New Glycosides from *Capsicum annuum* L. Var. *acuminatum*. Isolation, Structure Determination, and Biological Activity

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Investigation of polar extracts from ripe fruits of *Capsicum annuum* L. var. *acuminatum* yielded three new glycosides, capsosides A (**1**) and B (**2**) and capsianoside VII (**3**), along with seven known compounds (**4**−**10**). The chemical structures were elucidated mainly by extensive nuclear magnetic resonance methods and mass spectrometry, and the biological activities of icariside E_5 (**4**) were tested by different assays. Icariside E_5, in contrast to capsaicin, neither induces an increase in the intracellular levels of reactive oxygen species nor affects the mitochondria permeability transition, and it does not signal through the vanilloid receptor type 1. Interestingly, this compound protects Jurkat cells from apoptosis induced by the oxidative stress mediated by serum withdrawal. These results suggest that icariside E_5 may have antioxidant properties that strengthen the importance of peppers in the Mediterranean diet.

Keywords: *Capsicum annuum L.; Solanaceae; red pepper; glycosides; ROS; vanilloid receptor 1; apoptosis*

INTRODUCTION

One of the main goals of the chemistry of naturally occurring compounds is screening for promising biologically active substances of plant origin. *Capsicum* species are very important plants used as vegetable foods, spices, and external medicine, and capsaicinoids are the group of compounds responsible for the "heat" sensation of *Capsicum* fruits. These metabolites are also used as pest repellants in agriculture, and there is interest in using them as synergists with organophosphate insecticides (*1*). Capsaicin, a prototypical capsaicinoid, is used to mitigate neurogenic pain; several products applied as creams and gels (e.g. Axsain and Zostrix) have appared on the market as topical analgesics, but irritance severely limits the pharmacological use of capsaicinoid.

It is generally accepted that specific vanilloid receptors mediate the effects of capsaicin in some cell systems. Thus, transient exposure of nociceptor terminals to capsaicin leads to excitation and desensitization in dorsal root ganglion neurons and an increase in intracellular calcium (*2*−*4*). The recent cloning of a vanilloid receptor, termed VR1 (vanilloid receptor subtype 1), has shown that this receptor belongs to the family of putative store-operate calcium channels and is expressed exclusively in trigeminal dorsal root sensory ganglia (*4*). The generation of *knock out* mice for VR1 gene has definitively proved the correlation between capsaicin pungency and this receptor in vivo (*5*).

In addition to these biological activities, capsaicin is a quinone analogue that can inhibit the NADH oxidase found in plasma membranes (*6*) and induce apoptosis by a vanilloid receptor-independent pathway in transformed cells and in activated T cells (*7*−*9*). We have shown recently that capsaicin-induced apoptosis in transformed cells was preceded by an increase in the reactive oxygen species (ROS) and a subsequent disruption of the transmembrane mitochondria potential ($\Delta\psi_m$), a process that can be related to the mitochondrial permeability transition pore opening (PT), because PT inhibitors (such as Bcl-2 and cyclosporine-A) inhibit this disruption (*8*, *9*).

Peppers are also a good source of vitamins A, C, and E, which are present in high concentration in various pepper types (*10*). Total flavonoid and phenolic components are important dietary antioxidants (*11*) and, with vitamins, play an important role in cancer chemoprevention (*12*).

Although carotenoids, lipids, and capsaicins have been studied in depth, there are few reports on the highly polar components of *Capsicum* species. We have examined the chemical composition of the butanol-soluble fraction of *C. annuum* L. var. *acuminatum* (ripe fruits), and here we report the structure elucidation of 10 compounds, 3 of which (**1**−**3**) are based on a new structural type. For one of them (**4**), we have tested some of the biological activities in vitro that have been attributed to the prototypical capsaicinoid, capsaicin.

MATERIALS AND METHODS

General Methods. Fast atom bombardment mass spectra (FABMS) were recorded in a glycerol matrix on a VG PROSPEC

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10.1021/jf0013454 CCC: $20.00
Published on Web 03/20/2001

Table 1. DCCC Fractionation and HPLC Purification of the High-Polar Components from *C. annuum* L. Var *acuminatum*

fractions	amount (mg)	compound	MeOH/H_2O
71−100	33.7	**7**, **8**, **9**	3:7
101−130	19.3	**2**, **7**, **8**, **9**	3:7
131−150	13.7	adenosine	2:8
162−176	38.3	**5**, **6**	6:4
177−181	19.0	**6**	6:4
182−190	28.7	**3**, **6**	7:3
191−199	13.3	**3**	7:3
200−220	36.8	**4**	35:65
237−240	25.0	**10**	3:7
245−253	6.8	**1**	8:2

instruments (Cs^+ ions of energy of 4 kV). High-resolution (HR) FABMS spectra were recorded in a glycerol matrix on a VG AUTOSPEC instrument. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Kratos Kompact MALDI IV (Shimadzu), reflectron mode (20 kV accelerating voltage), laser power pulse 105, matrix system 2,5-dihydroxybenzoic acid (DHB); the single charged $(M + H)^+$ of gramicidin S (FW = 1142.5) was used as external standard for the mass calibration. Optical rotations were determined on a Perkin-Elmer 141 polarimeter; infrared spectra were recorded on a Bruker IFS-48 spectrometer. GLC analyses were performed on a Carlo Erba Fractovap 4160 for capillary column (SPB-1, 25 m, 152 °C, helium carrier, flow = 5 mL min^{-1}); GC-MS was determined with an HP 5890 by employing a capillary column (RTX-1 Chemtek, 30 m × 0.25 mm i.d., 0.25 μm df). ^1H and ^{13}C NMR spectra were recorded at 500.13 and 125.76 MHz, respectively, on a Bruker AMX-500 spectrometer equipped with a Bruker X32 computer, using the UXNMR software package. Chemical shifts were referenced to the residual solvent signal (CD_3OD δ_H 3.34, δ_C 49.0; $CDCl_3$ δ_H 7.26, δ_C 77.0; D_2O δ_H 5.00). The multiplicities of ^{13}C resonances were determined by DEPT experiments. The ^1H-detected one-bond and multiple-bond ^{13}C multiple-quantum coherence experiments (HMQC and HMBC, respectively) utilized a 5-mm probe with reverse geometry and the sample was not spun. The magnitude of delay for optimizing one-bond correlation in the HMQC spectrum and suppressing them in the HMBC spectrum was 3.5 ms, and the evolution delay for long-range couplings in the latter was set to 60 ms. A DCC-A (droplet counter current chromatography) apparatus equipped with 300 tubes was manufactured by Buchi. HPLC was performed on a Waters model 6000A pump equipped with U6K injector and a differential refractometer, model 401, with a reversed phase C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.).

Plant Material. Ripe fruits of *C. annuum* L.var *acuminatum* were collected at Campobasso (Italy) in July 1998 and identified at the Dipartimento di Scienze Animali, Vegetali e dell'Ambiente (Università del Molise). A voucher specimen is preserved at Dipartimento di Scienze e Tecnologie Agro-Alimentari, Ambientali e Microbiologiche dell'Università del Molise, Campobasso, Italy.

