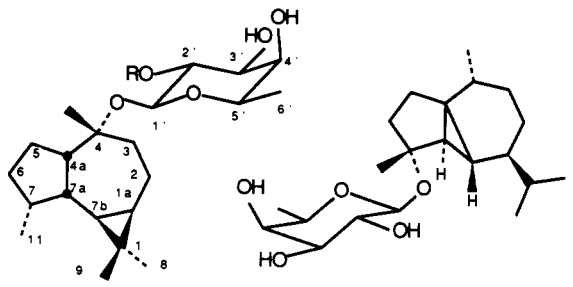
Up to now no data are available on the antiviral action of glycosides of epicubebol and of alloaromadendrol, both showing a cyclopropane ring; therefore, we conducted an investigation on the possible action of these sesquiterpene glycosides against two RNA viruses: a minus-strand RNA virus, vesicular stomatitis virus (VSV), and a plus-strand RNA virus, rhinovirus (HRV type 1B). The compounds were found to be active mainly against VSV.

RESULTS AND DISCUSSION

Compounds **1–5** were identified on the basis of spectral data and comparison with literature data (1,2).

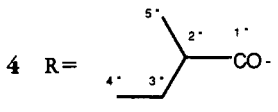
The new glycosides **6** and **7** are esters of arvoside B [4-*O*-(β-D-fucopyranosyl)-4-alloaromadendrol] [**2**] at the hydroxyl in C-2′ position of the fucose unit. Compound **6** is the 2″-methylpropanoyl derivative and compound **7** the 2″-methyl-2″-butenoyl derivative.

Substitution at C-2′ in **6** and **7** was deduced from the ^{13}C-nmr spectra (Table 1) and the ^{1}H-nmr spectra (Table 2) (H-2′, δ 4.98 and 5.11, respectively, dd, $J = 7.6, 9.8$ Hz

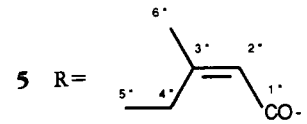


1

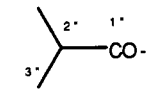
2 R=H
3 R=Ac-



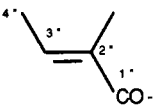
4 R=



5 R=



6 R=



7 R=

The relatively strong shielding effect observed on these signals, due to the spatial orientation of the double bond, was possible only if the aglycone was based on the alloaromadendrol skeleton with the C-2′ (2″-methyl-2″-butenoyl)fucosyl residue in **7** in a cis relationship with H-1a and H-7b.

was shifted downfield about 1.6 and 1.7 ppm if compared with **2**). Comparison of the chemical shift of the ^{13}C-nmr signals assigned to C-1′, C-2′, C-3′ in compounds **6** and **7** with the corresponding signals in **2** indicated that C-2′ was deshielded by 1.5 ppm, while C-1′ and C-3′ were deshielded (γ effect) by 2.3 and 2.2–2.0 ppm, respectively, as expected for an ester bond (5). The relative stereochemistry of the aglycone moiety in compounds **6** and **7** was ascertained by ^{1}H nOe difference spectra (NOEDS) by methods reported in our previous paper (2).

Further evidence for the cis junction and relative stereochemistry at C-4 was obtained by comparison of the ^{1}H-nmr spectra of compounds **2** and **7** (Table 2). Signals of the C-11 methyl, C-8 methyl, H-1a, and H-7b in **7** were shifted to high field by 0.06, 0.10, 0.02, and 0.046 ppm, respectively. Inspection of molecular models showed that

Preliminary experiments were performed to identify the maximum nontoxic concentration of compounds **1** to **7** for HeLa and chicken embryo related (CER) cells. While the alloaromadendrol glycosides were found to be toxic at concentrations higher than 20 μg/ml, the epicubebol glycoside **1** was less toxic; it was possible to use this compound at concentrations up to 100 μg/ml. Because no significant differences were observed for the toxicity of compounds in both cell lines examined, the glycosides were tested on HRV and VSV infections at the same doses.

In the experiments performed with HRV type 1B in HeLa cells, glycoside **1** at 100 μg/ml gave more than 25% reduction of cytopathic effect (CPE), while all the other compounds at 20 μg/ml were inactive. On the contrary, in VSV-infected CER cells all the glycosides tested were found to be active, although to a different extent. The most

TABLE 1. ^{13}C-nmr Spectral Data of Compounds **2**, **6**, and **7** (62.9 MHz, CD_3OD, TMS internal standard).

