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Short communication

Synthesis of compounds with antiproliferative activity as analogues of prenylated natural products existing in Brazilian propolis

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Abstract

This work describes the syntheses and antitumoral properties of five prenyl compounds from which antiproliferative activities were predicted by using the TOPS-MODE approach, a computational method for drug design. The syntheses of 2-(3-methylbut-2-enyloxy)acetophenone (**2**), 2-hydroxy-5-(3-methylbut-2-enyl)acetophenone (**3**), 2-hydroxy-3-(1,1-dimethylallyl)acetophenone (**4**), and 5-(3-methylbut-2-enyl)-2-(3-methylbut-2-enyloxy)acetophenone (**5**) were realized by *O*-prenylation of phenolic compounds with prenyl bromide and by Claisen rearrangement, respectively. Reaction of 2-hydroxy-5-(3-methylbut-2-enyl)acetophenone **3** under Vilsmeier–Haack conditions with phosphoryl chloride and *N*, *N*-dimethylformamide yielded 6-(3-methylbut-2-enyl)chromone-3-carbaldehyde (**6**). The compounds were tested for their cytotoxicity toward a diverse panel of cultured human tumor cell lines. Compound **3** showed significant selective cytotoxic activity ($IC_{50} < 9$ μg/ml).

Keywords: o-Hydroxy-acetophenone; Prenylation; Claisen rearrangement; Brazilian propolis; Antiproliferative activity

1. Introduction

Brazilian propolis, a natural resinous product collected by bees, consists of a mixture of more than 100 compounds. Many of them are aromatic, in particular phenolic compounds. Among the main classes of compounds found in Brazilian propolis are the prenylated derivatives of *p*-coumaric acid and *o*-hydroxy-acetophenone, respectively. These substances only exist in Brazilian propolis and not in propolis from other regions [1,2]. In recent years, the synthesis and pharmacology of some Brazilian propolis compounds have been extensively investigated due to their wide range of biological activities (antimicrobial, antiproliferative, antiinflammatory, antiviral, etc) [3–6]. Hori and colleagues (Uto et al. [6]) reported the first total synthesis of Artepillin C, 3-{4-hydroxy-3,5-di(3-methylbut-2-enyl)phenyl}-2(*E*)-propenoic acid, which is a biologically active constituent of propolis. Recently, it was reported that Artepillin C has antitumor, apoptosis inducing, immunomodulating and antioxidative activities. It shows that this compound may be one of the most important active principles of Brazilian propolis [3] and that the synthesis of prenylated compounds analogous of it deserves attention.

2. Results and discussion

The search for anticancer compounds has always been on the desktop of medicinal chemists and a great number of different approaches have been used in this search [7–10]. However, the discovery of selective antitumor compounds has remained a largely elusive goal of cancer research. Consequently, novel approaches are needed for the efficient search for suitable candidates to be assayed as anticancer drugs [11,12].

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2.1. TOPS-MODE approach

In the last years we have developed an approach to QSAR/QSPR based on the use of spectral moments of the bond matrix as molecular descriptors. It is known as TOPS-MODE approach, which is the acronym of Topological Sub-Structural Molecular Descriptors/Design [13,14]. This TOPS-MODE approach is based on the calculation of spectral moments of molecular bond matrices [15–18] appropriately weighted to account for hydrophobic, electronic and geometric molecular features. A bond matrix is a square symmetric matrix in which non-diagonal entries are ones or zeroes if the corresponding bonds have a common atom or not, respectively [18]. These matrices represent the molecular skeleton without taking into account hydrogen atoms. Bond weights are placed as diagonal entries of such matrices and represent quantitative contributions to different physicochemical properties. The spectral moment of order k is defined as the trace of the kth power of the matrix and it is designated here as μ_k.

The starting point for our approach is to calculate TOPS-MODE descriptors for the series of molecules under study. Then, we develop a quantitative model describing the property under study in terms of the spectral moments. In general, this model can be of the following form:

$$P = b_0 + \sum_{j=1}^{L} b_j \mu_j \quad (1)$$

where P is the property under study, b_j are the coefficients of the quantitative model (linear regression or discriminant analysis) and b_0 is the error.

The jth spectral moment of the bond matrix can be expressed as a sum of bond moments, which are simply the corresponding entries of the jth power of the bond matrix:

$$\mu_j = \sum_{i}^{m} \mu_j(i) \quad (2)$$

where $\mu_j(i)$ is the bond moment of the ith bond in a molecule with m bonds. Then, model (2) can be written as:

$$P = b_0 + \sum_{j=1}^{L} b_j \sum_{i=1}^{m} \mu_j(i) = b_0 + \sum_{i=1}^{m} \sum_{j=1}^{L} b_j \mu_j(i) \quad (3)$$

where the right-handed term in Eq. (3) represents the contribution of bond i to the property P and will be called the "bond contribution" and represented by $P(i)$:

$$P(i) = \sum_{j=1}^{L} b_j \mu_j(i) \quad (4)$$

and the property P is expressed as an additive function of bond contributions:

$$P = \sum_{i=1}^{m} P(i) \quad (5)$$

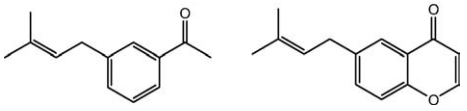
In spite of TOPS-MODE descriptions of molecular structure are defined in a global way, this approach permits one to find the contribution of any fragment in the molecular structure to the property under study. This is possible because spectral moments can be expressed as linear combinations associated to molecular fragments.