Extraction and Isolation. The fresh ripe fruits (pericarp, 400 g) were separated from the seeds, chopped, and soaked in MeOH at room temperature (2.5 L). Evaporation of MeOH extracts afforded 7.5 g of a glassy material, which was then subjected to Kupchan's partitioning methodology (*13*) to give four extracts: *n*-hexane (2.65 g), CCl_4 (0.780 g), $CHCl_3$ (1.48 g), and *n*-BuOH (1.97 g). The *n*-BuOH extract (1.97 g) was chromatographed by DCCC using *n*-BuOH/Me_2CO/H_2O (3:1:5) in the descending mode (the upper phase was the stationary phase), flow rate = 6 mL/h; 3 mL fractions were collected and monitored by thin-layer chromatography (TLC) on SiO_2 with *n*-BuOH/HOAc/H_2O (12:3:5) and $CHCl_3$/MeOH/H_2O (80:18:2). The results are summarized in Table 1. Each fraction was then purified by HPLC on a C_{18} column (30 cm × 3.9 mm i. d.) with MeOH/H_2O in different percentages as eluant.

*Capsoside A (**1**).* Yield 2.5 mg; $[\alpha]_D^{25}$ +30° (H_2O, *c* 0.2); FABMS (−ve ion) *m/z* 693 $[M - H]^-$; HRFABMS, found *m/z* 694.3794, calcd for $C_{33}H_{58}O_{15}$ *m/z* 694.3776; ^1H and ^{13}C NMR, Table 2.

Table 2. ^1H and ^{13}C NMR Data ($CDCl_3$) for Compound 1a[a]

C	δ_H	δ_C	C	δ_H	δ_C
	glycerol			**acyl group at C-2**	
1*a*	4.00 dd (11.0, 4.4)	67.6	1		172.4
1*b*	3.67 dd (11.0, 5.8)		2	2.82 br t	30.9
2	5.20 (m)	71.0	3	5.37[b]	125.6
3*a*	4.16 dd (11.7, 6.6)	62.3	4	5.36[b]	129.9
3*b*	4.31 dd (11.7, 2.2)		5	2.10 (m)	20.3
			6	0.98 t (7.6)	14.3
	galactose				
1′	4.50 d (8.0)	100.8		**acyl group at C-3**	
2′	5.19[b]	69.5	1		173.0
3′	5.01 dd (2.9, 10.3)	70.8	2	2.31 br t	34.1
4′	5.43 br d (2.9)	67.7	3	1.62	28.5
5′	3.86 br t (6.6)	70.6	4−9	1.26	30.2
6′	3.44 dd(10.3, 2.8)	66.9	10	1.30	31.7
	3.79 dd(10.3, 5.2)		11	1.26	22.4
			12	0.88 t (7.6)	14.5
	galactose				
1″	4.96 d (2.9)	97.5		**acetates** $\underline{CH_3}CO$	$CH_3\underline{CO}$
2″	5.13 dd[b]	69.0			169.3
3″	5.30 dd (11.0, 2.9)	68.6		1.99, 2.07, 2.08,	169.5
4″	5.48 br d (2.9)	69.0		2.09, 2.10, 2.15 (×2)	169.7
5″	4.22 br t (6.6)	68.0			169.7
6″	4.11[b]	62.0			169.9
	4.13[b]				170.1
					170.2

[a] *J* are given in hertz. [b] Signals overlapped with other signals.

Table 3. ^1H NMR Data (D_2O, 500 MHz) of Capsoside B (2)[a]

H	δ_H	H	δ_H	H	δ_H
			glucose		**glucose**
1*a*	3.83 dd (12.7, 5.9)	1′	4.68 d (8.1)	1″	4.63 d (7.8)
1*b*	4.05 dd[b]	2′	3.47 dd (8.1, 8.8)	2″	3.41 dd (7.8, 8.8)
2	1.77 m	3′	3.64[b]	3″	3.64[b]
3	1.39 m	4′	3.62[b]	4″	3.56 t (8.8)
4	1.72 m	5′	3.77 m	5″	3.60[b]
5	1.04 d (6.9)	6′	4.36 dd (11.8, 2.4)	6″	4.07 dd (12.0, 2.6)
6	1.04 d (6.9)		4.02 dd (11.8, 5.8)		3.88 dd (12.0, 5.9)

[a] *J* are given in hertz. [b] Signals overlapped with other signals.

*Acetylation of **1** To Give **1a**.* Compound **1** (1.5 mg) was treated with 0.2 μL of Ac_2O in 0.5 mL of anhydrous pyridine overnight at room temperature. The mixture was evaporated to dryness to yield the hepta-acetate **1a**: FABMS (−ve ion), *m/z* 987 $[M - H]^-$.

*Alkaline Treatment of **1**.* A solution of **1** (1.0 mg) in anhydrous MeOH (1 mL) was treated with 2.8% NaOMe/MeOH (1 mL) and stirred at 25 °C for 2 h. The reaction mixture was neutralized with NH_4Cl and partitioned with Et_2O and then with *n*-BuOH. The Et_2O phase was evaporated under N_2 to give the mixture of fatty acid methyl esters.

Gas Chromatography−Mass Spectrometry (GC-MS) Analysis of Fatty Acid Methyl Esters. The fatty acid methyl ester mixture was subjected to GC-MS (column temperature of 140−270 °C, rate of temperature increase = 3 °C min^{-1}). The results were as follows: t_R = 21.9 min, methyl dodecanoate [*m/z* 214 (M^+), *m/z* 199 ($M^+ - CH_3$)]; t_R = 9.8 min, methyl 3-hexenoate [*m/z* 128 (M^+)].

*Capsoside B (**2**).* Yield 0.7 mg; $[\alpha]_D^{25}$−17° (MeOH, *c* 0.07); HRFABMS, found *m/z* 426.2120, calcd for $C_{18}H_{34}O_{11}$ *m/z* 426.2101; ^1H NMR (D_2O), Table 3.

Methanolysis and Sugar Analysis. A solution of **2** (0.5 mg) in anhydrous 2 M HCl/MeOH (0.5 mL) was heated at 80 °C in a stoppered reaction vial for 8 h. Once cooled, the reaction mixture was neutralized with Ag_2CO_3 and centrifuged, and the supernatant was evaporated to dryness under N_2. The residue was trimethylsilylated with 1-(trimethylsilyl)imidazole and pyridine for 15 min at 70 °C. GLC analysis (25 m, SPB-1 capillary column; 158 °C; helium carrier flow = 10 mL min^{-1}) gave peaks that coeluted with those of silylated methylglucoside as standard.

