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Original article

Synthesis and biological properties of new α-methylene-γ-butyrolactones and α,β-unsaturated δ-lactones

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Abstract

The synthesis of a series of α-methylene-γ-butyrolactones (compounds **4a**, **4b**, **6**–**12, 16, 17**) and α,β-unsaturated-δ-lactones (compounds **19**–**23**, **25**, **26**) starting from 4,4-dimethyldihydrofuran-2,3-dione (**1**) has been described. Their chemical structures were assigned by spectroscopic evidence. These new compounds exhibited significantly different antiproliferative properties against cultured human tumor cell lines with their IC_{50} values ranging from 0.88 to > 20.00 μM.

Keywords: α-Methylene-γ-butyrolactones; α,β-Unsaturated-δ-lactones; Cytotoxic activity; Antiproliferative activity

1. Introduction

The α-methylene-γ-butyrolactone structural unit characterizes a rapidly expanding group of sesquiterpenes, which are known to possess significant biological activity. Cytotoxic, anti-inflammatory, phytotoxic, allergenic and antimicrobial properties are shown not only by highly functionalized, complex sesquiterpene lactones but also simple representatives have been studied for their biological effects [1].

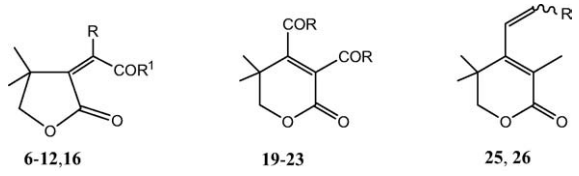
Because of their broad range of biological activities and their interesting structural features, α-methylene-γ-butyrolactones present a scientific challenge which is reflected in an increasing number of investigations and syntheses of these heterocycles [2].

Also α,β-unsaturated-δ-lactones are widely distributed in both plants and fungi and possess a diverse range of biological activity. They have been reported as plant growth inhibitors, insect antifeedants, antifungal and antitumor agents [3].

Recently we have synthesized and evaluated the cytotoxic activity of a series of substituted α-methylene-γ-butyrolactones [4], the anti-inflammatory activity of a series of α,β-unsaturated-δ-lactones substituted with a pentyl chain at the 3- position of the ring [5] and of 3-unsubstituted δ-lactones [6].

These investigations prompted us to develop three series of new compounds whose characteristic structural features are 3-alkylidene-4,4-dimethyldihydrofuran-2-ones **6**–**12**, **16**, **17**, 5,5-dimethyl-2-oxo-5,6-dihydro-2H-pyran-3,4-dicarboxyalkylesters **19**–**22** and amide **23** and 5,5-dimethyl-4-alkylidene-3-methyl-2-oxo-5,6-dihydro-2H-pyrane **25**, **26** moieties in order to value the role of the lactone unit in cytotoxic activity (Fig. 1).

In the present paper we describe the synthesis of α-methylene-γ-lactones **4a**, **4b**, **6**–**12**, **16** and **17**, starting from 4,4-dimethyldihydrofuran-2,3-dione **1** and of α,β-unsaturated-δ-lactones **19**–**23** starting from 5,5-dimethyl-2-oxo-5,6-dihydro-2H-pyran-3,4-dicarboxylic acid **18**. Besides, the synthesis of δ-lactones **25** and **26** has been described starting from 5,5-di-



6-12,16 19-23 25, 26

Fig. 1. General structures of the synthesized compounds.

Abbreviations: MEM, minimum essential medium; SRB, sulforhodamine B.

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methyl-2-oxo-3-methyl-5,6-dihydro-2H-pyran-4-carboxyaldehyde **15b**. In order to determine the role of lactone units and alkyl or phenyl substituents as pharmacophores responsible for cytostatic activity, alkyl saturated, unsaturated side chains, cyano, benzoyl and phenyl derivatives of unsaturated γ- and δ-lactones have been prepared and tested in vitro.

All the compounds have been tested against human epithelial tumor cell lines KB and human neuroblastoma cell lines, IMR-32, showing different cytotoxic effects. Since two compounds (**20** and **23**) exhibited a pharmacological interesting cell toxicity profile in both cell lines, the modification of mitochondrial functionality induced by the two compounds, was evaluated in KB and IMR-32 cell lines exposed for 4 and 6 h to different concentrations of the above compounds, to investigate the possible initial step of their cytotoxic effect.

2. Chemistry

The unsaturated γ- and δ-lactones were obtained starting from γ-butyrolactone **1** according to the procedure described in the preceding papers [5,6]. It was found that the reaction of **1** with the triethyl phosphonoacetates **2** and **2a** was accompanied by the formation of two isomers of the 4,4-dimethyl-2-oxo-tetrahydrofuran-3-methylen-carboxyethylesters (**3a**–**d**), E and Z, respectively, easily separated by "flash chromatography" (Fig. 2).

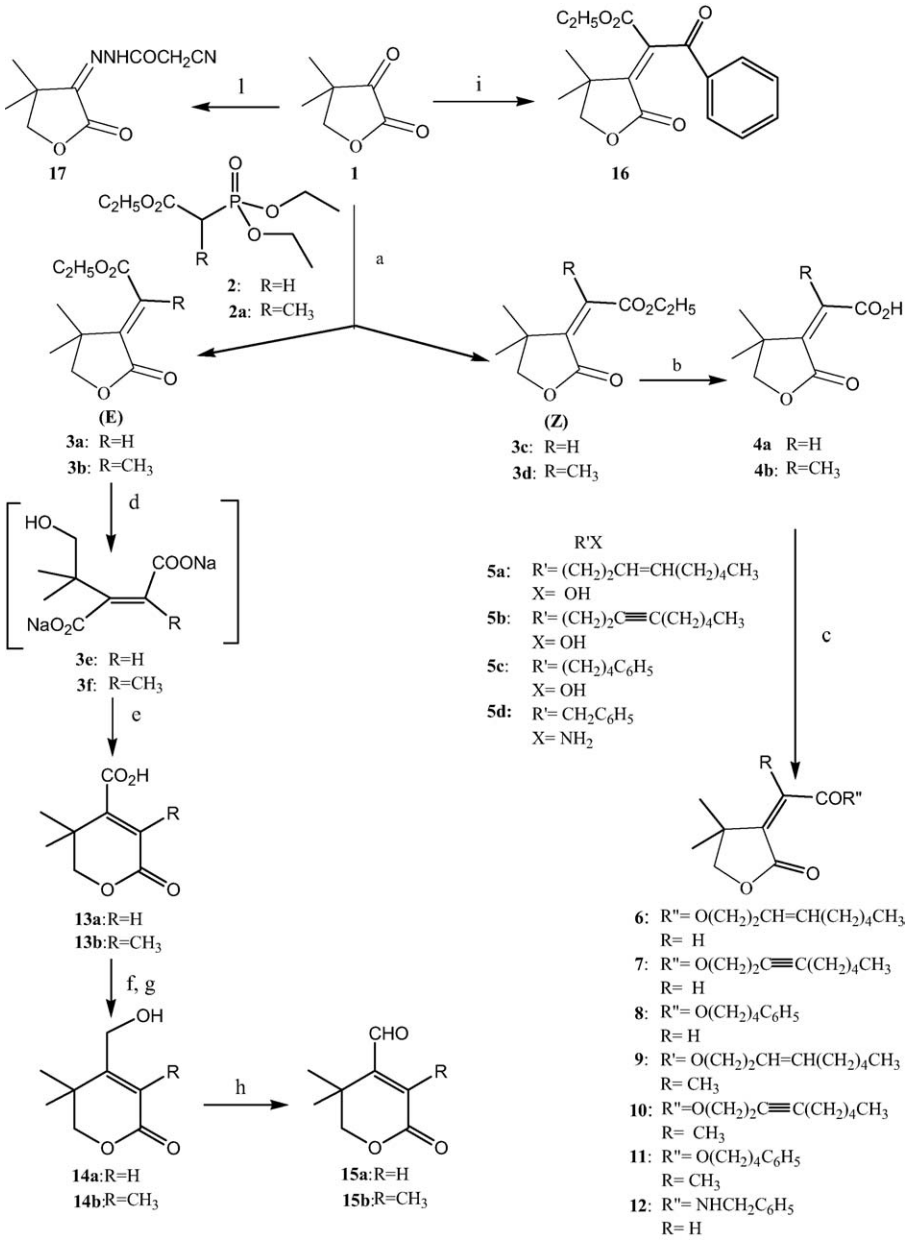

Fig. 2. (a) NaH, THF; (b) LiOH/THF/MeOH; (c) DCC, DMAP/dry CH_2Cl_2; (d) NaOH 1 N; (e) HCl 1 N; (f) CO_2Cl_2/DMF/CH_2Cl_2; (g) LiAl(tOBu)$_3$H/THF; (h) PCC/dry CH_2Cl_2; (i) $EtO_2CCH_2COC_6H_5$, $TiCl_4$/dry THF; (l) $H_2NNHCOCH_2CN$, CH_3CO_2H, HCl conc./ETOH, reflux.

