GEIPARVARIN REGIOISOMERS

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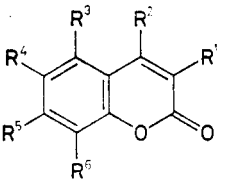
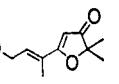
Dedicated to Dr Miroslav Protiva on the occasion of his 70th birthday.

The synthesis and the cytotoxicity of Geiparvarin (7-[(*E*)-3-(4,5-dihydro-5,5-dimethyl-4-oxo-2-furanyl)-2-butenyloxy]-coumarin) regioisomers are reported.

Geiparvarin *V*, a naturally occurring coumarin derivative[1] since some years has become the subject of a number of research works aimed to exploit its promising antineoplastic properties[2–7]. Being expecially our area of interest the structure/activity relationships of *V*, were solved to study its regioisomers. Indeed, from a structural point of view, *V* represents just one of its six possible regioisomers, and not necessarily the best one. With this aim we prepared and tested all the other regioisomers *I*–*IV* and *VI*.



	R^1	R^2	R^3	R^4	R^5	R^6
I	X	H	H	H	H	H
II	H	X	H	H	H	H
III	H	H	X	H	H	H
IV	H	H	H	X	H	H
V	H	H	H	H	X	H
VI	H	H	H	H	H	X

In formulae *I*–*VI*: X = 

EXPERIMENTAL

Melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. NMR spectra were measured on Varian EM-390 apparatus (90 MHz for ^{1}H) in deuteriochloroform with tetramethylsilane as internal reference. Chemical shifts are given in ppm (δ-scale), coupling constants (J) in Hz. Mass spectra were recorded on VG 7070 spectrometer, energy of ionizing electron 70 eV.

General Procedure for Preparation Compounds *I*—*IV*, *VI*

Mixture of selected hydroxycoumarin (0·276 g; 1·65 mmol) 5-[(*E*)-3-methanesulfonyloxy-1-methylpropenyl]-2,2-dimethylfuran-3(2*H*)-one[8] (0·43 g; 1·65 mmol), potassium carbonate (0·228 g; 1·65 mmol), anhydrous lithium bromide (0·03 g; 0·35 mmol) and acetone (50 ml) was refluxed under stirring for 3 h. The solvent was removed under reduced pressure and the residue was partitioned between dichloromethane and 10% aqueous ammonia. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic phases were washed with aqueous ammonia, brine and dried over anhydrous magnesium sulfate. The solvent was evaporated in vacuo and the residue was chromatographed on column of silica gel (50 g) in petroleum ether–ethyl acetate (3 : 2).

3-[(*E*)-3-(4,5-*Dihydro*-5,5-*dimethyl*-4-*oxo*-2-*furanyl*)-2-*butenyloxy*]-*coumarin* (I). 3-Hydroxycoumarin afforded 0·32 g (60%) of compound *I*, m.p. 197—200°C (ethyl acetate). ^{1}H NMR spectrum: 1·48 (s, 6 H); 2·05 (d, 3 H, J = 1·0); 4·85 (d, 2 H, J = 6); 5·64 (s, 1 H); 6·8—7·6 (m, 6 H). Mass spectrum, m/z (%); 326 (M^+, 67), 165 (100), 69 (97). For $C_{19}H_{18}O_5$ (326·4) calculated: 69·93% C, 5·56% H; found: 69·85% C, 5·61% H.

4-[(*E*)-3-(4,5-*Dihydro*-5,5-*dimethyl*-4-*oxo*-2-*furanyl*)-2-*butenyloxy*]-*coumarin* (II). 4-Hydroxycoumarin gave 0·27 g (50%) of compound *II*, m.p. 196—198°C (ethyl acetate). ^{1}H NMR spectrum: 1·42 (s, 6 H); 2·1 (d, 3 H, J = 1·0); 4·96 (d, 2 H, J = 6); 5·68 (s, 1 H); 5·76 (s, 1 H); 6·7—8·0 (m, 5 H). Mass spectrum, m/z (%); 326 (M^+, 26), 162 (88), 120 (100). For $C_{19}H_{18}O_5$ (326·4) calculated: 69·93% C, 5·56% H; found: 69·99% C, 5·44% H.