Table 4. ^{1}H and ^{13}C NMR (CD_3OD, 500 MHz) of Capsianoside VII (3)[a]

C	δ_H	δ_C	C	δ_H	δ_C	C	δ_H	δ_C
1*a*	5.21 dd (1.5. 18)	112.1	17*a*	4.36 d (11.7)	67.8		**glucose**	
1*b*	5.06 dd (11, 1.5)		17*b*	4.16 d (11.7)		1″	4.23 d (7.8)	102.3
2	5.94 dd (11, 18)	146.4	18	1.64 s	16.1	2″	3.25 dd (7.8, 8.8)	75.3
3		73.9	19	1.64 s	16.3	3″	3.29[b]	76.8
4	1.54 m	43.5	20	1.27 s	27.3	4″	3.66[b]	72.4
5	2.08 m	23.7				5″	3.41[b]	75.5
6	5.15 br t	125.8				6″	3.95 dd (11, 2.3)	66.9
7		135.5					3.64 dd[b]	
8	2.02 m	40.9						
9	2.11 m	27.7		**rhamnose I**			**rhamnose II**	
10	5.15 br t	125.9	1′	4.74 br s	101.7	1‴	4.85 br s	102.8
11		136.0	2′	3.88 br d (3.2)	72.6	2‴	3.86 br d (3.2)	72.3
12	2.02 m	41.0	3′	3.65 dd[b]	79.3	3‴	3.71 dd (9.6, 3.2)	72.3
13	2.20 m	27.3	4′	3.45 t (9.6)	73.8	4‴	3.38 t[b]	73.9
14	5.43 t (7)	131.3	5′	4.02 dt (6.2, 9.6)	70.7	5‴	3.75 dt (9.6, 6.2)	69.7
15		132.5	6′	1.28 d (6.2)	17.9	6‴	1.29 d (6.2)	18.2
16	1.80 s	22.0						

[a] *J* are given in hertz. [b] Signals overlapped with other signals.

*Capsianoside VII (**3**).* Yield 1.5 mg; $[\alpha]_D^{25}$ −32° (MeOH, *c* 0.1); ^{1}H and ^{13}C NMR, Table 4.

Methanolysis and Sugar Analysis. Methanolysis of **3** (0.5 mg) and subsequent GLC analysis of the silylated sugar compounds (152 °C on a 25 m SPB-1 capillary column; helium carrier flow = 10 mL min^{-1}) was carried out as previously described for capsoside B (**2**). The identification was based on cochromatography with silylated methylglucoside and silylated methylrhamnoside as standards.

*Icariside E_5 (**4**).* Yield 3.0 mg; $[\alpha]_D^{25}$ −116.3° (MeOH, *c* 0.2); FABMS (+ve ion), *m/z* 545 $[M + Na]^+$, *m/z* 383 $[(M + Na) - 162]^+$; ^{1}H NMR (CD_3OD) δ_H 6.97 (1H, d, *J* = 1.6 Hz, H-2′), 6.94 (1H, d, *J* = 1.6 Hz, H-6′), 6.61 (1H, overlapped, H-5), 6.60 (1H, overlapped, H-7′), 6.59 (1H, overlapped, H-2), 6.51 (1H, dd, *J* = 8.0, 1.6 Hz, H-6), 6.33 (1H, dt *J* = 16.0, 5.8 Hz, H-8′), 4.25 (2H, d, *J* = 5.8 Hz, H_2-9′), 4.00 (1H, m, H-8), 3.86 (3H, s, OCH_3-3′), 3.79 (1H, overlapped, H-9), 3.72 (1H, s, OCH_3-3), 3.69 (1H, overlapped, H-9), 3.00 (1H, dd, *J* = 13.5, 5.9 Hz, H-7), 2.75 (1H, dd, 13.5, 9.3 Hz, H-7); glucose δ_H 4.72 (1H, d, *J* = 7.3 Hz, H-1″), 3.81 (1H, dd, *J* = 11.8, 2.3 Hz, H-6″), 3.70 (1H, dd, *J* = 11.8, 5.1 Hz, H-6″), 3.47 (1H, t, *J* = 8.4 Hz, H-2″), 3.43 (1H, t, *J* = 8.4 Hz, H-3″), 3.41 (1H, t, *J* = 8.4 Hz, H-4″), 3.14 (1H, m, H-5″); ^{13}C NMR (CD_3OD) δ_C 133.2 (C-1), 116.0 (C-2), 148.3 (C-3), 145.3 (C-4), 114.1 (C-5), 122.8 (C-6), 39.1 (C-7) 43.0 (C-8), 66.5 (C-9), 135.4 (C-1′), 108.7 (C-2′), 153.4 (C-3′), 145.0 (C-4′), 139.0 (C-5′), 118.7 (C-6′), 131.5 (C-7′), 129.6 (C-8′), 63.5 (C-9′), 56.0 (OMe-3), 55.9 (OMe-3′); glucose δ_C 106.0 (C-1″), 75.7 (C-2″), 77.5 (C-3″), 71.0 (C-4″), 77.8 (C-5″), 62.3 (C-6″); ^{1}H NMR (D_2O) δ_H 7.39 (1H, d, *J* = 1.6 Hz, H-2′), 7.24 (1H, d, *J* = 1.6 Hz, H-6′), 6.90 (1H, dd, *J* = 8.0, 1.6 Hz, H-5), 6.83 (1H, d, *J* = 16.0 Hz, H-7′), 6.79 (1H, dd, *J* = 8.0, 1.6 Hz, H-6), 6.67 (1H, d, *J* = 1.6 Hz, H-2), 6.61 (1H, dt, *J* = 16.0, 5.1 Hz, H-8′), 4.45 (2H, d, *J* = 5.8 Hz, H_2-9′), 4.00 (1H, m, H-8), 3.97 (3H, s, OCH_3-3′), 3.82 (2H, overlapped, H_2-9), 3.73 (3H, s, OCH_3-3), 3.18 (1H, dd, *J* = 11.0, 4.8 Hz, H-7), 3.01 (1H, t, *J* = 11.0 Hz, H-7); glucose δ_H 5.00 (1H, d, overlapped, H-1″), 3.90 (2H, overlapped, H_2-6″), 3.62 (1H, t, *J* = 8.8 Hz, H-3″), 3.55 (1H, t, *J* = 9.5 Hz, H-4″), 3.43 (1H, t, *J* = 8.8 Hz, H-2″), 2.86 (1H, m, H-5″).

*Enzymatic Hydrolysis of Icariside E_5 (**4**) To Give **4a**.* Compound **4** (1 mg) in a citrate buffer (1 mL; pH 4.5) was incubated with a glycosidase mixture (2.5 mg) of *Charonia lampas* (Shikagaku Kogyo) at 37 °C. After 3 days, the TLC analysis showed that the starting material had disappeared and displayed one major spot. The mixture was passed through a C-18 Sep-Pak cartridge, washed with H_2O, and eluted with MeOH. The MeOH was evaporated to dryness, and the residue was submitted to HPLC (C_{18} μ-Bondapak column, 30 cm × 3.9 mm i.d.; MeOH/H_2O 3:7) to give compound **4a**.