Fragment contributions are of general significance, and as a consequence they can be of limited applicability in some particular cases. For instance, one can find that a fragment F has a positive contribution to a property, which can be interpreted as the presence of this fragment enhances such property. However, it is mainly the case that this fragment has a positive contribution ONLY when it is embedded into a particular class of structural framework. In consequence, the interpretation of the results obtained by using fragment contributions should be taken with care and general interpretations of the sort "this fragment enhances this property/activity" should be made with care. It is preferred to refer such contributions to particular classes of compounds where these fragments can be part of certain pharmacophores.

3. Selection of anticancer compounds

In a previous work we developed an approach for the rational selection of candidate compounds for anticancer activity. This approach is based on the application of the TOPS-MODE methodology to a series of anticancer and not anticancer compounds by finding a discriminant model that permits the selection of compounds with high probability of showing this activity. This model was built by using bond dipole moments to differentiate the different bonds in the molecules studied. The model was developed by using 224 compounds and it was able to classify correctly 88.3% of compounds in the training series and 91.6% of compounds in the external prediction set (see Ref. [14] for details and the model).

Using this model we evaluated a series of compounds analogues of prenylated natural products existing in Brazilian propolis. Several of these compounds were selected by considering a basic framework and adding different substituents at different positions according to simple rules that permit their synthesis in a short number of steps. From these series of compounds we selected two structural motifs as promising candidates for anticancer activity. They correspond to the 1-[3-(3-methylbut-2-enyl]ethanone and 6-(3-methylbut-2-enyl)chromen-4-one and their structures are given below:



These two frameworks were selected as they appeared in several compounds evaluated by TOPS-MODE as active anticancer compounds. Some of these structures as well as their derivatives with posteriori probabilities of being anticancer are given in Fig. 1. As can be seen, the first series of compounds shows less homogeneity in the prediction of anticancer activity. It can be interpreted that compounds with higher hydrophobicity are predicted as active while the more hydrophilic ones are predicted to be negative. However, it is necessary to remark that these hydrophobic compounds can be metabolized

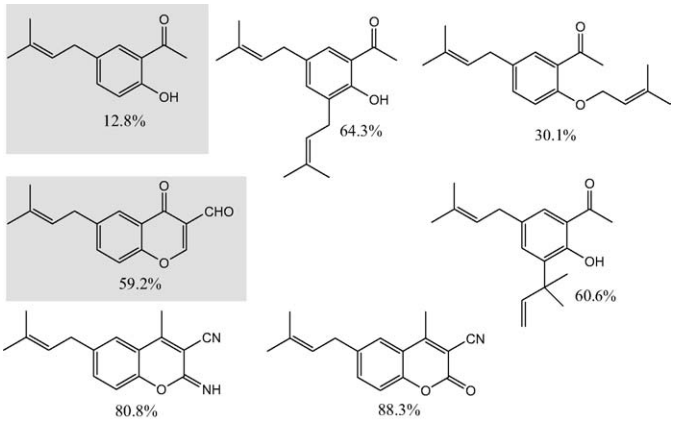


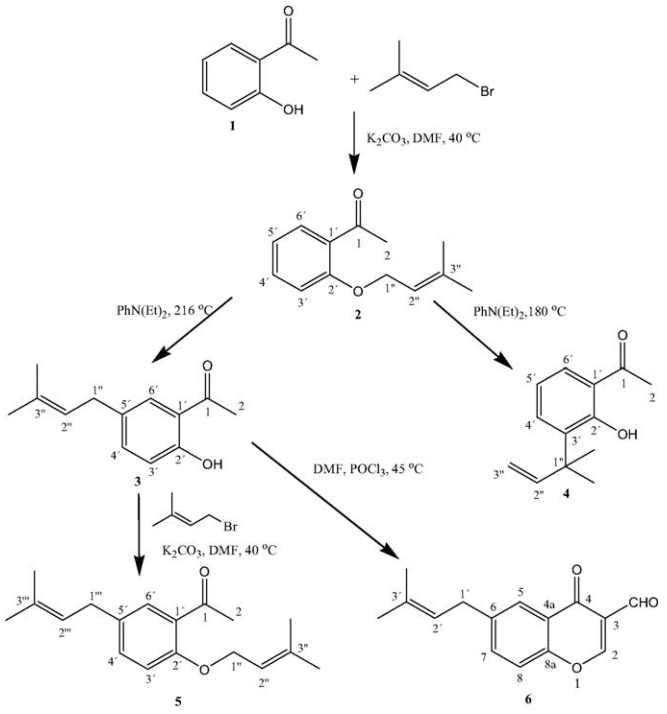
Fig. 1. Molecular structures of some of the compounds predicted by TOPS-MODE for anticancer activity having the molecular frameworks of 1-[3-(3-methylbut-2-enyl]ethanone and 6-(3-methylbut-2-enyl)chromen-4-one.