5-[(*E*)-3-(4,5-*Dihydro*-5,5-*dimethyl*-4-*oxo*-2-*furanyl*)-2-*butenyloxy*]-*coumarin* (III). 5-Hydroxycoumarin gave 0·24 g (45%) of compound *III*, m.p. 195—197°C (ethyl acetate). ^{1}H NMR spectrum: 1·42 (s, 6 H), 2·04 (d, 3 H, J = 1·0); 4·88 (d, 2 H, J = 6); 5·64 (s, 1 H); 6·32—8·24 (m, 6 H). Mass spectrum, m/z (%); 326 (M^+, 26), 165 (100), 69 (66). For $C_{19}H_{18}O_5$ (326·4) calculated: 69·93% C, 5·56% H; found: 69·86% C, 5·61% H.

6-[(*E*)-3-(4,5-*Dihydro*-5,5-*dimethyl*-4-*oxo*-2-*furanyl*)-2-*butenyloxy*]-*coumarin* (IV). 6-Hydroxycoumarin afforded 0·22 g (40%) of compound *IV*, m.p. 166—168°C (ethyl acetate). ^{1}H NMR spectrum: 1·42 (s, 6 H); 2·02 (d, 3 H, J = 1·0); 4·84 (d, 2 H, J = 6); 5·66 (s, 1 H); 6·38—7·84 (m, 6 H). Mass spectrum, m/z (%): 326 (M^+, 35), 165 (31), 69 (100). For $C_{19}H_{18}O_5$ (326·4) calculated: 69·93% C, 5·56% H; found: 69·78% C, 5·71% H.

8-[(*E*)-3-(4,5-*Dihydro*-5,5-*dimethyl*-4-*oxo*-2-*furanyl*)-2-*butenyloxy*]-*coumarin* (VI). 8-Hydroxycoumarin gave 0·32 g (60%) of compound *VI*, m.p. 102—104°C (ethyl acetate). ^{1}H NMR spectrum: 1·43 (s, 6 H); 2·02 (d, 3 H, J = 1·0); 4·96 (d, 2 H, J = 6); 5·62 (s, 1 H); 6·42—7·88 (m, 6 H). Mass spectrum, m/z (%); 326 (M^+, 13), 165 (90), 162 (100). For $C_{19}H_{18}O_5$ (326·4) calculated: 69·93% C, 5·56% H; found: 69·77% C, 5·67% H.

CYTOTOXIC ACTIVITY

Growth Test in Suspension Cell Culture

LS cells were cultured in RPMI 1640 medium, with Hepes buffer and L-glutamine, supplement with 5% newborn calf serum, penicillin and streptomycin (GIBCO liquid medium and products). To the medium Pluronic F-68 at 0·1% concentration was added in order to reduce cell attachment. After preliminary experiments, two series of experimental cultures were set, with three flasks for each treatment; about $0·3-0·5 \cdot 10^5$ cells/ml were seeded in NUNC plastic flasks for monolayer cultures and incubated at 37°C. 24 h later the compounds to be tested were added.

The coumarins were dissolved in ethanol to a concentration of 10^{-2} mol l^{-1} and the ethanolic solutions diluted in the medium to obtain ethanol concentrations of 10^{-2} mol l^{-1} or less, and drug concentrations of $10^{-5}-10^{-6}$ mol l^{-1}. Just after coumarin addition and then after 24 and 48 h, 1·3 ml of cell suspension was sampled

TABLE I

Growth rates per day of LS cells in suspension culture with Geiparvarin *V* regioisomers. Menas and standard deviations of triplicate cultures in parallel

Compound	Specific growth rate	
	$t_2 - t_0$	$t_2 - t_1$
	1st Experiment[a]	
Ethanol	0·51 ± 0·40	0·65 ± 0·44
Geiparvarin	0·82 ± 0·40	0·54 ± 0·20
I	2·58 ± 1·16	1·46 ± 1·17
II	0·07 ± 0·05	3·14 ± 0·75
III	−0·22 ± 0·22	2·56 ± 0·65
IV	0·85 ± 0·84	2·00 ± 1·25
VI	−0·35 ± 0·10	2·35 ± 0·17
	2nd Experiment[b]	
Ethanol	1·27 ± 0·44	0·74 ± 0·27
Geiparvarin	0·97 ± 0·86	0·72 ± 0·45
I	0·93 ± 0·16	0·93 ± 0·09
II	0·22 ± 0·13	1·68 ± 0·86
III	−0·17 ± 0·10	3·51 ± 1·50
IV	0·14 ± 0·04	3·28 ± 0·86
VI	0·24 ± 0·10	1·78 ± 0·16

Concentration of compounds: [a] 10^{-5} mol l^{-1}; [b] 10^{-6} mol l^{-1}.

from each flask and the cell number per ml measured by the Coulter Counter Mod. ZBI. The specific growth rate per day was numerically estimated with the equatin $X_{t1} - X_{t0}/X_{t0}$, where X is the number of cells per ml in the culture at time t. In Table I the means and standard derivations of specific growth rate in each 24 h interval are reported. The effects of coumarins are evidenced through comparison of growth rates within each experiment and time interval.