*Compound **4a**.* $[\alpha]_D^{25}$ −18.4° (MeOH, *c* 0.05); FABMS (−ve ion), *m/z* 359 $[M - H]^-$; ^{1}H NMR (CD_3OD) δ_H 6.97 (1H, d, *J* = 1.6 Hz, H-2′), 6.95 (1H, d, *J* = 1.6 Hz, H-6′), 6.61 (1H, overlapped, H-7′), 6.59 (1H, overlapped, H-5), 6.58 (1H, overlapped, H-2), 6.51 (1H, dd, *J* = 8, 1.6 Hz, H-6), 6.33 (1H, dt, *J* = 15.8, 5.5 Hz, H-8′), 4.26 (2H, d, *J* = 5.5 Hz, H_2-9′), 4.00 (1H, m, H-8), 3.86 (3H, s, OMe-3′), 3.79 (1H, overlapped, H-9), 3.72 (1H, s, OMe-3), 3.69 (1H, overlapped, H-9), 3.01 (1H, dd, *J* = 13.5, 5.9 Hz, H-7), 2.78 (1H, dd, *J* = 13.5, 9.3 Hz, H-7).

*Capsianoside III (**5**).* Yield 3.2 mg; ^{1}H and ^{13}C NMR data are identical to those reported in the literature (*14*).

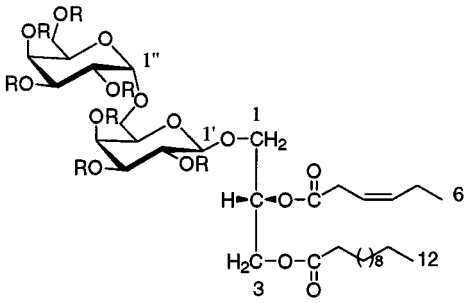
*Capsianoside II (**6**).* Yield 4.2 mg; ^{1}H and ^{13}C NMR data are identical to those reported in the literature (*14*).

*cis-p-Coumaric Acid 4-O-β-D-Glucoside (**7**).* Yield 2.0 mg; $[\alpha]_D^{25}$ −35° (MeOH, *c* 0.2); FABMS (+ve ion), *m/z* 349 $[M + Na]^+$; MALDI-TOF MS, *m/z* 349 $(M + Na)^+$ and *m/z* 371 $(MNa + Na)^+$; ^{1}H NMR (CD_3OD) δ_H 7.55 (2H, each d, *J* = 8.1 Hz, H-2 and H-6), 7.03 (2H, each d, *J* = 8.1 Hz, H-3 and H-5), 6.32 (1H, d, *J* = 12.5 Hz, H-7), 5.99 (1H, d, *J* = 12.5 Hz, H-8), 5.00 (1H, d, overlapped, H-1′ glucose), 3.90 (1H, d, *J* = 11.8 Hz, H-6′ glucose), 3.74 (1H, dd, *J* = 11.8, 3.8 Hz, H-6′ glucose), 3.50 (1H, t, overlapped H-2′ glucose), 3.48−3.45 (3H, overlapped H-3′, H-4′, H-5′ glucose); ^{13}C NMR (CD_3OD) δ_C 132.4 (C-1), 131.1 (C-2 and C-6), 117.3 (C-3 and C-5), 158.6 (C-4), 130.0 (C-7), 127.5 (C-8), 177.1 (C-9); glucose δ_C 102.1 (C-1′), 75.0 (C-2′), 78.2 (C-3′), 71.4 (C-4′), 78.1 (C-5′), 62.5 (C-6′).

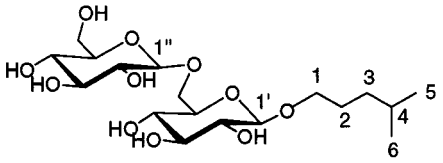
*trans-Sinapoyl β-D-Glucoside (**8**).* Yield 0.4 mg; $[\alpha]_D^{25}$ 0° (MeOH, *c* 0.04); FABMS (+ve ion), *m/z* 419 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ_H 7.74 (1H, d, *J* = 15.6 Hz, H-7), 6.69 (2H, s, H-2 and H-6), 6.45 (1H, d, *J* = 15.6 Hz, H-8), 5.60 (1H, d, *J* = 8.0 Hz, H-1′ glucose), 3.91 (6H, s, OMe), 3.90 (1H, d, *J* = 12.0 Hz, H-6′ glucose), 3.72 (1H, dd, *J* = 12.0, 4.0 Hz, H-6′ glucose), 3.41−3.50 (3H, overlapped H-3′, H-4′, H-5′ glucose); ^{13}C NMR (CD_3OD) δ_C 126.5 (C-1), 108.7 (C-2 and C-6), 147.6 (C-3 and C-5), 140.0 (C-4), 144.6 (C-7), 115.5 (C-8), 168.2 (C-9), 56.8 (OMe); glucose δ_C 98.3 (C-1′), 75.0 (C-2′), 78.5 (C-3′), 71.4 (C-4′), 78.1 (C-5′), 62.4 (C-6′).

*Vanilloyl β-D-Glucoside (**9**).* Yield 0.7 mg; $[\alpha]_D^{25}$ −16.5° (MeOH, *c* 0.07); FABMS (+ve ion), *m/z* 353 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ_H 7.67 (1H, dd, *J* = 8.0, 1.6 Hz, H-6), 7.63 (1H, d, *J* = 1.6 Hz, H-2), 6.87 (1H, d, *J* = 8.0, Hz, H-5), 5.70 (1H, d, *J* = 7.8 Hz, H-1′ glucose), 3.93 (3H, s, OMe), 3.88 (1H, dd, *J* = 11.8, 2.3 Hz, H-6′ glucose), 3.73 (1H, dd, *J* = 11.8, 5.0 Hz, H-6′ glucose), 3.52 (1H, dd, overlapped H-2′ glucose), 3.41−3.50 (3H, overlapped H-3′, H-4′, H-5′ glucose); ^{13}C NMR δ_C 122.3 (C-1), 114.7 (C-2), 148.5 (C-3), 154.0 (C-4), 116.3 (C-5), 125.5 (C-6), 167.0 (C═O), 56.5 (OMe); glucose δ_C 97.5 (C-1′), 74.6 (C-2′), 78.3 (C-3′), 71.4 (C-4′), 78.2 (C-5′), 62.4 (C-6′).