to more hydrophobic ones, which can show the anticancer activity. For instance, the first compound can be methoxylated in vivo to give a more hydrophobic derivative. Consequently, we decided to focus on the simplest representatives of these two series of compounds, mainly the members highlighted in Fig. 1. The synthesis of these compounds has a twofold objective. On the first hand, we would like to evaluate the anticancer potential of these simple compounds with the perspective of developing them for such use. On the other hand, we are interested in using these simple compounds as leads for the synthesis of new and more complex compounds with improved anticancer activity.

4. Synthesis strategy

According to the TOPS-MODE method the 6-(3-methylbut-2-enyl)chromone-3-carbaldehyde (**6**) was predicted to have antitumoral properties. The probability of antitumoral activity of +59.2 refers to the percentage assigned by the model for a compound to show anticancer activity. Using the information provided by TOPS-MODE design we planned a synthetic strategy based on the development of simple compounds having the frameworks shown below. This synthetic strategy is given in Scheme 1. Herein we present the first total synthesis of 2-hydroxy-5-(3-methylbut-2-enyl)acetophenone (**3**). This compound was found and identified in Brazilian propolis by Marcucci and Bankova [1], but its biological properties were not studied.

The synthesis of (3,3-dimethylallyloxy)acetophenone (**2**) can be realized following Appendino et al. [19] by starting from *o*-hydroxy-acetophenone (**1**) and prenyl bromide in the presence of sodium hydride/dimethyl sulfoxide at room temperature during 24 hours. The strong basic medium used in this reaction caused the formation of side products and so the desired product was formed only in poor yield (25%). Therefore, we reacted *o*-hydroxy-acetophenone, prenyl bromide and anhydrous potassium carbonate as base in a 1:2:3 molar ratio in dry *N,N*-dimethylformamide at 40 °C. After 8 hours the prenyl ether **2** could be isolated in an excellent yield (85%, Scheme 1).



Scheme 1. Synthesis of prenylated compounds.

Furthermore, we prepared the natural product **3** starting from compound **2** under Claisen rearrangement conditions [20,21] using *N*,*N*-diethylaniline as solvent. The reaction was completed after 8 hours reaction time under an argon atmosphere at a temperature of 216 °C and the *C*-prenylated *o*-hydroxy-acetophenone **3** was obtained in 70% yield. The 5′-position of the prenyl group in this compound was determined by NMR spectroscopy. When the compound **2** in *N*,*N*-diethylaniline was heated only up to 180 °C, the 2-hydroxy-3-(1,1-dimethylallyl)acetophenone (**4**) was formed which could be isolated in 28% yield as a colorless oil. Furthermore, compound **3** was treated again with prenyl bromide using the same procedure described for **2** to obtain 5-(3-methylbut-2-enyl)-2-(3-methylbut-2-enyloxy)acetophenone (**5**) in 80% yield as colorless liquid.

The Vilsmeier-Haack reaction of 2-hydroxy-5-(3-methylbut-2-enyl)acetophenone (**3**) with phosphoryl chloride and *N*,*N*-dimethylformamide afforded the 6-(3-methylbut-2-enyl)chromone-3-carbaldehyde (**6**) in 11% yield as a white solid. In the ^{1}H- and ^{13}C-NMR spectra of this compound the signal for the formyl group were found at $\delta = 10.31$ and $\delta = 188.7$, respectively. On the other hand, for the aromatic protons only one coupling between H-7 and H-8 ($^{3}J = 8.5$ Hz) was determined, thus excluding any other alternative structures.

5. Cytotoxic activity of the synthesized compounds

Three of the synthesized compounds have been examined for in vitro cytotoxic activity tests.

The *O*-prenyl derivative **2** showed antiproliferative non-selective activity for all human carcinoma cell lines tested and induced cell death for human lung carcinoma (NCI460), mela-

Table 1
Cytotoxicity of some synthesized compounds

Compounds	Human cell lines tested IC_{50} (μg/ml)			
	NCI460	UACC62	MCF7	NCIADR
2	25.0	45.0	25.0	8.0
3	9.0	4.2	6.0	6.0
6	30.0	32.0	11.0	34.0

Table 2
Cytotoxicity of two synthesized compounds (**2** and **3**; MTT assay)

Cell line	Compound **2** (μg/ml)			Compound **3** (μg/ml)		
	100	50	25	100	50	25
Colo205 (colonic)	0.3	49.7	97.8	0.6	0.3	50.8
T47D (breast)	1.2	2.8	33.7	7.7	3.2	12.5
MIAPaCa2 (pancreatic)	0.0	51.8	98.9	0.0	4.0	62.9
WI38 (fibroblast)	0.0	64.8	66.1	3.3	52.8	79.6

Data are shown as percentage of proliferation of the tumor cell lines in the presence of the test compounds, with medium control without compounds set to 100%. Since compound **3** showed much higher activity against the tumor cell lines and low activity against WI38 fibroblasts, compound **3** was chosen for further evaluation.

noma (UACC62), MCF-7 (breast) and human breast expressing the multidrug resistance phenotype (NCI-ADR) (see Tables 1 and 2). The chromone-3-carbaldehyde **6** also showed antiproliferative and cytotoxic activities against all investigated human carcinoma cells lines starting at a concentration of 2.5 μg/ml.