Geiparvarin in suspension cultures did not show any effect on culture growth. A strong inhibition in the first 24 h of culture after drug addition was displayed by regioisomers *II*, *III* and *VI*. However, in the following 24 h of treatment, rates of growth were enhanced, resulting in a much higher numerical density of cells compared to the controls. Observations made on cultures by light microscopy showed no differences among cells from different experimental cultures.

Toxicity on Cells Monolayer Cultures

The toxicity test was performed according to rapid cell culture assay devised by Sauter et al.[9], on monolayer cultures of Vero cells raised in Dulbecco's MEM with Hepes and L-glutamine, supplemented with 5% or 10% newborn calf serum, penicillin and streptomycin (GIBCO liquid medium and products).

The cells, at a density of about $3 . 10^5$ cells/ml were seeded in sterile flat bottom NUNC plastic plates (24 wells, 16 mm each in diameter) and incubated in 5% CO_2 at 37°C for 24 h, until a good cell monolayer was formed. The medium was then completely changed with a fresh one containing the coumarins. Only compounds *I*, *III* and *VI* were tested, having shown some inhibition of growth rate in the previous experiments. The coumarins were dissolved in dimethylsulfoxide (DMSO) to a concentration of 10^{-3} mol l^{-1} and then the coumarin–DMSO solution dissolved

TABLE II

Effects of some Geiparvarin regioisomers on growth of Vero cell cultures. Means and standard deviations of DNA content, in μg per flask, 24 and 48 h after drug addition

Compound	Time, h		
	0	24	48
Control		8·6 ± 0·36	13·5 ± 0·47
DMSO		9·2 ± 0·05	12·5 ± 0·06
I	5·5 ± 0·38	8·8 ± 0·26	12·6 ± 0·47
III		9·3 ± 0·41	11·5 ± 0·17
IV		9·1 ± 0·17	10·6 ± 0·12
VI		8·6 ± 0·27	10·5 ± 0·33

in the culture medium to get a final concentration of DMSO 10^{-3} mol l^{-1}, non toxic to cells, and coumarin concentration of 10^{-6} mol l^{-1}. After 24 and 48 h the cell layers were washed with phosphate buffer solution (PBS), fixed in formalin, stained with Giemsa, washed with distilled water and dried. The cell layers were permanently stained a pale blue colour. No difference were found among wells; no toxic effect was detected with tested compounds at 10^{-5} mol l^{-1} concentration.

Growth Test on Cell Monolayer Cultures

For the anti-growth test on cell monolayer, Vero cells were seeded at the density of about $3-5 . 10^{-5}$ per ml, in NUNC plastic flasks with the above mentioned medium and incubated at 37°C. After 24 h the medium was poured out and replaced with a fresh one containing the compounds to be tested, diluted in the medium as described for the toxicity test. At the time of drug addition, for each tested coumarin, these flasks were sampled and again three flasks after 24 and 48 h of growth. The culture growth was estimated by measuring the amount of DNA per flask with a Kontron 25 spectrofluorimeter, by a modified method after Birnboim and Jevcak[10]. The flasks with the cell monolayer were washed with PBS and stored at −20°C. At the end of experiments, the cell monolayer from each flask was lysed in 1 ml of the following solution: 0·01 mol l^{-1} Tris, 0·01 mol l^{-1} ethylendiaminetetracetic acid (EDTA), 0·01 mol l^{-1} NaCl, 0·2% sodium dodecyl sulphate (SDS), pH 8·0. No cell debris or damaged cells were seen under a light microscope.

To 1 ml of the clear lysis solution, 3 ml of 0·008 mg/ml ethidium bromide solution were added and the relative fluorescence was measured with a Kontron 25 spectrofluorimeter. A standard curve was obtained from known scalar concentrations of calf thymus DNA in PBS, lysed and stained as for cell monolayers and measured at the same time intervals. Table II shows the results: a slight inhibition on culture growth by regioisomers *I* and *VI* can be seen, confirming the previous results.

CONCLUSIONS

Geiparvarin regioisomers, tested at concentrations as low as for therapeutical applications, showed no toxicity and a slight antigrowth effect on mammalian cells in vitro. The results of the experiments here described confirm the findings by Gawron and Glowniak[11] and Carrara et al.[7] when drug concentration of the same order are compared. The most effective derivatives were compounds *III*, *IV* and *VI*, corresponding to the 5-, 6- and 8-regioisomers of *V*.

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