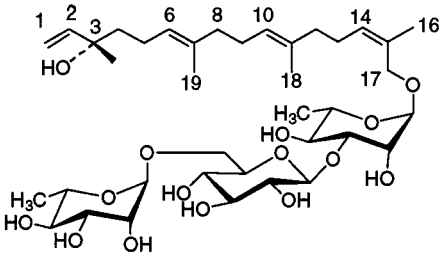
*Quercetin 3-O-Rhamnoside (**10**).* Yield 2.8 mg; FABMS (−ve ion), *m/z* 447 $[M - H]^-$; ^{1}H NMR (CD_3OD) δ_H 7.36 (1H, d, *J* = 1.6 Hz, H-2′), 7.34 (1H, dd, *J* = 8.0, 1.6 Hz, H-6′), 6.94 (1H, d, *J* = 8.0 Hz, H-5′), 6.40 (1H, d, *J* = 1.6 Hz, H-8), 6.23 (1H, d, *J* = 1.6 Hz, H-6), 5.37 (1H, br s, H-1″), 4.25 (1H, br d, *J* = 3.2 Hz, H-2″), 3.77 (1H, dd, *J* = 3.2, 9.4 Hz, H-3″), 3.43 (1H, m, H-5″), 3.36 (1H, t, overlapped H-4″), 0.96 (3H, d, *J* = 6.2 Hz, CH_3-6″); ^{13}C NMR (CD_3OD) δ_C 158.5 (C-2), 136.2 (C-3), 179.6 (C-4), 163.2 (C-5), 99.8 (C-6), 166.0 (C-7), 94.7 (C-8), 159.2 (C-



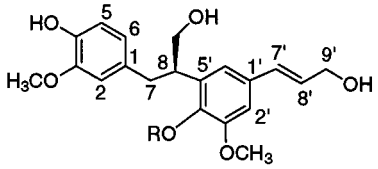
Capsoside A (1) R=H
1a R=Ac



Capsoside B (2)



Capsianoside VII (3)



Icariside E_5 (4) R=β-D-glucose
4a R=H

Figure 1. New glycosides and the rare icariside E_5 from *C. annuum* L. var. *acuminatum*.

9), 105.8 (C-10), 122.8 (C-1′), 116.3 (C-2′), 146.4 (C-3′), 149.8 (C-4′), 116.9 (C-5′), 122.8 (C-6′); rhamnose δ_C 103.5 (C-1″), 72.0 (C-2″), 72.0 (C-3″), 72.1 (C-4″), 71.9 (C-5″), 17.6 (C-6″).

Cell Lines and Reagents. Jurkat cells (ATCC, Rockville, MD) were maintained in exponential growth in RPMI-1640 medium (Bio-Whittaker, VerViers, Belgium) and the human embryonic kidney-derived 293T cells in DMEM (Bio-Whittaker). The culture media were supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and the antibiotics penicillin and streptomycin (Gibco, Paisley, Scotland). Capsaicin was purchased from Sigma.

Determination of Mitochondrial Transmembrane Potential and ROS Generation. To study the mitochondrial transmembrane potential ($\Delta\psi_m$) and the superoxide anion generation (ROS), we incubated the cells (10^6/mL) in phosphate-buffered saline (PBS) with 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (green fluorescence) (20 nM) (Molecular Probes Europe) and dihydroethidine (HE) (red fluorescent after oxidation) (2 μM) (Sigma) for 20 min at 37 °C, followed by analysis on an Epics XL analyzer (Coulter, Hialeah, FL). When indicated, the percentages of dead and live cells were determined by incubating an aliquot of the cells with 10 μg/mL of propidium iodide (PI) for 2 min at room temperature. Cells permeable to PI (dead cells) appeared as red fluorescence by flow cytometry.

Determination of Nuclear DNA Loss and Cell Cycle Analysis. The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined by ethanol fixation (70%, for 24 h at 4 °C). Then, the cells were washed twice with PBS containing 4% glucose and subjected to RNA digestion (RNase-A, 50 units/mL) and PI (20 μg/mL) staining in PBS for 1 h at room temperature. The cell cycle was analyzed by cytofluorometry as previously described (*15*). With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and subsequent staining allow determination of the percentage of subdiploid cells (sub-G_0/G_1 fraction).

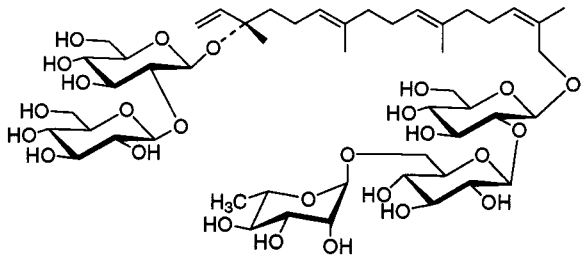
Transient Transfections and Cell Death Assays. 293T cells (10^5/mL) were transiently cotransfected with the pEGFP-C1 plasmid encoding a brighter fluorescent variant of the *green fluorescence protein* (Clontech, Palo Alto, CA) and either the plasmid encoding the complete rat cDNA VR1 or the empty vector, pcDNA3 (Invitrogen, Leek, The Netherlands). The transfections were made using the lipofectamine reagent (Life Technologies, Madrid, Spain) according to the manufacturer's recommendations. Forty-eight hours after transfection, the cells were stimulated with the different compounds for 6 h and the percentage of adherent green fluorescent cells (live cells) ws measured by flow cytometry.

RESULTS AND DISCUSSION

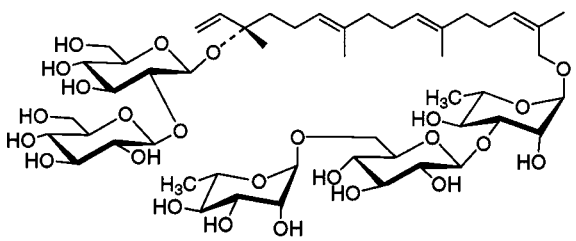
Chromatographic separation of *n*-butanol extracts of *C. annuum* L. var. *acuminatum* yielded three new glycosides (**1**−**3**) in very minute amounts and seven other known compounds (**4**−**10**) (Figures 1 and 2).

Galactolipids occur widely in the plant kingdom and form the major lipid in photosynthetic tissues. The new diacyldigalactosyl glycerol **1**, named capsoside A, is chemically uncommon for the atypical short alkyl chain. During the green stage of the fruits the activation of β-galactosidase enzyme releases free galactose from membrane and seems to play an important role in the ripening of fruits (*16*). Capsoside B (**2**), an aliphatic diglucoside, could be considered a cell wall polysaccharide. Disaccharides and trisaccharides esterified with ferulic acid and *p*-coumaric acid have been identified as components of the cell wall of higher plants (*17*). The glycoside **3** is related to the known capsianoside II (*14*) and named capsianoside VII.