It was found that compound **3** exhibited the most potent activity against the four cancer cell lines at five different concentrations ranging from 0.5 to 250 μg/ml. Stronger antiproliferative and cytotoxic activities were observed against all investigated human carcinoma cells lines starting from a concentration of 2.5 μg/ml showing IC_{50} values equal or less than 9 μg/ml. Following these results, a screening of antiproliferative activity of compound **3** was done with some other cell lines: U87MG, CRO2B, MIAPaCa2 and SIM at concentrations ranging from 1.5 to 50 μg/ml. Later, these antiproliferative activity tests were extended to the following cell lines:

Colon: SW620, CaCo-2 and Colo 205, Ovary: SKOV3, Prostate: LNCaP, Pancreas: MIAPaCa2, Bladder: J82, Breast: T47D, Fibroblasts: WI38 and NIH3T3, not pathogenic lines as well as to some other cells lines as showed in Table 3.

Table 3
Cytotoxic activity shown by compound **3**—additional cell lines (MTT assay)

Cell line	Origin	IC_{50} μg/ml
U87MG	Astrocytoma	15.7
CRO2B	Carcinoid	5.0
SIM	Sarcoma	6.25
KAL	Sarcoma	6.1
SKOV3	Ovarian	9.5
CaCo-2	Colonic	6.8
J82	Bladder	8.3
SW620	Colonic	4.5
EW-7	Sarcoma	15.4
LAN1	Neuroblastoma	6.7
LNCaP	Prostatic	8.3
NIH3T3	Fibroblast	7.7

All of these cell lines were strongly affected by compound **3** proving its high antiproliferative and cytotoxic activities. Only in the case of the not pathogenic line (Fibroblast WI38) low activity was found. These results showed that this compound was selective for the studied tumor cell lines.

Remarkably, the compound **3** shows a powerful anticancer activity despite it was predicted to be inactive by TOPS-MODE classification model. This fail could be due to several reasons. The first was previously advanced in this work as this compound can be metabolically activated to the more hydrophobic derivative in which the OH group is transformed to OCH_3. Another possible explanation is that these compounds develop their anticancer activity due to their antioxidant properties, a feature probably not well accounted by the TOPS-MODE model previously developed. However, we have used this approach to evaluate the antioxidant activity of compounds in the Brazilian propolis and we have identified the OH group as an important pharmacophore for this activity. Compound **5** shows inactivity as anticancer as predicted by TOPS-MODE, which probably support our second hypothesis about the role of the OH group at this position. Also, compound **6** shows the same activity as predicted by the model, confirming that the strategy used for selecting these compounds, which can be further improved, resulted appropriated.

6. Experimental

6.1. General procedures

TLC was carried out on silica gel 60 GF_{254} (Merck) with detection by UV light ($\lambda = 254$ nm) and/or by charring with iodine. Silica gel 60 (63–200 mesh, Merck) was used for column chromatography. IR spectra were recorded with a Nicolet 205 FT-IR spectrometer. ^{1}H-NMR (300.13 and 250.13 MHz, respectively) and ^{13}C-NMR (75.5 MHz and 62.9 MHz, respectively) spectra were recorded on Bruker instruments ARX 300 and AC 250 with Deuterochloform as solvent. The calibration of spectra was carried out on the signal of TMS (internal, ^{1}H) and $CDCl_3$ (^{13}C) [$\delta\ ^1H_{TMS} = 0$; $\delta\ ^{13}C_{CDCl3} = 77.0$]. The ^{1}H- and ^{13}C-NMR signals were assigned by DEPT and two-dimensional ^{1}H,^{1}H COSY and ^{1}H,^{13}C correlation experiments (HETCOR, HMBC). The mass spectra were recorded on an AMD 402/3 spectrometer (AMD Intectra GmbH). Elemental analysis was performed on a Leco CHNS-932 instrument.

6.1.1. Methods A: general O-alkylation procedure of phenolic compounds with prenyl bromide

To a stirred solution of the phenolic compound (2 mmol) in dry DMF (10 ml), anhydrous potassium carbonate (828 mg, 6 mmol) was added and the solution stirred for 30 min at 40 °C under argon. Prenyl bromide (596 mg, 4 mmol) was added drop-wise and the resulting mixture was heated and stirred at the same temperature for 8 h under argon. Then the mixture was poured into cold water (50 ml) and extracted with chloroform. The combined organic layers were washed with

diluted sodium hydrogen sulfate solution, then with water, dried with Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel 60 (63–200 mesh, Merck) with the appropriate eluent.