Configurational assignments were made using the diagnostic deshielding effect of the carbonyl group exerted on the cis-oriented vinyl proton [7]. In the ^{1}H NMR spectra of the compounds **3a** and **3c**, the signal at $\delta = 6.76$ ppm, due to the olefinic proton, confirms the stereochemistry E for the compound **3a**, while the signal at $\delta = 6.15$ ppm confirms the configuration Z for the compound **3c**. The chemical shift to low fields of the olefinic exacyclic proton of the compound **3a** is due either to the magnetic anisotropic effect of the C=O group or to the intramolecular bond of the olefinic proton with the C=O bond of the lactonic ring [6].

In the ^{1}H NMR spectra of the compounds **3b** and **3d** the singlet at $\delta = 2.38$ ppm, ascribed to the exacyclic methyl group, confirms the stereochemistry E for the compound **3b**, while the signal at $\delta = 2.05$ ppm is due to the Z isomer **3d**.

As described previously, the hydrolysis of the E isomers of the 4,4-dimethyl-2-oxo-tetrahydrofuran-3-methylencarboxyethylester derivatives with sodium hydroxide and subsequently with hydrochloric acid led to the ring opening giving the fumaric acid derivatives **3e** and **3f** as intermediates which after rearrangement gave the δ-valerolactones **13a**, **b** (Fig. 2) [5,6]. The acids **13a**, **b**, derived from the hydrolysis of the compounds (E)—**3a**, **b**, treated with oxalyl chloride and DMF in CH_2Cl_2 gave the corresponding chlorides which were submitted, without further purification, to reduction to the corresponding alcohols **14a**, **b** with $LiAl(tOBu)_3H$ in THF. The compounds **14a**, **b** in presence of PCC in dry CH_2Cl_2 gave the corresponding aldehydes **15 a**, **b** [5,6].

However, the Z isomers of the 4,4-dimethyl-2-oxo-tetrahydrofuran-3-methylenecarboxyethylester derivatives, under the same conditions of hydrolysis of the E isomers were unable to give the corresponding δ-lactones, but the final products were the acids **4a**, **b**. In order to confirm this observation, the compounds **3c** and **3d,** were hydrolyzed under mild conditions (LiOH in THF−MeOH), yielding the corresponding acids **4a**, **b**. The comparison of the FT-IR and ^{1}H NMR spectra confirm the synthesis of the acids **4a**, **b** (Fig. 2).

Following the sequence of reactions depicted in Fig. 2, the starting compounds for the synthesis of the lactones **6**−**12** were the corresponding acids **4a** and **4b**. The compounds **6**−**12** have been prepared by reaction of the acids **4a** and **4b** with the corresponding alcohols **5a**−**c** and amine **5d** in presence of DCC and DMAP. Purification and separation of these mixtures by distillation and column chromatography afforded pure γ-lactones **6**−**12**.

The Knoevenagel condensation reaction of **1** with benzoyl acetic acid ethyl ester occurred in presence of $TiCl_4$ in THF and yielded the corresponding unsaturated derivative of lactone **16** (Fig. 2) [8]. The condensation reaction of **1** with cyanoacetohydrazide was carried out in the presence of acetic acid and hydrochloric acid under reflux to give the compound **17** in 40% yield (Fig. 2).

Concerning the synthesis of δ-lactone derivatives, the compound **18** represents the starting material for the synthesis of the compounds **19**–**23**. Molecule **18** has been prepared as described previously [8] and has been isolated as calcium salt from the aqueous phase of the coagulated latex sap of the plant *Euphorbia biglandulosa* Desf. [9].

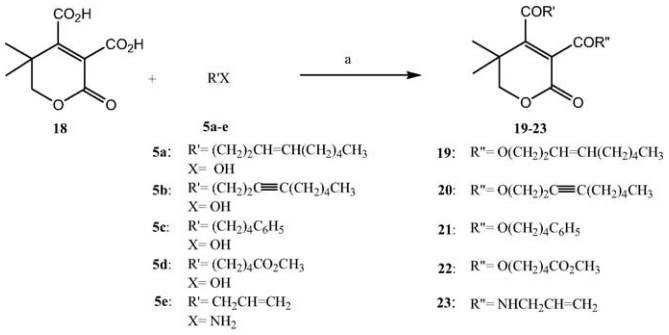

Fig. 3. (a) DCC, DMAP/dry CH_2Cl_2.

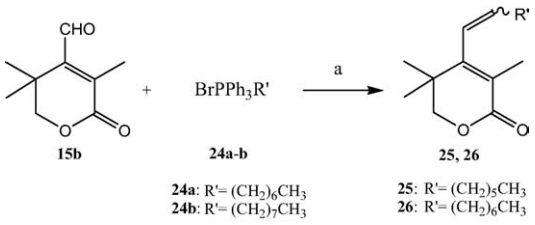

Fig. 4. (a) BuLi 1.6 M/THF dry.

We used mild conditions for the preparation of esters **19**−**22** and amide **23** derivatives of the compound **18** with various alcohols **5a–d** and amine **5e** derivatives utilizing DCC and DMAP for the coupling reaction. The esterification was carried out in one step and below room temperature to keep the reaction under control (Fig. 3). Purification and separation of these mixtures by column chromatography afforded pure δ-lactones **19**−**22** and **23**.

The aldehyde **15b** has been obtained starting from 2-(4,4-dimethyl-2-oxo-dihydrofuran-3-ylidene) propionic acid ethyl ester **3b** which was submitted to hydrolysis in the presence of sodium hydroxide followed by hydrochloric acid leading to a rearrangement of the molecule to δ-valerolactone and following the sequence of reactions depicted in Fig. 2 [5,6].

In order to study the structure−activity relationships, we have prepared the compounds **25** and **26** (bearing a methyl group at the 3-position and an alkyl chain at the 4-position of the lactone ring), starting from aldehyde **15b** and from the Wittig salts **24a** or **24b**, respectively (Fig. 4) [6].

3. Biological activity

Cytotoxic assay was carried out using a reported procedure [10,11]. The results are shown in Table 1.

Cellular MTT reduction activity by compounds **20** and **23** on two cell lines, was measured using a reported procedure [12]. The data are shown in Fig. 5.

Concentration-dependent reduction of cell survival was evaluated for the compounds **20** and **23** after 24 h contact with two different cell lines. The data are reported in Fig. 6.

Table 1
IC_{50} values for compounds **4a**, **4b**, **6**–**12, 16, 17, 19**–**23**, **25** and **26** against KB and IMR-32 cell lines

Compound	KB IC_{50} (μM)	IMR-32 IC_{50} (μM)
4a	> 20	> 20
4b	> 20	> 20
6	> 20	> 20
7	10.24	9.06
8	> 20	> 20
9	15.78	12.36
10	> 20	> 20
11	> 20	> 20
12	> 20	> 20
16	> 20	> 20
17	> 20	> 20
19	> 20	> 20
20	11.34	0.88
21	> 20	> 20
22	> 20	2.02
23	4.96	1.02
25	> 20	> 20
26	> 20	> 20

> 20 = value higher than 20 μM, lacking in pharmacological interest.