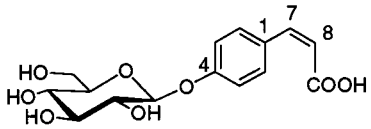
The lignan glycoside icariside E_5 (**4**), which was previously isolated from *Epimedium diphyllum* (*18*), Albizziae Cortex (*Albizzia julibrissin)* (*19*), and *Ehretia ovalifolia* (*20*), is very unusual. All of these plants, typical of China and Japan, have been used since ancient times as tonics or dyestuffs (*20*). There is no report of icariside E_5 from European plants, and this is the first occurrence in *Capsicum* species. Icariside E_5 shares a vanillyl moiety (4-hydroxy-3-methoxybenzyl), which is essential for the biological activities detected in capsaicin and resiniferatoxin (*21, 22*). This finding prompted us to evaluate the biological activity of compound **4** on VR1 on induction of apoptosis by a



Capsianoside III (5)



Capsianoside II (6)



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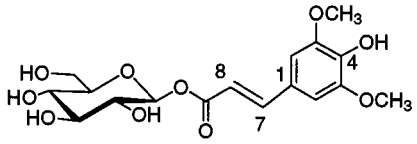

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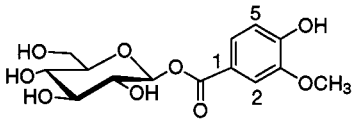

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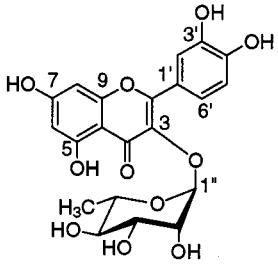

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Figure 2. Glycosides isolated from *C. annuum* L. var. a*cuminatum*.

vanilloid receptor-independent pathway and for its possible antioxidant activity.

Capsoside A (**1**) displayed in the HRFABMS a molecular ion peak at *m/z* 694.3794 in accordance with the empirical formula $C_{33}H_{58}O_{15}$. The infrared spectrum ($CHCl_3$) showed the presence of hydroxyl groups and ester functions (3404, 1726 cm^{-1}). The ^1H NMR spectrum of **1** in CD_3OD displayed a resonance at δ 1.32, a series of overlapping signals between δ 3.0 and 4.0, and two doublets at δ 4.88 ($J = 2.9$ Hz) and δ 4.27 ($J = 7.8$ Hz) attributable to two anomeric protons. These features suggested the glycolipidic nature of the compound. A better proton dispersion was obtained in the proton spectrum of the peracetate **1a**, by treatment with Ac_2O/pyridine at room temperature. This derivative was used for all NMR structural studies. The ^1H NMR spectrum of **1a** in $CDCl_3$ revealed two methyls at δ 0.88 and 0.98, each resonating as a triplet, and seven distinct methyl singlets between δ 1.99 and 2.15, corresponding to seven acetoxy groups, whereas the corresponding carbonyl carbon atoms resonated from δ 169.3 to 170.2 in the ^{13}C NMR spectrum. The region of the proton NMR spectrum between δ 3.4 and 5.5 contained a number of well-resolved multiplets. Interpretation of an $^1H-^1H$ COSY experiment with **1a** clearly indicated that signals belonged to five separate spin systems. The first was characterized by a methine proton (δ 5.20, m) coupled with two pairs of methylene protons and indicative of a glycerol moiety in which CH_2-1 and CH_2-3 resonate at rather different chemical shifts (Table 2). The second and third spin systems relate to two sugar units, and the NMR data of monosaccharides were identical to those of 3-*O*-(α-D-galactopyranosyl-(1→6)-*O*-*β*-D-galactopyranosyl)-*sn*-glycerol nona-acetate obtained by degradation of digalactosyl diacylglycerol previously isolated from American *Sonchus arvensis* (*23*). The HMBC cross-peak H-1′ (δ 4.50)/C-1 (67.6 ppm) implied that the sugar residue is bonded to C-1 of glycerol, and the correlation H-1″ (δ 4.96)/ C-6′ (66.9) showed a terminal galactose linked to C-6′ of *β*-D-galactose. The remaining two spin systems, evidenced by a COSY experiment, represented the two acyl groups linked to the glycerol moiety. Treatment of **1** with NaOMe/MeOH furnished a mixture of fatty acid methyl esters. This last mixture was determined by GC-MS analysis to be a ratio 1:1 of a methyl dodecanoate *m/z* 214 $(M)^+$ and methyl 3-hexenoate *m/z* 128 $(M)^+$. The locations of these fatty acid residues on the glycerol moiety were defined by interpretation of HMBC and $^1H-^1H$ COSY experiments on **1a** derivative. The HMBC cross-peak (^3J) H-3 (δ 4.16)/ $\underline{CO}$-$(CH_2)_{10}CH_3$ (δ 173.0) and $-CO\underline{CH_2}(CH_2)_9CH_3$ (δ 2.31)/$\underline{CO}(CH_2)_{10}CH_3$ (173.0) showed that the C_{12} alkyl chain is bonded to C-3 of glycerol. Similarly, an HMBC experiment revealed long-range couplings between the carbonyl signal (172.4 ppm)/H-2 proton signal (δ 5.20) and C-2 (71.0 ppm)/$CO\underline{CH_2}(CH)_2CH_2CH_3$ (δ 2.82). The position of the double bond on the C_6 fatty acid was established from a COSY sequence, whereas the *Z* stereochemistry was assigned on the basis of the high-field chemical shift of the allylic methylene carbons in the ^{13}C NMR spectrum (*24*). We propose the *2S* stereochemistry at capsoside A (**1**) on the basis of NMR and $[\alpha]_D$ comparison with data of digalactolipid from *Sonchus arvensis* (*23*). Thus, it has been concluded the chemical structure of capsoside A (**1**) is (2*S*)-2-*O*-(3-*Z*-

hexenoyl)-3-*O*-(dodecanoyl)glyceryl 6′-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside.

Capsoside B (**2**) is the minor component of the mixture obtained from *n*-butanol extracts (1.0 mg). It showed a quasimolecular ion peak at *m/z* 449 $[M + Na]^+$ in the FABMS spectrum (positive ion). The structure was mostly obtained by interpretation of a $^1H-^1H$ COSY experiment and by HRFABMS. The ^1H NMR spectrum exhibited signals of two overlapped methyl groups resonating as doublets (δ 1.04), two anomeric protons (δ 4.68 and 4.63), and sugar methylene proton signals partially overlapped in the region between δ 4.10 and 3.40. Inspection of the COSY allowed assembly of the C_6 chain starting from the doublets at δ 1.04 (Table 3). Using the anomeric proton at δ 4.63 (d, $J = 7.8$ Hz) as a starting point, analysis of the COSY experiment allowed the identification, in sequence, of four oxymethine groups and one oxymethylene group. The sugar moiety was identified as β-glucose in the pyranose form, on the basis of large couplings observed for all of the oxymethine protons implying their axial position. The second anomeric proton at δ 4.68 (d, $J = 8.1$ Hz) proved to belong to a β-glucopyranose unit. In confirmation, acid methanolysis and GLC analysis afforded methyl glucoside as the only component. The low-field chemical shift of H_2-6′ (δ 4.02 dd and δ 4.36 dd), in the ^1H NMR spectrum, indicated that the hydroxyl group at C-6′ was glycosylated with the second monosaccharide. The downfield shift of H_2-1 of the C_6 chain (δ 3.83 dd and δ 4.05 dd), with respect to a primary alcohol (δ 3.36−3.46), suggests the location of a sugar residue at C-1. Thus, capsoside B (**2**) can be defined as (4-methylpentyl-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