6.1.2. Methods B: general Claisen rearrangement procedure

The allylphenylether (1 mmol) was added to *N*,*N*-diethylaniline (10 ml) and the resulting mixture was stirred at 180 °C or at 216 °C for 5 or 8 hours, respectively. Then, it was extracted with chloroform. The combined organic extracts were washed with a hydrochloric acid-solution (15%) and finally with water, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in methanol (20 ml). A small amount of Amberlit IR-120 was added to the solution. The resulting mixture was then stirred at room temperature for 20 min and evaporated under reduced pressure. Purification was carried out by column chromatography on silica gel 60 (63–200 mesh; Merck).

6.1.2.1. 2-(3-Methylbut-2-enyloxy)acetophenone ***(2)***. A mixture of *o*-hydroxy-acetophenone (**1**), (272 mg, 2 mmol), anhydrous potassium carbonate (828 mg, 6 mmol) and dry DMF (10 ml) was reacted with prenyl bromide (596 mg, 4 mmol) as described under Methods A. The resulting residue was purified by chromatographic column on silica gel (toluene/ethylacetate 9:1). Yield 347 mg (85%); colorless oil. — ^{1}H-NMR (250 MHz, $CDCl_3$): $\delta = 7.72$ (dd, 1H, $^3J_{5',6'} \approx 7.8$ Hz, $^4J_{4',6'} \approx 2.0$ Hz, H-6′), 7.42 (ddd, 1H, $^3J_{3',4'} \approx 8.5$ Hz , $^3J_{4',5'} \approx 7.5$ Hz, $^4J_{4',6'} \approx 2.0$ Hz, H-4′), 6.96 ("dt", 1H, $^4J_{3',5'} \approx 1.0$ Hz, H-5′), 6.95 (br d, 1H, $^3J_{3',4'} \approx 8.5$ Hz, H-3′), 5.49 (m, 1H, H-2′′), 4.6 (d, 2H, $^3J_{1'',2''} \approx 6.5$ Hz, H-1′′), 2.61 (s, 3H, H-2), 1.79 (br m, 3H, CH_3-prenyl), 1.74 (br s, 3H, CH_3-prenyl). – ^{13}C-NMR (62.9 MHz, $CDCl_3$): $\delta = 200.0$ (C=O), 158.3 (C-2′), 138.2 (C-3′′), 133.5 (C-4′), 130.3 (C-6′), 128.5 (C-1′), 120.4 (C-5′), 119.1 (C-2′′), 112.7 (C-3′), 65.3 (C-1′′), 32.0 (C-2), 25.7 (CH_3-prenyl), 18.2 (CH_3-prenyl). — IR (film): $\nu = 3072$, 3028 (=CH), 2976 (CH_3), 2879 (CH_2), 1674 (C=O), 1236 (C–O) cm^{-1}. — $C_{13}H_{16}O_2$ (204,26): calcd. C 76.44, H 7.89; found C 76.43, H 7.81.

6.1.2.2. 2-Hydroxy-5-(3-methylbut-2-enyl)acetophenone ***(3)***. Compound **2** was reacted according to Methods B at 216 °C for 8 h. The resulting residue was purified by column chromatography on silica gel (toluene/ethylacetate 9.5:0.5). Yield 143 mg (70%); colorless liquid. — ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 12.12$ (s, 1H, OH), 7.49 (d, 1H, $^4J_{4',6'} \approx 2.2$ Hz, H-6′), 7.29 (dd, 1H, $^3J_{3',4'} \approx 8.5$ Hz, $^4J_{4',6'} \approx 2.2$ Hz, H-4′), 6.9 (d, 1H, $^3J_{3',4'} \approx 8.5$ Hz, H-3′), 5.28 (m, 1H, H-2′′), 3.29 (d, 2H, $^3J_{1'',2''} \approx 7.5$ Hz, H-1′′), 2.61 (s, 3H, H-2), 1.76 (br s, 3H, CH_3-prenyl), 1.72 (br s, 3H, CH_3-prenyl). — ^{13}C-NMR (62.9 MHz, $CDCl_3$): $\delta = 204.4$ (C=O), 160.5 (C-2′), 136.8 (C-4′), 133.1 (C-3′′), 132.1 (C-5′), 129.7 (C-6′), 122.8 (C-2′′), 119.4 (C-1′), 118.3 (C-3′), 33.4 (C-1′′), 26.6 (C-2), 25.7 (CH_3-prenyl), 17.8 (CH_3-prenyl). — MS (70 eV): $m/z = 204$, 189 (M^+ –15 (CH_3)), 161 (M^+ –43 (CH_3CO)), 69 (C_5H_9 prenyl), 43 (CH_3CO). — IR (film): $\nu = 3437.6$ (OH), 3029 (=CH aromat.), 2971, 2916 (CH_3), 2857 (CH_2), 1642.8 (C=O), 1589 (C=C) cm^{-1}. — $C_{13}H_{16}O_2$ (204,26): calcd. C 76.44, H 7.89; found: C 76.45, H 7.82.