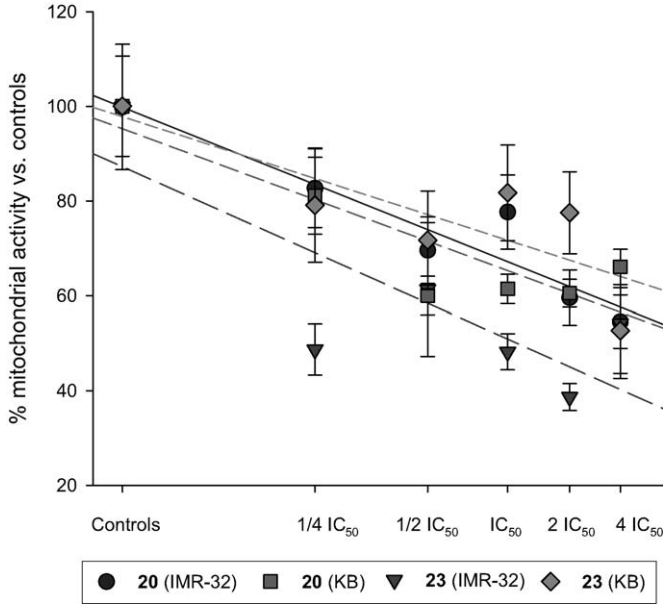

Fig. 5. Concentration-dependence of mitochondrial activity in KB and IMR-32 cell lines after 6 h exposure to compounds **20** and **23**.

4. Results and discussion

The antiproliferative activity of all obtained compounds was tested in vitro against two tumor cell lines (human KB and IMR-32) and expressed as IC_{50} values. IC_{50} is the concentration (μM) required to inhibit tumor cell proliferation by 50% after 72 h of exposure of the cells to a tested compound. The measured IC_{50} values for 3-alkylidene-4,4-dimethyldihydrofuran-2-ones **6**–**12**, **16**, **17**, 5,5-dimethyl-2-oxo-5, 6-dihydro-2H-pyran-3,4-dicarboxyalkylesters **19**–**22** and amide **23** and 5,5-dimethyl-4-alkylidene-3-methyl-2-oxo-5,6-dihydro-2H-pyrane **25**, **26** are summarized in Table 1. As can be seen from the

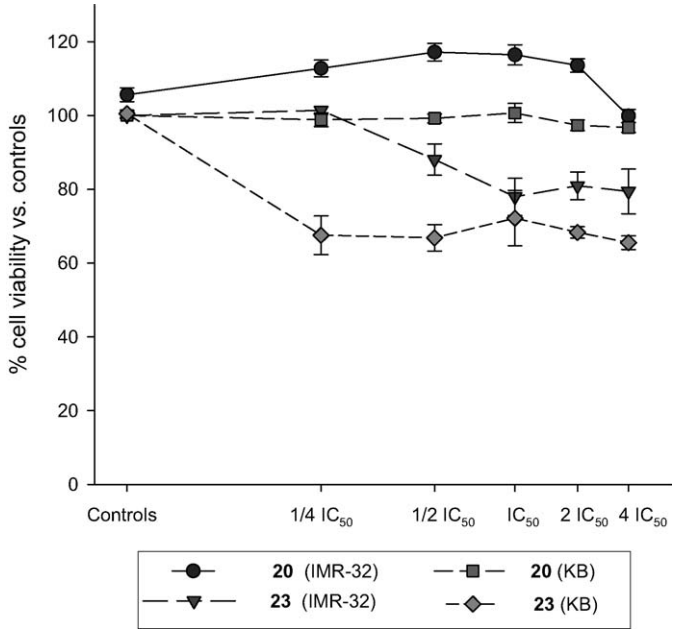

Fig. 6. Concentration-dependence of cell viability in KB and IMR-32 cell lines after 24 h exposure to compounds **20** and **23**.

presented data, cytotoxicity of 3-alkylidene-4,4-dimethyldihydrofuran-2-ones **6**–**12**, **16**, **17** is low with two exceptions for compound **7** and **9** only for which it has been possible to obtain pharmacologically interesting IC_{50} values. Cisplatinum was used as a reference compound [10]. All other compounds gave IC_{50} values > 20 μM. The compounds **7** and **9** exhibited cell toxicity in both cell lines with IC_{50} values 100 times higher than antiproliferative activity reference compound, cisplatinum, which IC_{50} value in KB cell lines is 0.37 μM. They bear an unsaturated alkyl chain at the 3-position of the lactone ring, but also the presence of an additional methyl group adjacent to the exacyclic methylene seems to play a role in the activity.

In the case of compound **7**, the absence of a methyl group between the γ-lactone ring and the ester function bearing an alkyl chain with a triple bond let to a cytotoxicity against KB (IC_{50} = 10.24 μM) and IMR-32 (IC_{50} = 9.26 μM) cell lines, while the presence of the methyl group and of an alkyl chain with a cis double bond adjacent to the ester function in the compound **9** gave an IC_{50} value of 15.78 μM against KB and 12.36 μM against IMR-32 cell lines, respectively.

As concerning the compounds **16** and **17**, the cytotoxicity values show clearly that substitution of the methylene group with the benzoyl or hydrazyl substituent decrease the activity. Steric hindrance introduced by the substituent (**8**, **11**, **12**, **16**), probably makes the double bond less vulnerable to nucleophilic attack.

There is no clear relationship between the cytotoxicity of the γ-lactones **6**–**12**, **16** and **17** against KB and IMR-32 cell lines and the nature of the R and R″ substituents (Fig. 2). In fact, the compound **10** differs from **7** only in the presence of an additional methyl group adjacent to the exacyclic methylene of the ring and some consideration can be made for the compounds **9** and **6** but nevertheless, the compounds **6** and **10** present IC_{50} values > 20 μM.

Comparison of the cytotoxicities of **6**–**12**, **16**, **17** and **19**–**23**, **25** and **26** shows that replacement of the γ-lactone with the α,β-unsaturated δ-lactone increases the activity.

In order to analyze the effects of the α,β-unsaturated-δ-lactones **19**–**23, 25** and **26**, on the viability of KB and IMR-32 cell lines, we have conducted the sulforhodamine B assay (SRB) [11]. KB and IMR-32 cells were exposed at 1.25, 2.50, 5.00 and 10 μg ml^{-1} solutions of each compound. Values of IC_{50} were reported in Table 1 for both cell lines. After 72 h of treatment, all derivatives, except compound **25**, exhibited growth inhibition at 10 μg ml^{-1}. As illustrated in Table 1, both cell lines showed different cytotoxic profiles when treated with each product. Compounds **20** and **23** exhibited cell toxicity in both cell lines, while compound **22** appeared efficient only in IMR-32 cell line. On the basis of the pharmacological data obtained, a modification of mitochondrial functionality, measured by MTT test, was evaluated in KB and IMR-32 cell lines exposed for 4 and 6 h to different concentrations (1/4, 1/2, 1, 2 and 4 IC_{50}) of compounds **20** and **23**.

No modifications in mitochondrial activity [12] were highlighted after 4 h exposure to two compounds. Instead, after 6 h exposure to both compounds was highlighted, in both cell lines, a concentration dependent decrease in mitochondrial activity (Fig. 5). The reduction is significant ($P < 0.01$) for both compounds tested at two higher concentrations excluding compound **23** in KB cell line. At lower concentration used, only compound **23** in IMR-32 cell line appears to reduce statistically mitochondrial activity with respect to respective control.

To verify if modification in mitochondrial functionality couldn't be ascribed to a reduction in cell number, an analysis of KB and IMR-32 cell viability after 24 h exposure to two compounds was carried out. No modification in viability of both cell lines was evidenced for compound **20**, while a decrease of about 30% ($P < 0.01$) in KB cell number at all concentrations, and about 20% ($P < 0.05$) in IMR-32 cell number for all three higher concentrations, was found for compound **23** (Fig. 6).