Carbon	DEPT	Compound		
		2	**6**	**7**
Aglycone				
C-1	C	19.7	19.7	19.6
C-1a	CH	30.1	30.0	30.0
C-2	CH_2	20.0	19.4	19.6
C-3	CH_2	38.0	39.3	38.9
C-4	C	82.9	82.6	82.8
C-4a	CH	56.1	56.0	56.5
C-5	CH_2	26.7	26.5	26.5
C-6	CH_2	30.0	30.2	30.0
C-7	CH	39.3	39.5	39.6
C-7a	CH	40.9	41.0	40.9
C-7b	CH	23.8	23.7	23.7
C-8	Me	16.6	16.8	16.8
C-9	Me	29.1	29.1	29.1
C-10	Me	27.5	26.8	26.9
C-11	Me	16.5	16.8	16.8
Sugar unit				
C-1′	CH	98.7	96.4	96.4
C-2′	CH	72.8	74.2	74.2
C-3′	CH	75.6	73.4	73.6
C-4′	CH	73.1	73.4	73.4
C-5′	CH	71.4	71.4	71.4
C-6′	CH_2	16.8	16.8	16.8
R				
C-1″	C			168.5
C-2″	C			129.1
C-3″	CH			140.1
C-4″	Me			16.8
C-5″	Me			20.9
R				
C-1″	C		177.6	
C-2″	CH		42.5	
C-3″	Me		16.9	
C-4″	Me		18.0	

effective was **2**, with an MIC_{50} of 14 μg/ml; the acyl derivatives at the 2′-hydroxy group were less active, especially in the case of the acetyl derivative **3**. Compound **1** showed an MIC_{50} of 36 μg/ml and completely inhibited virus plaque formation at 100 μg/ml (Table 3).

The results indicate, in general, that this type of glycoside is more active towards RNA-enveloped viruses as compared to RNA-naked viruses, as already found with the triterpene glycosides from *Uncaria tomentosa* and *Guettarda platypoda* (6). The most active compounds were those with the highest polarity, presenting three free hydroxy groups instead of two, as all the other glycosides. However, taking into account that the inhibitory effect was observed only at relatively high concentrations of drugs, we did not carry out further investigations.

EXPERIMENTAL

APPARATUS.—^{1}H- and ^{13}C-nmr spectra were obtained on a Bruker MW-250 spectrometer; chemical shifts are relative to TMS. The distortionless enhancement by polarization transfer (DEPT) experiments, NOEDS, and negative ion fabms were obtained as described earlier (1,2).

TABLE 2. ^{1}H-nmr Spectral Data of Compounds **2**, **6**, and **7** (500 MHz, CD_3OD, TMS internal standard).[a]

Proton	2	6[b]	7
Aglycone			
H-1a	0.62 ddd	0.62	0.60
H-2α	1.49 ddd	[c]	1.49
H-2β	1.57[d]	[c]	1.56
H-3α	1.78 br dd	[c]	1.76
H-3β	1.65[d]	[c]	[d]
H-4a	1.62[d]	[c]	1.62
H-5α	[c]	[c]	[c]
H-5β	[c]	[c]	[c]
H-6α	1.32 m	[c]	1.32
H-6β	1.88 m	[c]	1.82
H-7β	1.99[d]	[c]	1.95
H-7a	1.73 ddd	[c]	1.71
H-7b	0.156 dd	0.15	0.11
H-8	1.04 s	0.99	0.94
H-9	1.04 s	1.04	1.01
H-10	1.195 s	1.20	1.21
H-11	0.96 d	0.98	0.90
Fucosyl			
H-1′	4.45 d	4.45	4.71
H-2′	3.42 dd	5.02	5.1
H-3′	3.50 dd	3.65	3.65
H-4′	3.60 dd	3.65	3.65
H-5′	3.66 dq	3.65	3.65
H-6′	1.28 d	1.28	1.28
R			
H-2″		2.58 dq	
H-3″		1.22 d	
H-4″		1.18 d	
H-3″			6.22 dq
H-4″			2.05 dq
H-5″			1.92 m

[a]J values (Hz), compound **2**: $J_{1a\text{-}7b}=9.5$, $J_{1a\text{-}2\beta}=9.5$, $J_{1a\text{-}2\alpha}=6$, $J_{2\alpha\text{-}2\beta}=13.8$, $J_{2\alpha\text{-}3\alpha}=7.2$, $J_{2\alpha\text{-}3\beta}=0$, $J_{3\alpha\text{-}3\beta}=13.8$, $J_{7b\text{-}7a}=9.5$, $J_{11Me\text{-}7\beta}=7$, $J_{1'\text{-}2'}=7.6$, $J_{2'\text{-}3'}=9.8$, $J_{3'\text{-}4'}=3.4$, $J_{4'\text{-}5'}=0.9$, $J_{5'\text{-}6'}=6.4$; compound **6**: $J_{2''\text{-}3''}=7.5$, $J_{2''\text{-}4''}=7.5$; compound **7**: $J_{3''\text{-}4''}=7.5$, $J_{3''\text{-}5''}=1.5$, $J_{4''\text{-}5''}=1.5$.