Capsianoside VII (**3**) showed a quasimolecular ion $[M - H]^-$ at *m/z* 759 in the negative FABMS and fragment ion peaks at *m/z* 613 $[M - H - 146]^-$ and *m/z* 451 $(613 - 162)^-$ due to the sequential losses of a deoxyhexose and a hexose. A comparative analysis of ^1H and ^{13}C NMR and $^1H-^1H$ COSY spectra of **3** with those of capsianoside II (*14*) suggested the presence of a 6*E*,-10*E*,14*Z*,17-hydroxygeranyllinalool moiety along with an oligosaccharide chain. The glycoside **3** is a derivative of capsianoside II, also isolated from *C. annuum* var. *acuminatum*. The differences between the NMR spectra of the two compounds are due to the lack of signals of the diglucosyl residue linked at the C-3 position in the known capsianoside II (Table 4). The ^1H NMR spectrum, in addition to the sugar moiety, disclosed four methyl signals (δ 1.27 s, 1.64 s × 2, 1.80 s), one terminal vinyl group (ABX-type δ 5.06 dd, δ 5.21 dd, δ 5.94 dd), and three olefinic proton signals at δ 5.15 (2H) and δ 5.43 (t). We observed a good coincidence in the chemical shifts from C-6 to C-19 of the aglycon part, whereas significant differences were detectable for the data from C-1 to C-5 and C-20 with respect to the reference capsianoside II. Acid methanolysis and gas−liquid chromatographic (GLC) analysis yielded glucose and rhamnose in the ratio 1:2. The ^{13}C NMR spectrum showed three anomeric carbon signals at 102.8, 102.3, and 101.7 ppm, which were correlated, in the HMQC experiment, with the corresponding anomeric protons at δ 4.85, 4.23, and 4.74. The ^{13}C NMR also revealed signals of β-glucose and α-rhamnose (×2) and the presence of 20 carbon signals, including one oxygenated methylene carbon (67.8 ppm) and one quaternary carbon bearing an oxygen atom (73.9 ppm). The location of sugar units and interglycosidic linkages were determined by the aid of an HMBC experiment. Cross-peaks between anomeric proton (δ 4.74) of rha I/ C-17 (67.8 ppm), H-1″ β-glucose (δ 4.23)/C-3′ rha I (79.3 ppm) and H-1‴ of rha II (δ 4.85)/C-6 of glucose (66.9 ppm) clarified the location of the sugar chain at C-17 and the interglycosidic linkages, which were identical to those observed for capsianoside II. A significant HMBC correlation was detected for H-2 (δ 5.94) with C-3 (73.9 ppm), thus indicating the presence of a hydroxyl group at the C-3 position.

The absolute configuration at C-3 was suggested to be *3S* on the basis of the optical rotation ($[\alpha]_D^{25}$ −32.0°) when compared with those of capsianoside II ($[\alpha]_D$ −35.5°) and its partially hydrolyzed derivative ($[\alpha]_D$ 16−37.5°) (*14*). Therefore, the structure of capsianoside VII (**3**) was defined as 17-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside-6*E*,10*E*,-14*Z*-3(*S*)-geranyllinalool.

Icariside E_5 (**4**) (*18*−*20*) has a molecular formula of $C_{26}H_{34}O_{11}$ that was deduced from FABMS, ^{13}C NMR, $^1H-^1H$ COSY, and HMQC spectra. Enzymatic hydrolysis of **4** with the glycosidase mixture of *Charonia lampas* gave compound **4a**, $[\alpha]_D^{25}$ −18.4°. The *8R* configuration is proposed by comparison of the optical rotation of **4a** with those of synthetic models (*S*)- and (*R*)-2-(2-methoxyphenyl)-3-(4-methoxy-2-*O*-methoxymethylphenyl)-1-propanols. These compounds were obtained by stereoselective synthesis and used for the preparation of (*S*)- and (*R*)-isoflavans (*25*).

We have previously shown that capsaicin and other phorboid homovanillates induce cell death through VR1 (*26*). To evaluate the effects of icariside E_5 in the signaling mediated through VR1, we cotransfected 293T cells either with an expression plasmid containing the entire VR1 cDNA or with the empty vector (pcDNA3) and with an equimolar concentration of the plasmid pEGFP-C1. Twenty-four hours after transfection, the cells were stimulated with either capsaicin (3 μM) or icariside E_5 (5 μM) for 6 h, and the percentage of green fluorescent cells was detected by flow cytometry. We found that this cell type is transiently transfected with a high efficiency, and in Figure 3 it is shown that >50% of the cells express the green fluorescence protein. The percentage of positive cells did not change with capsaicin in the pcDNA3 cotransfected cells, whereas a clear reduction in this percentage was observed in cells cotransfected with the plasmid pcDNA3-VR1. In contrast, icariside E_5 practically did not affect the viability of 293T cells expressing ectopically the VR1, and this result strongly suggests that this capsianoside does not bind and activate the capsaicin receptor VR1.

Next, we were interested in examining whether icariside E_5 was able to induce ROS generation and apoptosis in transformed cells lacking VR1. Thus, Jurkat cells were incubated with either capsaicin or icariside E_5 (50 μM), and after 6 h, half of the cells were collected and ROS generation and $\Delta\psi_m$ dissipation detected by double-staining experiments, using HE (nonfluorescent), which becomes ethidium (Eth, red fluorescent) after its oxidation via ROS, and $DiOC_6(3)$ (green fluorescent), a cationic probe that accumulates into mitochondria as a function of its potential (*27*). After 18 h of treatment, hypodiploidy (loss of fragmented DNA) as a marker for apoptosis was also analyzed by PI staining in the remaining cells of the culture. In these experiments, untreated cells were taken as background data, considered as having a high $\Delta\psi_m$ $[DiOC_6(3)^{high}]$

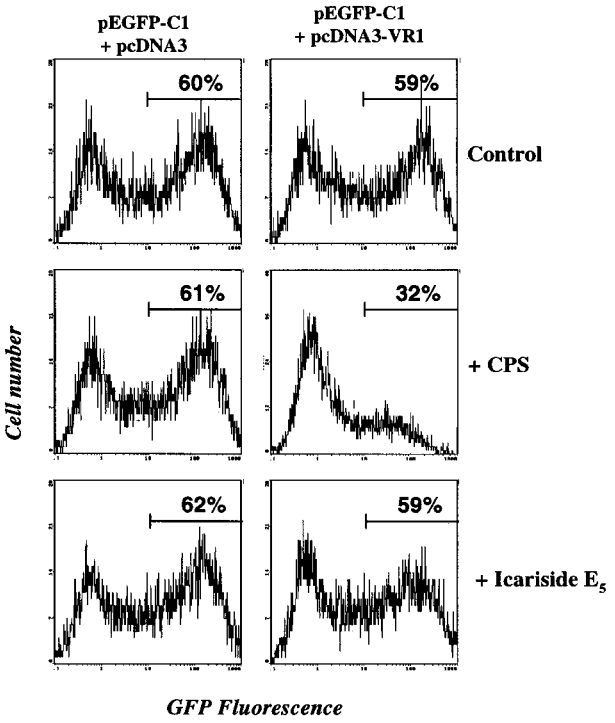

Figure 3. Capsaicin but not icariside E_5 induced cell death in VR1-transfected cells. 293T cells were cotransfected with the plasmid pEGFP-C1 and either pcDNA-3 or pcDNA3-VR1 plasmids. After 48 h, the cells were stimulated with either capsaicin or icariside E_5 (5 μM) for 6 h and the adherent live cells analyzed for the expression of the green fluorescent protein by cytometry. Results are representative of three different experiments.