5. Conclusions

In conclusion, we have developed a general and straightforward route to 3-alkylidene-4,4-dimethyldihydrofuran-2-ones **6**–**12**, **16**, **17**, 5,5-dimethyl-2-oxo-5, 6-dihydro-2H-pyran-3,4-dicarboxyalkylesters **19**–**22** and amide **23** and 5,5-dimethyl-4-alkylidene-3-methyl-2-oxo-5,6-dihydro-2H-pyrane **25**, **26** starting from easily available common intermediate, 4,4-dimethyldihydrofuran-2,3-dione **1**.

For all obtained compounds, cytotoxic activity against the KB and IMR-32 cell lines was determined. Various derivatives of γ-lactones and of unsaturated δ-lactones show different response in their ability to influence tumor cell growth (Table 1). Two of the prepared 3-alkylidene-4,4-dimethyldihydrofuran-2-ones **7** and **9** exhibited significant cytotoxic activity against KB and IMR-32 cell lines. δ-Lactones possessed more pronounced antiproliferative activity than γ-lactones, in particular two of the prepared δ-lactones **20** and **23** exhibited remarkable cytotoxicity toward KB and IMR-32 cell lines with IC_{50} values only 10-times higher than cisplatinum, but seem to act with a different action mechanism. The comparison of cytotoxic activity data between compound **20** and **23** showed that the 3,4-diamide derivative of δ-lactone plays significant role in enhancing the cytotoxic properties of the compounds.

No clear correlation between cytotoxicity and structures in these series of compounds was found, but it seems that observed differences in activity can be better rationalized in terms of steric and electronic effects.

Activities are generally enhanced by the presence of further alkylating groups (α,β − unsaturated esters and amides), which may represent reactive receptor sites for biological nucleophiles, in particular thiol and amino groups.

Preliminary studies have been conducted in order to highlight the biological targets of antiproliferative properties of compounds **20** and **23.** Further studies are required to propose a plausible action mechanism, in particular a possible proapoptotic effect. Our current efforts are directed toward improving the antiproliferative and of mitochondrial activity inhibition properties, hypothesizing that 3,4-diesters and -diamides δ-lactones derivatives are potential tumor cell growth inhibitors.

6. Experimental protocols

6.1. Chemistry

Nuclear magnetic resonance spectra were recorded with a Varian Gemini 200 MHz spectrometer. ^{13}C NMR: 90.5 MHz, Gemini 200 spectrometer. NMR spectra were obtained by using $CDCl_3$ and DMSO as solvents; chemical shifts are expressed as δ units (ppm) relative to tetramethylsilane (TMS) as internal standard. The abbreviations s, d, dd, t, q, m and sb refer to singlet, doublet, doublet of doublet, triplet, quartet, multiplet and singlet broad signal, respectively. The EI-MS spectra were measured with a VG−ZAB 2F spectrometer. The ionizing energy was 70 eV in all cases and compounds were introduced by direct insertion. Elementary analyses were carried out on a Carlo Erba model 1016 analyzer. The column chromatography was performed by using silica gel (Kieselgel 60, 230–400 Mesh, 60 Å Merck) or aluminum oxide (150 mesh, 58 Å Merck).

TLC: Kieselgel 60 F_{254} (20 × 20 cm; 0.2 mm, Merck). Melting points were determined on a Büchi 510 micromelting point apparatus and are uncorrected. Infrared spectra were recorded as film or KBr in cm^{-1} on a Jasco FT/IR−200 model spectrophotometer. Reagents used were AR grade and all solvents for synthesis, extraction and column chromatography were distilled and dried before use.

6.1.1. (4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)acetic acid ***4a***

A 0.1 N aqueous solution of LiOH (75 ml, 37.6 mmol) was added to a solution of the ester **3c** [6] (1 g, 5.88 mmol) in 2:1 (v/v) THF−MeOH (130 ml). The mixture was stirred at room temperature for 2 h, then concentrated under vacuum. The solution was acidified up to pH 2–3 with 2 N aqueous HCl satu-

rated with NaCl and extracted with ether. This process was repeated twice. The combined ethereal extracts were dried (Na_2SO_4) and the solvents were evaporated to give the crude acid **4a** as a white semi-solid which was used, without further purification, in the next step. TLC: ethyl acetate/acetic acid (8:0.1, v/v), $R_f = 0.12$. Yield: 0.89 g (90%). FT-IR (film): 2898, 1778, 1712, 1644 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.25 (s; 6H, 2C*H*$_3$−4); 4.29 (s; 2H, C*H*$_2$−5); 6.35 (s; 1H, C=C*H*); 12.0 (br s; 1H, COO*H*); ^{13}C NMR ($CDCl_3$): δ 172.5 (C-2); 163.4 (*C*O_2H); 146.6 (C-3); 130.3 (=*C*H); 80.2 (C-5); 41.4 (C-4); 26.6 (2*C*H_3−4); MS (EI): *m/z* 170 (M^+, 18%); 152 (M^+−H_2O, 100%); 125 (M^+−45, 60%). Anal. Calcd. for $C_8H_{10}O_4$: C, 56.47; H, 5.88; found: C, 56.74; H, 5.92.

6.1.2. 2-(4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)propionic acid ***4b***

Obtained as a yellow oil from **3d** (1 g, 5.43 mmol) according to the procedure described above. TLC: petroleum ether/diethyl ether (8:3, v/v), $R_f = 0.19$. Yield: 0.5 g (58%). FT-IR (film): 2898, 1779, 1715, 1644 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.25 (s; 6H, 2C*H*$_3$−4); 2.10 (s; 3H, = −C*H*$_3$); 4.0 (s; 2H, C*H*$_2$−5); 12.0 (br s; 1H, COO*H*); ^{13}C NMR ($CDCl_3$): δ 172.5 (C-2); 163.4 (*C*O_2H); 146.6 (C-3); 123.4 (=*C*−CH_3); 80.2 (C-5); 41.4 (C-4); 26.6 (2*C*H_3−4); 20.4 (*C*H_3); MS (EI): *m/z* 184 (M^+, 20%); 166 (M^+−H_2O, 100%); 139 (M^+−45, 60%); 169 (M^+−CH_3, 22%). Anal. Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.52; found: C, 58.92; H, 6.73.

6.1.3. General procedure for the preparation of compounds ***6–12***

Carboxylic acid **4** (1.18 mol) was dissolved in dry CH_2Cl_2 (20 ml) and the solution was cooled at 0 °C while DCC (1.39 mol) and DMAP (26 mmol) were added in one portion. The mixture was stirred for 30 min at room temperature and cooled at 0 °C and a solution of the appropriate alcohol **5a–c** (1 mol) and amine **5d** (1 mol) were added. The mixture was stirred overnight at room temperature. The reaction was monitored by TLC and, after the consumption of the starting material, the resulting precipitate of DCU was filtered off and washed with CH_2Cl_2. The obtained solution was dried (Na_2SO_4) then evaporated under vacuum to yield a residue that was subjected to distillation under reduced pressure (0.1 mbar) to remove the unreacted starting material and column chromatography (eluent, petroleum ether/ethyl acetate = 8:2 for **6**, *n*-hexane/ethyl acetate = 1:1 for **7**, **8**, petroleum ether/ethyl acetate = 1:1 for **9, 12**, *n*-hexane/ethyl acetate = 7:3 for **10** and *n*-hexane/diethyl ether = 1:1 for **11**).

6.1.3.1. (4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)acetic acid non-3′-enyl ester ***6***. Semi-solid. TLC: petroleum ether/ethyl acetate (8:2, v/v), Rf = 0.69. Yield: 134 mg (39%). FT-IR (film): 3008, 2958, 1773, 1733, 1650 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.9 (t; 3H, C$\underline{H}_3$−9′); 1.2−1.4 (br s; 12H, 2C$\underline{H}_3$−4, C$\underline{H}_2$−6′, 7′, 8′); 2.05 (m; 2H, C$\underline{H}_2$−5′); 2.48 (m; 2H, C$\underline{H}_2$−2′); 4.07 (s; 2H, C$\underline{H}_2$−5); 4.23 (t; 2H, C$\underline{H}_2$−1′); 5.34−5.59 (m; 2H, C$\underline{H}$−3′, 4′); 6.19 (s; 1H, =C$\underline{H}$−3); ^{13}C NMR ($CDCl_3$): δ 167.1 (C$\underline{O}$O); 165.1 (C-2); 142.6 (C-3); 132.9 (C$\underline{H}$-3); 125.0 (C−3′), 123.7 (C-4′); 77.7 (C-5); 64.9 (C-1′); 39.7 (C-4); 27.6 (C-5′); 27.1 (C−2′); 29.0−22.4 (C-6′, C-7′, C-8′); 24.5, 24.9 (2C$\underline{H}_3$−4); 13.9 (C-9′); MS (EI): *m/z* 294 (M^+, 37%); 279 (M^+−15, 100%); 264 (M^+−2CH_3, 47%); 153 (M^+−$C_9H_{17}O$, 95%); 125 (M^+−$C_{10}H_{17}O_2$, 58%). Anal. Calcd. for $C_{17}H_{26}O_4$: C, 69.38; H, 8.84; found: C, 69.46; H, 8.63.