6.1.2.3. 2-Hydroxy-3-(1,1-dimethylallyl)acetophenone ***(4)***. Compound **2** (1 mmol) was reacted at 180 °C for 5 h according to Methods B. The resulting residues were purified by chromatographic column on silica gel (toluene/ethylacetate 9.5:0.5). $C_{13}H_{16}O_2$ (204,26); Yield 57 mg (28%); colorless liquid. — ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 13.10$ (s, 1H, OH), 7.64 (dd, 1H, $^3J_{5',6'} \approx 8.0$ Hz, $^4J_{4',6'} \approx 1.5$ Hz, H-6′), 7.49 (dd, 1H, $^3J_{4',5'} \approx 8.0$ Hz, H-4′), 6.83 (t, 1H, H-5′), 6.25 (dd, 1H, $J_{cis} \approx 10.8$ Hz, $J_{trans} \approx 17.5$ Hz, H-2′′), 5.10–4.95 (m, 2H, H-3′′), 2.63 (s, 3H, H-2), 1.50 (s, 6H, $(CH_3)_2$). — ^{13}C-NMR (75.5 MHz, $CDCl_3$): $\delta = 205.1$ (C=O), 161.7 (C-2′), 147.2 (C-2′′), 136.8 (C-3′), 134.1 (C-4′), 129.1 (C-6′), 119.6 (C-1′), 118.0 (C-5′), 110.5 (C-3′′), 40.6 (C-1′′), 27.0 (C-2), 26.8 ($(CH_3)_2$). — MS (70 eV): $m/z = 204$ (M^+), 189 (M^+ –15 (CH_3)), 161 (M^+ –43 (CH_3CO), 43 (CH_3CO). — IR (film): $\nu = 3435$ (OH), 3030 (=CH aromat.), 2970, 2915 (CH_3), 2855 (CH_2), 1644 (C=O), 1587 (C=C) cm^{-1}. $C_{13}H_{16}O_2$ (204,26): calcd. C 76.44, H 7.89; found: C 76.41, H 7.85.

6.1.2.4. 5-(3-Methylbut-2-enyl)-2-(3-methylbut-2-enyloxy)acetophenone ***(5)***. Compound **3** was reacted according to Methods A. The resulting residue was purified by column chromatography on silica gel (toluene/ethylacetate 9:1). $C_{18}H_{24}O_2$ (272.37); Yield 435 mg (80%); colorless liquid. — ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 7.52$ (d, 1H, $^4J_{4',6'} \approx 2.5$ Hz, H-6′), 7.23 (dd, 1H, $^3J_{3',4'} \approx 8.5$ Hz, H-4′), 6.86 (d, 1H, $^3J_{3',4'} \approx 8.5$ Hz, H-3′), 5.47 (m, 1H, H-2′′), 5.26 (m, 1H, H-2′′′), 4.57 (d, 2H, $^3J_{1'',2''} \approx 6.5$ Hz, H-1′′), 3.27 (d, 2H, $^3J_{1''',2'''} \approx 7.0$ Hz, H-1′′′), 2.6 (s, 3H, H-2), 1.77 (br m, 3H, CH_3-prenyl), 1.72 (br s, 3H, CH_3-prenyl), 1.71 (br m, 3H, CH_3-prenyl), 1.69 (br s, 3H, CH_3-prenyl). — ^{13}C-NMR (62.9 MHz, $CDCl_3$): $\delta = 200.2$ (C=O), 156.6 (C-2′), 138.0 (C-3′′), 133.8 (C-5′), 133.2 (C-4′), 132.6 (C-3′′′), 129.9 (C-6′), 128.5 (C-1′), 123.0 (C-2′′′), 119.4 (C-2′′), 112.9 (C-3′), 65.5 (C-1′′), 33.2 (C-1′′′), 32.0 (C-2), 25.7 (CH_3-prenyl), 25.7 (CH_3-prenyl), 18.2 (CH_3-prenyl), 17.8 (CH_3-prenyl). — IR (film): $\nu = 3070$, 3026 (=CH), 2970 (CH_3), 2879 (CH_2), 1678 (C=O), 1236 (C–O) cm^{-1}. — $C_{18}H_{24}O_2$ (272.37): calcd. C 79.37, H 8.88; found: C 79.32, H 8.85.