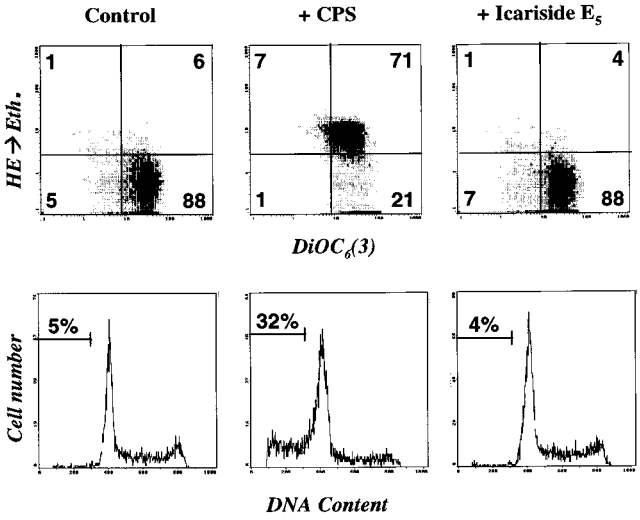

Figure 4. Icariside E_5 does not induce ROS generation or apoptosis in Jurkat cells. Jurkat cells were treated with either capsaicin or icariside E_5 (50 μM). After 6 h of treatment, half of the cells were collected and the simultaneous $\Delta\Psi_m$ disruption and ROS generation detected by cytofluorometry (top). Results represent the percentage of cells obtained in biparametric histograms delimited by four compartments, namely, $\Delta\Psi_m{}^{high}$ (normal cells, bottom right), $\Delta\Psi_m{}^{low}$ (bottom left), $(HE\rightarrow Eth)^{high}$ (ROS generating cells, top right), and $(HE\rightarrow Eth)^{high}/\Delta\Psi_m{}^{low}$ (preapoptotic cells, top left). Apoptosis was measured after 18 h of treatment by cell cycle analysis with PI staining (bottom). Results are representative of three independent experiments.

and low levels of intracellular ROS $(HE\rightarrow Eth)^{low}$ (Figure 4, control). As expected, capsaicin induced an increase in the percentages of $DiOC_6(3)^{high}/(HE\rightarrow Eth)^{high}$ cells,

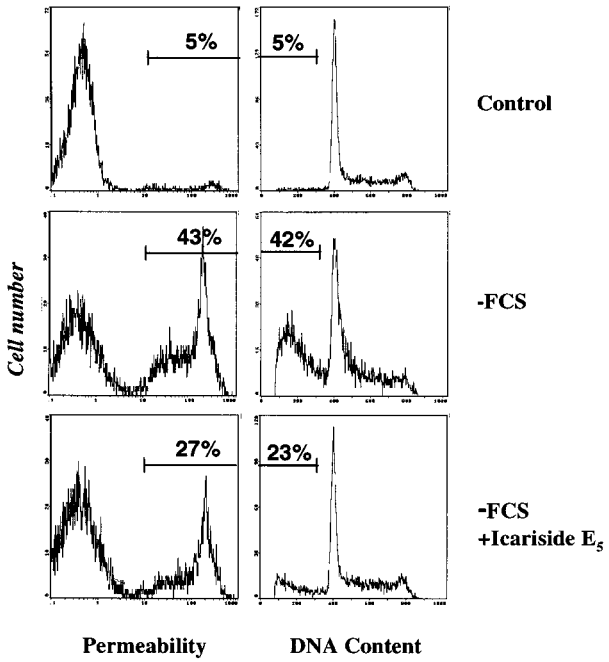

Figure 5. Icariside E_5 prevents apoptosis in Jurkat cells. Exponentially growing cells were shifted from 10% to 0.1% FCS in the presence or absence of icariside E_5 (50 μM), harvested after 24 h, and subjected to cytometric analysis. Apoptosis was measured by cell cycle analysis with PI staining and the percentage of subdiploid cells detected by flow cytometry. The percentage of dead cells was also determined by PI staining as described under Material and Methods.

reflecting the pro-oxidant potential of capsaicin in transformed cells. Again, icariside E_5 was ineffective as capsaicin-like activity and neither changed the redox state of the cells nor induced apoptosis in Jurkat cells (Figure 4).

Because icariside E_5 did not show capsaicin-like activities, we were interested in studying the possible antioxidant activity of this compound. To perform these studies, Jurkat cells were shifted from 10% FCS to 0.1% FCS cultures. This serum withdrawal in Jurkat cells affects the intracellular redox state of the cells and increases the percentage of apoptosis after 24 h. In Figure 5 we show that icariside E_5 significantly prevents the apoptosis induced by serum withdrawal in Jurkat cells, indicating a potential antioxidant role of this compound in cultured cells.

Compounds **5** and **6** were identified as capsianoside III (**5**) and capsianoside II (**6**) (*14*) with respect to the physical and NMR data. Acyclic diterpene glycosides, as dimeric esters (*14*, *28*), were not detected in *C. annuum* L. var. *acuminatum*. This species of *Capsicum* also contains various phenolic compounds, and it is suggested that the antioxidative activity is related to their conjugated rings and hydroxyl groups (*29*). The chemical structures were determined mainly by spectral methods and literature data to be *cis*-*p*-coumaric acid 4-*O*-β-D-glucoside (**7**) (*30*), *trans*-sinapoyl β-D-glucoside (**8**) (*31*), vanilloyl β-D-glucoside (**9**) (*32*), and quercetin 3-*O*-rhamnoside (**10**) (*33*).

ACKNOWLEDGMENT

We thank Dr. David Julius (University of California, San Francisco, CA) for the rat VR1 cDNA. MS and NMR were provided by Centro di Servizio Interdipartimentale

di Analisi Strumentale, (CRIAS), Università di Napoli Federico II.

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Received for review November 14, 2000. Revised manuscript received February 2, 2001. Accepted February 7, 2001. Chemical work was supported by Università degli Studi del Molise (ex quota 60%). Biological work was supported by a grant from the Spanish Comisión Interministerial de Ciencia y Tecnología (1FD97-0683-C05-03) to E.M.

JF0013454