6.1.3.2. (4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)acetic acid non-3′-ynl ester ***7***. Semi-solid. TLC: *n*-hexane/ethyl acetate (1:1, v/v), Rf = 0.71. Yield: 180 mg (53%). FT-IR (film): 2929, 2854, 2117, 1770, 1735, 1670 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.9 (t; 3H, C$\underline{H}_3$−9′); 1.30 (br s; 12H, 2C$\underline{H}_3$−4, C$\underline{H}_2$−6′, 7′, 8′); 2.1 (t; 2H, C$\underline{H}_2$−5′); 2.4 (t; 2H, C$\underline{H}_2$−2′); 4.1 (s; 2H, C$\underline{H}_2$−5); 4.28 (t; 2H, C$\underline{H}_2$−1′); 6.10 (s; 1H, C$\underline{H}$−3); ^{13}C NMR ($CDCl_3$): δ 167.4 (C$\underline{O}$O); 165.0 (C-2); 143.2 (C-3); 124.9 (=C$\underline{H}$−3); 82.3 (C-3′); 77.9 (C-5); 75.2 (C-4′); 64.0 (C-1′); 39.9 (C-4); 31.1−22.3 (C-6′, C-7′, C-8′,); 24.7, 24.8 (2C$\underline{H}_3$−4); 19.0 (C-2′); 18.7 (C-5′); 14.1 (C-9′); MS (EI): *m/z* 292 (M^+, 13%); 277 (M^+−15, 29%); 262 (M^+−2CH_3, 10%); 263 (M^+−C_2H_5, 20%); 249 (M^+−C_3H_7, 12%); 235 (M^+−C_4H_9, 18%); 221 (M^+−C_5H_{11}, 20%); 207 (M^+−$C_4H_5O_2$, 100%). Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.86; H, 8.22; found: C, 70.01; H, 8.39.

6.1.3.3. (4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)acetic acid 4′-phenylbutyl ester ***8***. White crystals (m.p. 66 °C). TLC: *n*-hexane/ethyl acetate (1:1, v/v), Rf = 0.58. Yield: 150 mg (43%). FT-IR (film): 3026, 2961, 1769, 1730, 1660 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.23 (br s; 6H, 2C$\underline{H}_3$−4); 1.75 (m; 4H, C$\underline{H}_2$−2′, 3′); 2.70 (t; 2H, C$\underline{H}_2$−4′); 4.15 (s; 2H, C$\underline{H}_2$−5); 4.28 (t; 2H, C$\underline{H}_2$−1′); 6.20 (s; 1H, C$\underline{H}$−3); 7.19−7.40 (m; 5H, H-arom); ^{13}C NMR ($CDCl_3$): δ 167.8 (C$\underline{O}$O); 165.8 (C-2); 143.0 (C-3); 142.4 (C-1″); 128.8−125.3 (C-arom.); 125.6 (=C$\underline{H}$); 78.2 (C-5); 66.0 (C-1′); 40.25 (C-4); 35.6 (C-4′); 28.39 (C-3′); 28.0 (C-2′); 26.3, 26.6 (2C$\underline{H}_3$−4); MS (EI): *m/z* 302 (M^+, 35%); 225 (M^+−C_6H_5, 12%); 212 (M^+−C_7H_8, 10%); 154 (M^+−$C_{10}H_{13}O$, 100%); 125 (M^+−$C_{11}H_{13}O_2$, 15%). Anal. Calcd. for $C_{18}H_{12}O_4$: C, 71.52; H, 3.97; found: C, 71.31; H, 3.86.

6.1.3.4. 2-(4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)propionic acid non-3′-enyl ester ***9***. Oil. TLC: petroleum ether/ethyl acetate (1:1, v/v), Rf = 0.75. Yield: 60 mg (16%). FT-IR (film): 2929, 1758, 1729, 1668 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.9 (t; 3H, C$\underline{H}_3$−9′); 1.32 (m; 6H, C$\underline{H}_2$−6′, 7′, 8′), 1.40 (s; 6H, 2C$\underline{H}_3$−4); 2.15 (m; 2H, C$\underline{H}_2$−5′); 2.22 (s; 3H, = –C$\underline{H}_3$); 2.32 (m; 2H, C$\underline{H}_2$−2′); 4.0 (s; 2H, C$\underline{H}_2$−5); 4.22 (t; 2H, C$\underline{H}_2$−1′); 5.35−5.60 (m; 2H, C$\underline{H}$−3′, 4′); ^{13}C NMR ($CDCl_3$): δ 169.6 (C$\underline{O}$O); 167.3 (C-2); 140.0 (C-3); 132.8 (=C$\underline{C}$−CH_3); 124.4 (C-3′); 128.2 (C-4′); 78.1 (C-5); 69.4 (C-1′); 38.5 (C-4); 26.9 (C-2′); 26.8 (C-5′); 30.3−22.5 (C-6′, C-7′, C-8′); 24.5, 25.0 (2C$\underline{H}_3$−4); 13.6 (C-9′); 10.6 (C$\underline{H}_3$−C=); MS (EI): *m/z* 308 (M^+, 28%); 293 (M^+−15, 10%); 278 (M^+−2CH_3, 100%). Anal. Calcd. for $C_{18}H_{28}O_4$: C, 70.13; H, 9.09; found: C, 70.31; H, 9.24.

*6.1.3.5. 2-(4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)propionic acid non-3′-ynl ester **10***. Oil. TLC: *n*-hexane/ethyl acetate (7:3, v/v), Rf = 0.82. Yield: 99.5 mg (28%). FT-IR (film): 2958, 2929, 2117, 1733, 1668, 1100 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.9 (t; 3H, C<u>H</u>$_3$−9′); 1.30 (m; 6H, C<u>H</u>$_2$−6′, 7′, 8′); 1.35 (s; 6H, 2C<u>H</u>$_3$−4), 2.15 (m; 5H, C<u>H</u>$_2$−5′, C<u>H</u>$_3$−C=); 2.5 (m; 2H, C<u>H</u>$_2$−2′); 4.0 (s; 2H, C<u>H</u>$_2$−5); 4.28 (t; 2H, C<u>H</u>$_2$−1′); ^{13}C NMR ($CDCl_3$): δ 169.0 (<u>C</u>OO); 168.4 (C-2); 140.0 (C-3); 131.5 (=<u>C</u>−CH$_3$); 81.6 (C-3′); 78.0 (C-5); 74.8 (C-4′); 67.6 (C-1′); 40.9 (C-4); 30.6−21.7 (C-6′, 7′, 8′); 24.5, 24.7 (2<u>C</u>H$_3$−4); 19.0 (C-2′); 18.9 (C-5′); 13.5 (C-9′); 10.6 (<u>C</u>H$_3$C=); MS (EI): *m/z* 306 (M^+, 10%); 291 (M^+−15, 13%); 276 (M^+−2CH$_3$, 10%); 277 (M^+−C_2H_5, 23%); 263 (M^+−C_3H_7, 9%); 249 (M^+−C_4H_9, 13%); 218 (M^+−C_4H_9−30, 100%). Anal. Calcd. for $C_{18}H_{26}O_4$: C, 70.58; H, 8.49; found: C, 70.12; H, 8.69.