6.1.2.5. 6-(3-Methylbut-2-enyl)chromone-3-carbaldehyde ***(6)***. To a solution of compound **3** (817 mg, 4 mmol) in dry DMF (2 ml) was added phosphoryl chloride (1.23 g, 8 mmol) dropwise at 0 °C. The resulting mixture was heated at 45 °C for 1 h, poured into cold water (15 ml) and extracted with chloroform. The combined organic layers were dried with anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (*n*-hexane/ethylacetate 1:0,1). $C_{15}H_{14}O_3$ (242.26); Yield 106 mg (11%); white solid. — ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 10.36$ (s, 1H, CHO), 8.5 (s, 1H, H-2), 8.05 (d, 1H, $^4J_{5,7} \approx 2.2$ Hz, H-5), 7.54 (dd, 1H, $^3J_{7,8} \approx 8.5$ Hz, H-7), 7.42 (d, 1H, $^3J_{7,8} \approx 8.5$ Hz, H-8), 5.3 (m, 1H, H-2′), 3.44 (d, 2H, $^3J_{1',2'} \approx 7.5$ Hz, H-1′), 1.75 (br m, 3H, CH_3-prenyl), 1.71 (br s, 3H, CH_3-pre-

nyl). — ^{13}C-NMR (75.5 MHz, $CDCl_3$): $\delta = 188.7$ (CHO), 176.0 (C-4), 160.3 (C-2), 154.6 (C-8a), 140.8 (C-6), 135.2 (C-7), 134.0 (C-3′), 124.7 (C-5), 124.7 (C-4a), 121.6 (C-2′), 120.1 (C-3), 118.4 (C-8), 33.6 (C-1′), 25.6 (CH_3-prenyl), 17.8 (CH_3-prenyl). — MS (70 eV): $m/z = 242$ (M^+), 241 ($M^+ - 1$ (H)), 69 (prenyl), 29 (CHO). — IR (film): $\nu = 3040$, 3020 (=CH), 2975 (CH_3), 2870 (CH_2), 1695 (CHO), 1645 (C=O) cm^{-1}. — $C_{15}H_{14}O_3$ (242.26): calcd. C 74.36, H 5.82, found: C 74.33, H 5.79.

7. Cytotoxicity assay

The experiments [22,23] were performed using the following human cancer cell lines: NCI-460 (lung), UACC-62 (melanoma), MCF-7 (breast) and NCI-ADR (breast expressing the multidrug resistance phenotype). The National Cancer Institute, Frederick MA/USA (NCI), kindly donated these cell lines and stock cultures were kept in liquid nitrogen.

Cells were cultured in 25 cm^2 flasks (Nunc Brand Products) containing 5 ml of RPMI 1640 (Gibco BRL, Life Technologies.) with 5% fetal bovine serum (Gibco BRL, Life Technologies). After the twentieth serial passages the cells were discarded and new flasks were unfrozen for use. All the adherent cell lines were detached from the culture flasks by addition of 0.5 ml of trypsin (Nutricell Nutrientes Celulares). Thereafter, trypsin was inactivated by addition of 5 ml of 5% serum in RPMI 1640 medium. Cells were separated into single-cell suspensions by a gentle pipetting action. After counting, the cells were diluted into appropriate seeding densities and inoculated onto 96-wells microtiter plates (Nunc Brand Products). Cells plating volume was 100 μl per well. Seeding densities varied among the cell lines as follows: 4.0×10^4 (NCI-460), 3.0×10^4 (UACC62), 6.5×10^4 (MCF-7) and 5.0×10^4 (NCI-ADR) cells per ml. Microtiter plates containing cells were pre incubated for 24 hours at 37 °C in order to allow stabilization before the addition (100 μl) of the test substance (either crude extract, fractions or drugs). The plates were incubated with the test substance for 48 hours at 37 °C and 5% CO_2 [22]. The positive control of these experiments was doxorrubicin [(DOX) (Sigma Chemical Co.)] and tamoxifen [(TAM) (Sigma Chemical Co.)]. These agents were tested at five 10-fold concentrations, starting from with maximum concentration of 10^{-4} M for DOX and 5 μM for TAM in RPMI/FBS/gentamicin.

7.1. Solubilization and dilution of test substance

For initial screening, the substances were tested at 250 μg/ml. If antiproliferative activity was detected, the test substance was retested at four concentrations (0.25; 2.5; 25; 250 μg/ml), and each concentration was studied in triplicate wells. All samples were initially solubilized in dimethyl sulfoxide (Sigma Chemical Co.) at 400 times the desired final maximum test concentration. The synthesized compounds were stored frozen at –70 °C. The concentrates were diluted with complete medium containing 50 μg/ml gentamicin (Schering-Plough).

8. Antiproliferative–sulforhodamine B (SRB) assay

Sulforhodamine B (SRB) is an aminoxantine with a bright pink color that has two sulfonic groups. Since it is an anionic dye in weak acid solution it is capable of bonding to protein's amino acids basic terminals cells fixed with trichloroacetic acid. Therefore this non-clonogenic methodology permits a high sensitive protein with a straight relationship to cell culture [22].

The SRB assay was performed according to the assay described by Skehan [22]. Briefly, the cells were fixed by means of protein precipitation with 50% trichloroacetic acid (TCA) (Sigma Chemical Co.) at 4 °C (50 μl/well, final concentration 10%) for 1 h. The supernatant was then discarded and the plates were washed five times with tap water. The cells were stained for 30 min with 0.4% the SRB (Sigma Chemical Co.), dissolved in 1% acetic acid (50 μl/well) (Sigma Chemical Co.) and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were dried and bound protein stain was solubilized with 150 μl of 10 mM Trizma buffer (Sigma Chemical Co.). The optical density was read on an automated spectrophotometer plate reader (Molecular Devices Versa Max Microplate Reader) at 540 nm.