[b]^{1}H-nmr spectrum obtained at 250 MHz.

[c]Overlapped with other signals in the region 1–2 ppm.

[d]Partially overlapping signals.

EXTRACTION AND ISOLATION.—Plants of *C. arvensis* were collected near Naples, Italy, in the spring of 1985; a sample has been deposited in Dipartimento di Chimica delle Sostanze naturali, University of Naples. The aerial parts of air-dried plant material (800 g) were extracted in a Soxhlet apparatus, first with petroleum ether (40°–70° bp) (8.6 g) and then with $CHCl_3$ (7 g). A portion of the $CHCl_3$ extract (3 g) was chromatographed on a Si gel column (120 g) using $CHCl_3$ with increasing amounts of MeOH as elution solvent; fractions of 15 ml were collected. Fractions 70–83 (700 mg) eluted with $CHCl_3$:MeOH (8:2) were combined according to tlc on SiO_2, developed with $CHCl_3$-MeOH-H_2O (40:9:1), and submitted to hplc on a C_{18} μ-Bondapak column (30 cm × 7.8 mm i.d.), differential refractometer detector. Elution with MeOH-H_2O (8:2) (flow rate 5 ml/min) gave compounds **1–5** (1,2), **6**, and **7**. Compound **6** (10 mg): Rt 40 min, $[\alpha]_D$ −39.9 (MeOH). Compound **7** (8 mg): Rt 46 min, $[\alpha]_D$ −30.5 (MeOH). ^{1}H- and ^{13}C-nmr data, in comparison with those for compound **2**, are in Tables 1 and 2.

CELLS AND VIRUSES.—HeLa (Ohio) and CER cells were grown at 37° in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

TABLE 3. Effect of Sesquiterpene Glycosides from *Calendula arvensis* on Cell Culture in Cell Cultures Infected by Vasicular Stomatitis Virus (VSV) and Rhinovirus (HRV type 1B).

Compound	Concentration[a] (μg/ml)	VSV		HRV % CPE reduction
		% plaque reduction	MIC_{50}	
1	100	100	36	25
	50	90		0
	20	4		0
	4	0		0
2	20	73	14	0
	4	19		0
	0.8	8		0
3	20	10	>20	0
	4	7		0
	0.8	0		0
4	20	30	>20	0
	4	20		0
	0.8	5		0
5	20	22	>20	0
	4	18		0
	0.8	5		0
6	20	9	>20	0
	4	0		0
	0.8	0		0
7	20	22	>20	0
	4	13		0
	0.8	0		0

[a]The compounds were tested up to the highest nontoxic concentration for cell cultures, which was the dose not affecting cell morphology, viability, and growth after 48 h of incubation at 37°.

HRV type 1B was propagated as described previously (6) and titered in HeLa cells to determine its tissue culture infective dose ($TCID_{50}$).

The Indiana serotype of VSV was grown in CER cells as described before (6); virus infectivity was measured by plaque assay.

EFFECT OF COMPOUNDS ON UNINFECTED CELLS.—HeLa (10^4 cells/well) and CER (3.5×10^3 cells/well) cells were plated in 96-well tissue culture plates in the presence of serial fivefold dilutions of each compound to be tested. All doses were tested in triplicate dishes. After 48 h of incubation at 37° in 5% CO_2, cytotoxicity was monitored by microscope examination of cell morphology, counting the number of cells in control and drug-treated wells, and checking cell viability by neutral red uptake.

ESTIMATION OF ANTI-RHINOVIRUS ACTIVITY.—The procedure employed was essentially as described before (6). Briefly, HeLa cell suspensions were infected with 100 $TCID_{50}$ of HRV in the presence or the absence of drugs at different concentrations. After 48 h of incubation (33°, 5% CO_2) plates were searched for the appearance of viral-induced CPE.

The activity of the compounds was expressed as the minimal inhibitory concentration (MIC_{50}) necessary to reduce CPE by 50% with respect to control infected cells.

ESTIMATION OF ANTI-VSV ACTIVITY.—Monolayers of CER cells were infected (1 h, 37°) with about 100 plaque-forming units of virus and, after washing, overlaid with medium containing the compounds at different concentrations. After incubation for 48 h at 37°, the cells were stained with neutral red and the plaques counted.

The MIC_{50} value was the minimal inhibitory concentration reducing the plaque number by 50%. The value was calculated by plotting the percentage plaque number in treated cells versus compound concentration.

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