*6.1.3.6. 2-(4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)propionic acid 4′-phenylbutyl ester **11***. Oil. TLC: *n*-hexane/diethyl ether (1:1, v/v), Rf = 0.68. Yield: 63 mg (18%). FT-IR (film): 2859, 1754, 1731, 1668 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.30 (br s; 6H, 2C<u>H</u>$_3$−4); 1.8 (m; 4H, C<u>H</u>$_2$−2′, 3′); 2.18 (s; 3H, C<u>H</u>$_3$C=); 2.68 (t; 2H, C<u>H</u>$_2$−4′); 4.0 (s; 2H, C<u>H</u>$_2$−5); 4.22 (t; 2H, C<u>H</u>$_2$−1′); 7.19−7.37 (m; 5H, H-arom); ^{13}C NMR ($CDCl_3$): δ 167.8 (<u>C</u>OO); 165.8 (C-2); 141.7 (C-3); 140.0 (=<u>C</u>−CH$_3$); 128.3; 130.4−125.3 (C-arom.); 78.0 (C-5); 67.7 (C-1′); 40.0 (C-4); 35.0 (C-4′); 29.9 (C-3′); 28.5 (C-2′); 25.7, 25.1 (2<u>C</u>H$_3$−4); 10.6 (<u>C</u>H$_3$−C=); MS (EI): *m/z* 316 (M^+, 12%); 225 (M^+−C_7H_7, 12%); 168 (M^+−$C_{10}H_{13}O$, 100%); 153 (M^+−$C_{10}H_{13}O$ − 15, 10%); 122 (M^+−$C_{13}H_{22}O$, 90%). Anal. Calcd. for $C_{19}H_{24}O_4$: C, 72.15; H, 7.59; found: C, 71.98; H, 7.37.

*6.1.3.7. N-benzyl-2-(4,4-Dimethyl-2-oxodihydrofuran-3-ylidene) acetamide **12***. Oil. TLC: petroleum ether/ethyl acetate (1:1, v/v), Rf = 0.71. Yield: 109 mg (36%). FT-IR (film): 3310, 2925, 1763, 1629 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.27 (s; 6H, 2C<u>H</u>$_3$−4); 4.18 (s; 2H, C<u>H</u>$_2$−5); 4.58 (d; 2H, C<u>H</u>$_2$−1′); 6.22 (s; 1H, =C<u>H</u>); 7.19−7.40 (m; 5H, H-arom); 9.62 (br s; 1H, N<u>H</u>); ^{13}C NMR ($CDCl_3$): δ 170.7 (<u>C</u>ONH); 163.5 (C-2); 142.0 (C-3); 138.15 (=<u>C</u>CH); 133.0; 128.9−127.7 (C-arom.); 78.9 (C-5); 44.3 (C-1′); 42.0 (C-4); 27.0 (2<u>C</u>H$_3$−4); MS (EI): *m/z* 247 (M^+, 12%); 156 (M^+−C_7H_7, 23%); 141 (M^+−C_7H_8N, 100%). Anal. Calcd. for $C_{14}H_{17}O_3N$: C, 68.01; H, 6.88, N, 5.67; found: C, 68.27; H, 7.03, N, 5.55.

*6.1.4. 2-(4,4-Dimethyl-2-oxodihydrofuran-3-ylidene)-3-oxo-3-phenylpropionic acid ethyl ester **16***

To a stirred suspension of 0.2 mol of $TiCl_4$ (22 ml) in 400 ml of dry THF, under nitrogen, were added drop-wise, at 0 °C, 0.1 mol of **1** and 0.1 mol of ethyl benzoylacetate. After stirring for 1 h, 0.2 mol of pyridine in 50 ml of THF were added and the reaction mixture was stirred at room temperature for 36 h. The reaction mixture was washed with water (100 ml), and then extracted with ether (100 ml × 3). The ether extracts were combined and washed with brine (100 ml), dried over Na_2SO_4, filtered, and evaporated to give a crude solid which was crystallized from ether to afford **16** as white crystals. TLC: *n*-hexane/ethyl acetate (7:3, v/v), Rf = 0.61. Yield: 25 g (82%); m.p.: 77.5 °C; FT-IR (KBr): 2960, 1770, 1720, 1680, 1600 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.22 (t; 3H, C<u>H</u>$_3$CH$_2$); 1.43 (br s; 6H, 2C<u>H</u>$_3$−4); 4.10 (s; 2H, C<u>H</u>$_2$−5); 4.22 (q; 2H, C<u>H</u>$_2$OCO); 7.40−7.98 (m; 5H, H-arom); ^{13}C NMR ($CDCl_3$): δ 191.6 (<u>C</u>OC_6H_5); 168.9 (<u>C</u>O$_2C_2H_5$), 162.9 (C-2); 143.1 (C-3); 140.4 (=<u>C</u>); 135.6; 133.8−128.8 (C-arom.); 79.4 (C-5); 62.5 (C<u>H</u>$_2$OCO); 41.1 (C-4); 24.4 (2<u>C</u>H$_3$−4); 14.0 (<u>C</u>H$_3$CH$_2$); MS (EI): *m/z* 302 (M^+, 93%); 273 (M^+−C_2H_5, 100%); 257 (M^+−C_2H_5−15, 40%); 229 (M^+−$C_3H_5O_2$, 10%). Anal. Calcd. for $C_{17}H_{18}O_5$: C, 67.55; H, 5.96; found: C, 67.93; H, 6.01.

*6.1.5. Cyano-acetic acid (4,4-dimethyl-2-oxodihydrofuran-3-ylidene)hydrazide **17***

To a stirred suspension of 0.1 mol of **1** and 0.1 mol of cyanoacetohydrazide in 200 ml of ethanol, were added acetic acid (5 ml) and hydrochloric acid (1 ml). The reaction mixture was heated under reflux for 2 h. After the reaction was completed as judged by TLC the solid residue was collected by filtration and crystallized from ether to give **17** as white crystals. TLC: *n*-hexane/ethyl acetate (1:1, v/v), Rf = 0.55. Yield: 14.6 g (70%); m.p.: 90−93 °C; ^1H NMR (DMSO): δ 1.22 (s; 6H, 2C<u>H</u>$_3$−4); 3.37 (s; 2H, C<u>H</u>$_2$CN); 4.38 (s; 2H, C<u>H</u>$_2$−5); 9.8 (br s; 1H, N<u>H</u>); ^{13}C NMR (DMSO): δ 171.7 (<u>C</u>ONH); 163.9 (C-2); 157.0 (C-3); 115.4 (<u>C</u>≡N); 79.1 (C-5); 40.9 (C-4); 36.4 (<u>C</u>H$_2$CN); 25.1, 24.1 (2<u>C</u>H$_3$−4); MS (EI): *m/z* 209 (M^+, 100%); 141 (M^+−C_3H_2NO, 100%); 127 (M^+−$C_3H_3N_2O$, 13%). Anal. Calcd. for $C_9H_{11}O_3N_3$: C, 51.67; H, 5.26, N, 20.09; found: C, 51.82; H, 5.05, N, 20.32.

*6.1.6. General procedure for the preparation of compounds **19**–**23***

Dicarboxylic acid **18** [8] (0.9 mmol) was dissolved in dry CH_2Cl_2 (16 ml) and the solution was cooled at 0 °C while DCC (2.1 mmol) and DMAP (0.4 mmol) were added. The mixture was stirred at room temperature for 30 min, then cooled at 0 °C and a solution of the appropriate alcohol **5a**–**d** and amine **5e** (1.6 mmol) was added. The mixture was stirred overnight at room temperature. Usual work up gave the pure product.