8.1. Data calculations

The optical density data were calculated according to Excel® program (Microsoft Office Package) and the values for mean ± average standard error of data from replicated wells were calculated. The background optical measurements were subtracted from appropriated control well values and the appropriate drug-blank measurements. Cellular responses were calculated for growth stimulation, test substance effect and growth inhibition. Three measurements were run at: time zero (T0) value of SRB protein content at the beginning of test substance incubation, control value (C) at the end of the test substance incubation, and a set of test substance-treated test values (T) at the end of the test substance incubation period. If T was greater than or equal to T_0 (antiproliferative effect), the calculation was $100 \times [(T - T_0)/(C - T_0)]$. If T was less than T_0, cell killing (cytotoxic effect) occurred and was calculated from $100 \times [(T - T_0)/T_0]$. The IC_{50} values (drugs concentration eliciting 50% inhibition) were determined by non-linear regression analysis.

The results presented here refer to a representative experiment since all assays were run in triplicates the average standard error was always lower than 5%.

9. Cytotoxicity assay (MTT method)

Test substances were used as concentrated stock solutions in dimethyl sulfoxide (10 mg/ml) and used in a starting concentration of 50–100 μg/ml.

Except SIM, KAL Ewings sarcoma (established in Vienna) and LAN neuroblastoma (University of Los Angeles) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown to confluent monolayers

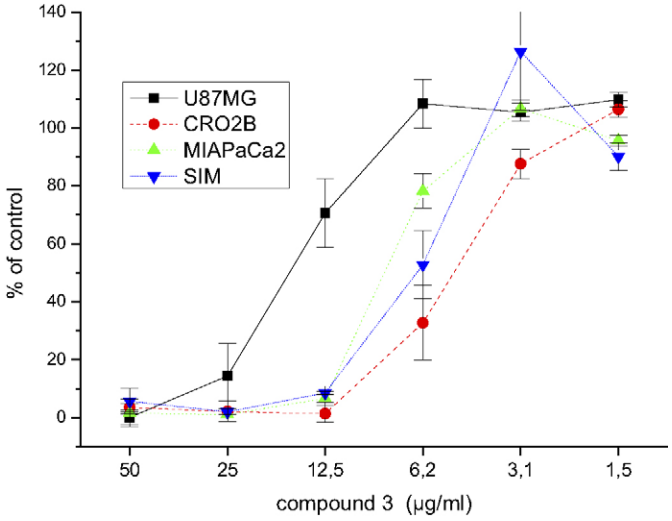


Fig. 2. Individual dose–response curve for some of the cell lines, showing typical experiments for compound **3** (MTT assay): values are shown as mean ± S.D.

in RPMI-1640 bicarbonate medium (Seromed, Berlin, Germany) in a humidified incubator (5% CO_2, 37 °C). With the single exception of the murine NIH3T3 fibroblasts all other cell lines used were of human origin. Cells were checked for mycoplasma contamination. The medium was supplemented with 10% heat-inactivated fetal bovine serum (Seromed) and 4 mM glutamine. The cells were subcultured by trypsination (0.03% trypsin containing 0.02% EDTA, three times a week). Trypsination was stopped using complete tissue culture medium. Cell numbers were counted using a TOA Sysmex microcellcounter (TOA, Tokyo, Japan).

10^4 cells/well were distributed to 96-well microtiter plates (100 µl medium) and compounds to be tested were added in a volume of another 100 µl. All compounds were diluted in five steps using twofold dilutions in triplicate or, in most cases, in quadruplicate. Plates were incubated under tissue culture conditions for 4 days, except for tests investigating the relationship between application time and response, and cell viability was measured using a modified MTT assay (EZ4U nonradioactive cell proliferation and cytotoxicity assay, Biomedica, Vienna, Austria; test manual at: www.bmgrp.com), accessing mitochondrial activity and thereby cell viability/cell numbers. In brief, the supplied substrate was dissolved in an activator solution and 20 µl of the mixture added per well (total volume 200 µl) for 2 h. The optical density was measured at 450 nm using an empty well as reference in a microplate reader (Eurogenetics, Brussels, Belgium). For each cell line eight wells were used to measure the MTT signal of the medium control without test substances and the proliferation in the test wells were calculated in relation to these control values set to 100%. Test results were recorded between 0.3 and 1.5 optical densities for slowly and rapidly proliferating cells, respectively. The concentrations of chemotherapeutic drugs given in the Fig. 2 are the initial concentrations used in the dilution sequence. These dose–response relationships were used to calculate IC_{50} (Inhibitory Concentration 50) using PRISM scientific software (GraphPad Inc., San Diego, CA, USA).

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