*6.1.6.1. 5,5-Dimethyl-2-oxo-5,6-dihydro-2H-pyran-3,4-dicarboxylic acid dinon-3′(Z)-enyl ester **19***. Oil. TLC: diethyl ether/*n*-hexane (8:2, v/v), Rf = 0.81.Yield: 166 mg (40%). FT-IR (film): 1731, 1729, 1633, 1031 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.92 (t; 6H, 2C<u>H</u>$_3$−9′); 1.2−1.32 (br s; 18H, 2C<u>H</u>$_3$−5, C<u>H</u>$_2$−6′, 7′, 8′); 2.10 (dd; 4H, C<u>H</u>$_2$−5′); 2.41 (dd; 4H, C<u>H</u>$_2$−2′); 4.0 (s; 2H, C<u>H</u>$_2$−6); 4.2 (t; 4H, C<u>H</u>$_2$−1′); 5.20−5.60 (m; 4H, C<u>H</u>−3′, 4′); ^{13}C NMR ($CDCl_3$): δ 164.9 (C-2); 163.9 (<u>C</u>OO); 160.9 (<u>C</u>OO); 156.3 (C-4); 134.3−134.0 (C-3′); 125.6 (C-3); 124.1 (C-4′); 78.4 (C-6); 66.4 (C-1′); 35.2 (C-5); 32.2 (C-2′, C-5′); 29.9−27.2 (C-6′, C-7′, C-8′); 23.2, 23.0 (2<u>C</u>H$_3$−5); 14.8 (C-9′); MS (EI): *m/z* 462 (M^+, 10%); 337 (M^+−C_9H_{17}, 12%); 212 (337−C_9H_{17}, 21%); 197 (212−CH_3, 12%); 124

(212−2C_2O_4, 100%); 109 (124−CH_3, 5%). Anal. Calcd. for $C_{27}H_{42}O_6$: C, 70.13; H, 9.09; found: C, 70; H, 8.99.

6.1.6.2. 5,5-Dimethyl-2-oxo-5,6-dihydro-2H-pyran-3,4-dicarboxylic acid dinon-3′-ynyl ester ***20***. Oil. TLC: diethyl ether/*n*-hexane (8:2, v/v), Rf = 0.85. Yield: 185 mg (45%). FT-IR (film): 2360, 2337, 2117, 1733, 1633, 1010 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.96 (t; 6H, 2C$\underline{H}_3$−9′); 1.20−1.40 (br s; 14H, 2C$\underline{H}_3$−5, C$\underline{H}_2$−6′, 7′, 8′); 2.2 (m; 4H, C$\underline{H}_2$−2′); 2.6 (m; 4H, C$\underline{H}_2$−5′); 4.05 (s; 2H, C$\underline{H}_2$−6); 4.25 (m; 4H, C$\underline{H}_2$−1′); ^{13}C NMR ($CDCl_3$): δ 163.7 (C-2); 162.6 (C$\underline{O}$O); 159.9 (C$\underline{O}$O); 155.5 (C-4); 124.6 (C-3); 82.3, 82.4 (C-3′); 76.1 (C-6); 74.7, 74.5 (C−4′); 64.3 (C-1′); 34.7 (C-5); 30.8−28.3 (C-6′, C-7′); 25.3, 24.5 (2C$\underline{H}_3$−5); 22.0 (C−8′); 18.9 (C-2′); 18.5 (C-5′); 13.8 (C-9′); MS (EI): *m*/*z* 458 (M^+, 10%); 335 (M^+−C_9H_{15}, 12%); 319 (M^+−C_9H_{15}−CH_3, 7%); 291 (M^+−$C_{10}H_{15}O_2$, 23%); 167 (M^+−$C_{10}H_{15}O_2$−C_9H_{15}, 15%); 123 (M^+−2$C_{10}H_{15}O_2$, 100%). Anal. Calcd. for $C_{27}H_{38}O_6$: C, 70.74; H, 8.29; found: C, 70.55; H, 8.42.

6.1.6.3. 5,5-Dimethyl-2-oxo-5,6-dihydro-2H-pyran-3,4-dicarboxylic acid bis-(4′-phenylbutyl) ester ***21***. Oil. TLC: diethyl ether/*n*-hexane (8:2, v/v), Rf = 0.65. Yield: 200 mg (45%). FT-IR (film): 3025, 1730, 1634, 1602, 1065 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.25 (s; 6H, 2C$\underline{H}_3$−5); 1.62−1.68 (m; 8H, C$\underline{H}_2$−2′, 3′); 2.62 (m; 4H, C$\underline{H}_2$−4′); 4.02 (s; 2H, C$\underline{H}_2$−6); 4.22 (m; 4H, C$\underline{H}_2$−1′); 7.02−7.22 (m; 10H, H-arom); ^{13}C NMR ($CDCl_3$): δ 163.8 (C-2); 162.8 (C$\underline{O}$O); 159.9 (C$\underline{O}$O); 155.2 (C-4); 141.4, 141.1 (C-1″); 127.9−125.3 (C-arom.); 124.5 (C-3); 75.8 (C-6); 65.6, 65.7 (C-1′); 34.9 (C-4′); 34.1 (C-5); 27.6−27.0 (C-2′, C-3′); 21.9 (2C$\underline{H}_3$−5); MS (EI): *m*/*z* 478 (M^+, 8%); 345 (M^+−$C_{10}H_{13}$, 15%); 330 (M^+−$C_{10}H_{13}$−15, 35%); 199 (M^+−2$C_{10}H_{13}$ − 15, 100%). Anal. Calcd. for $C_{29}H_{34}O_6$: C, 72.80; H, 7.11; found: C, 73.01; H, 6.97.

6.1.6.4. 5,5-Dimethyl-2-oxo-5,6-dihydro-2H-pyran-3,4-dicarboxylic acid 4-(4′-methoxycarbonylbutyl) ester ***22***. Oil. TLC: ethyl acetate/petrol ether (6:4, v/v), Rf = 0.68. Yield: 183 mg (46%). FT-IR (film): 1729, 1633, 1010 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.25 (s; 6H, 2C$\underline{H}_3$−5); 1.68 (m; 8H, C$\underline{H}_2$−2′, 3′); 2.27 (m; 4H, C$\underline{H}_2$−4′); 3.65 (s; 6H, C$\underline{H}_3$OCO); 4.10 (s; 2H, C$\underline{H}_2$−6); 4.25 (m; 4H, C$\underline{H}_2$−1′); ^{13}C NMR ($CDCl_3$): δ 172.7, 172.5 (C$\underline{O}$OCH$_3$); 163.5 (C-2); 162.4, 159.5 (C$\underline{O}$O); 155.0 (C-4); 124.1 (C-3); 75.5 (C-6); 64.9 (C-1′); 50.8, 50.9 (C$\underline{H}_3$OCO); 34.2 (C-5); 33.7, 32.7 (C-4′); 27.3−27.0 (C-2′, C-3′); 21.5; 20.5, 20.7 (2C$\underline{H}_3$−5); MS (EI): *m*/*z* 442 (M^+, 8%); 212 (M^+−2$C_6H_{11}O_2$, 18%); 124 (M^+−2$C_7H_{11}O_4$, 15%). Anal. Calcd. for $C_{21}H_{30}O_{10}$: C, 57.01; H, 6.79; found: C, 57.34; H, 6.91.

6.1.6.5. 5,5-Dimethyl-2-oxo-5,6-dihydro-2H-pyran-3,4-dicarboxylic acid bis-allylamide ***23***. Oil. TLC: methylene chloride/methanol (5:2, v/v), Rf = 0.58. Yield: 223 mg (85%). FT-IR (film): 3200, 1680, 1633, 1600 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.25 (s; 6H, 2C$\underline{H}_3$−5); 3.65 (m; 4H, C$\underline{H}_2$−1′); 4.10 (s; 2H, C$\underline{H}_2$−6); 5.13−5.20 (m; 4H, C$\underline{H}_2$−3′); 5.82 (m; 2H, C$\underline{H}$−2′); 8.2 (d; 2H, N$\underline{H}$); ^{13}C NMR ($CDCl_3$): δ 167.1, 166.5 (C$\underline{O}$NH); 163.7 (C−2); 155.6 (C−4); 134.5, 134.4 (C−2′); 124.6 (C−3); 115.1, 115.3 (C−3′); 75.6 (C−6); 46.9, 46.1 (C−1′); 34.0 (C−5); 21.8 (2C$\underline{H}_3$−5); MS (EI): *m*/*z* 292 (M^+, 12%); 237 (M^+−C_3H_6N, 25%); 182 (M^+−2C_3H_6N, 30%), 167 (M^+−2C_3H_6N−15, 100%). Anal. Calcd. for $C_{15}H_{20}O_4N_2$: C, 61.64; H, 6.85, N, 9.59; found: C, 61.43; H, 6.68, N, 9.35.

6.1.7. General procedure for the preparation of the compounds ***25*** *and* ***26***

To a stirred suspension of 30 mmol of Wittig salts **24** in 10.5 ml of dry THF, under nitrogen, at −10 °C, were added 32 mmol of a solution 1.6 M BuLi. After stirring for 30 min, 30 mmol of aldehyde **15b** [6] in 5 ml of dry THF, were added drop-wise. The resulting mixture was stirred for 3 h at −10 °C, quenched with water and extracted with ether (20 ml × 3). The extracts were combined, dried over Na_2SO_4, filtered and evaporated to give the compounds **25** and **26** mainly as E stereoisomers which were purified by 'flash chromatography' on silica gel (eluent, *n*-hexane/diethyl ether = 7:3 for **25** and **26**).

6.1.7.1. 3,5,5-Trimethyl-4-oct-1-enyl-5, 6-dihydro-2H-pyran-2-one ***25***. Ratio of stereoisomers E/Z = 90: 10, oil. TLC: *n*-hexane/diethyl ether (7:3, v/v), Rf = 075. Yield: 330 mg (60%). FT-IR (film): 1770, 1633, 1600, 1100 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.9 (s; 3H, C$\underline{H}_3$−8′); 1.05 (s; 6H, 2C$\underline{H}_3$−5); 1.22−1.40 (m; 8H, C$\underline{H}_2$−4′, 5′, 6′, 7′); 1.80 (s; 3H, C$\underline{H}_3$−3); 1.95 (m; 2H, C$\underline{H}_2$−3′); 4.02 (s; 2H, C$\underline{H}_2$−6); 5.70−5.90 (m; 2H, C$\underline{H}$−1′, 2′); ^{13}C NMR ($CDCl_3$): δ 165.9 (C-2); 158.9 (C-4); 136.2 (C-1′); 128.8 (C-2′); 120.6 (C-3); 75.6 (C-6); 35.2 (C-5); 31.9, 30.4, 29.8 (C-4′, C-5′, C-6′); 29.2 (C−3′); 23.1 (C-7′); 22.5, 22.7 (2C$\underline{H}_3$−5); 14.2 (C-8′); 11.2 (C$\underline{H}_3$=); MS (EI): *m*/*z* 250 (M^+, 15%); 152 (M^+−C_7H_{14}, 100%); 137 (M^+−C_7H_{14}−CH_3, 20%). Anal. Calcd. for $C_{16}H_{26}O_2$: C, 76.8; H, 10.4; found: C, 76.97; H, 10.51.

6.1.7.2. 3,5,5-Trimethyl-4-non-1-enyl-5,6-dihydro-2H-pyran-2-one ***26***. Ratio of stereoisomers E/Z = 90: 10, oil. TLC: *n*-hexane/diethyl ether (7:3, v/v), Rf = 081. Yield: 232 mg (40%). FT-IR (film): 1770, 1633, 1600, 1100 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.9 (s; 3H, C$\underline{H}_3$−9′); 1.05 (s; 6H, 2C$\underline{H}_3$−5); 1.22−1.40 (m; C$\underline{H}_2$−4′, 5′, 6′, 7′, 8′); 1.80 (s; 3H, C$\underline{H}_3$−3); 1.90 (m; 2H, C$\underline{H}_2$−3′); 4.02 (s; 2H, C$\underline{H}_2$−6); 5.70−5.90 (m; 2H, C$\underline{H}$−1′, 2′); ^{13}C NMR ($CDCl_3$): δ 165.4 (C−2); 155.7 (C-4); 135.7 (C-1′); 127.4 (C-2′); 120.3 (C-3); 75.5 (C-6); 34.6 (C-5); 33.1−22.9 (C-4′, 5′, 6′, 7′, 8′); 28.3 (C-3′); 22.1, 22.2 (2C$\underline{H}_3$−5); 14.1 (C-9′); 12.2 (C$\underline{H}_3$=); MS (EI): *m*/*z* 264 (M^+, 15%); 251 (M^+−15 + 2H, 75%); 166 (M^+−30−C_5H_{11} + 2H, 100%). Anal. Calcd. for $C_{17}H_{28}O_2$: C, 77.27; H, 10.60; found: C, 77.41; H, 10.83.

6.2. Cells and cytotoxic assays

6.2.1. Cell cultures

KB, human oral epidermoid carcinoma cell line (ECACC no. 86103004), and IMR-32, human adenocarcinoma cell line

(ECACC no. 86041809), were cultured according to standard procedure [10,13]. Vials of the original line were maintained in liquid N_2; cells were obtained, routinely subcultured once a week, and used for the experiment reported in the present work. The cell lines were maintained in Eagle's minimum essential medium (MEM) [14] supplemented with 10% newborn calf serum (Hyclone) for KB and fetal calf serum (Euroclone) for IMR-32, with 10 ml l^{-1} penicillin and streptomycin solution (Sigma Chemical Co., St. Louis, MO) (100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin) and buffered with 3 mM tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid, 3 nM *N*, *N*-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid, 3 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid and 3 mM Tricine (Sigma Chemical Co.). The cell population doubling time was ca. 24 and 20 h for KB and IMR-32, respectively. Cells from confluent monolayers were removed with 2−3 ml of 0.05% (KB cells) or 0.25% (IMR-32 cells) trypsin solution (Sigma Chemical Co.).

6.2.2. Growth inhibition of KB and IMR−32 cells derivatives

For the valuation of cytostatic activity, KB and IMR-32 cells were sown at a density of 2.5×10^4 cells per ml, in 0.2 ml per well in a 96-well plate (Corning Costar, Milano, Italy). After 24 h, derivatives were dissolved in sterile DMSO and solutions diluted in culture *medium* up to obtained opportune concentration (1.25, 2.50, 5.00 and 10.00 μg ml^{-1}); nutritive *medium* of every well was substituted with 0.2 ml of solution. After 72 h incubation at 37 °C, cellular vitality was evaluated with a colorimetric assay based on the quantification with sulforhodamine B (SRB − Sigma Chemical Co.) of cellular protein component [11]. Briefly, adherent cell cultures were fixed in situ by addition of 50 μl of cold 50% (v/v) trichloroacetic acid (TCA) and were kept for 60 min at 4 °C. The supernatant was then discarded and the plates were washed two times with bi-distilled water and air-dried. SRB solution (0.4% w/v in 1% acetic acid) was added and the cells were allowed to stain for 30 min at room temperature. Unbound SRB was removed by washing three times with 1% acetic acid. Then the plates were air-dried. Bound stain was dissolved with unbuffered 10 mM Tris base (tris-hydroxymethyl-aminomethane) (Sigma Chemical Co.) and the optical density was read at 570 nm with an automated microplate reader EL311s spectrophotometer (BIO-TEK Instruments, INC. Winooski, Vermount, USA). Each experiment was performed in quintuplicate and repeated twice. Cytostatic activity was evaluated as percentage of cellular growth inhibition in culture treated with compounds to respect to the growth observed in control culture.

IC_{50} and parallelism test were performed with the aim of PCS program [15].

6.2.3. MTT assay

Concentration-dependent cellular MTT reduction activity was measured after an incubation for 4 and 6 h with compounds **20** and **23** of KB and IMR-32 cells as described earlier [12,16]. MTT stock solution was added to each plate well so that the final concentration of tetrazolium salt in *medium* was 0.25 mg ml^{-1}. Because of two cell lines different metabolic mitochondrial activity, contact times with MTT solution were 150 min for KB cell line and 210 min for IMR-32 cell lines.

After that MTT formazan crystals formed were solubilized with DMSO. The absorbance was read at 570 nm using an automated microplate reader EL311s spectrophotometer (BIO −TEK Instruments, Inc., Winooski, Vermount, USA).

6.2.4. Statistical analysis

Data were analyzed using Student's *t*-test. Significance was accepted with $P < 0.05$. Values of IC_{50} were obtained with PCS program [15].

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