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Design, synthesis and in vitro antitumour activity of new goniofufurone and 7-*epi*-goniofufurone mimics with halogen or azido groups at the C-7 position

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Abstract

A series of new antitumour lactones containing the [3.3.0] bicyclic furano-lactone core and the halogen or azido group at the C-7 position have been designed, synthesized, and evaluated for their in vitro antitumour activity against a panel of human tumour cell lines. Some of the analogues displayed powerful antiproliferative effects to certain human tumour cells, but all of them were devoid of any cytotoxicity towards the normal foetal lung fibroblasts (MRC-5). A SAR study reveals the structural features of these lactones that may affect their antiproliferative activity. These are: the nature of substituent present at the C-7 position, stereochemistry at the C-7 position, the absence of phenyl group at the C-7 position. Flow cytometry data indicate that the cytotoxic effects of the synthesized analogues in a culture of K562 cells are mediated by apoptosis, additionally revealing that these molecules induced changes in cell cycle distribution of these cells. Results of Western blot analysis suggested that the most of synthesized compounds induce apoptosis in K562 cells in caspase-dependent way.

1. Introduction

Nature is an inexhaustible source of biologically active compounds. Particularly interesting are styryl lactones, which have been isolated from the *Goniothalamus* plant genus [1]. Special interest of medicinal chemists is directed to the styryl lactones with furano-furone scaffold, such as (+)-goniofufurone (**1**) and 7-*epi*-(+)-goniofufurone (**2**), which have been isolated from the stem bark of *Goniothalamus giganteus* [2,3]. As both natural products (**1** and **2**) and a number of their derivatives and analogues exhibited promising antitumour properties [1,4,5], they have been the target of many synthetic efforts [6–8]. We have also been involved in the synthesis of natural styryl lactones and their analogs [9–11]. Thus, we have recently reported a preliminary account [10] on a new and facile route to (+)-goniofufurone and 7-*epi*-(+)-goniofufurone mimics bearing chloro (analogues **3** and **4**, Scheme 1), or bromo functions (analogues **5** and **6**) at the C-7 position. The results of preliminary in vitro antitumour assay [12] confirmed that the replacement of hydroxyl group at the C-7 position with a halogen may increase the antitumour potency originally displayed by leads **1** and **2** against several human tumour cell lines [10]. On the other hand, a study carried out previously in our laboratory showed that 7-dephenyl analogue of **1** (compound **13**), and in particular the corresponding (3*S*,4*R*)-stereoisomer (not shown in the scheme) strongly inhibited the growth of certain human neoplastic cell lines [13]. Based upon these observations, we designed a series of

bicyclic lactones, **3**–**12**, as direct isosteres of **1** and **2**. Finally, several new (+)-goniofufurone and 7-*epi*-(+)-goniofufurone bioisosteres of type **14**–**18** have been designed by combining both isosterism and ring removal methods. Detailed design strategy is outlined in Scheme 1.

<Insert Scheme 1>

2. Results and discussion

2.1. Chemistry

As both titanium(IV) chloride and bromide efficiently catalyzed rapid nucleophilic replacement of benzoyloxy groups at C-7 with chloro or bromo nucleophiles to afford the corresponding isosteres **3**–**6** [10], we wanted to use titanium(IV) iodide and the similar methodology to prepare 7-iodo analogues of (+)-goniofufurone and 7-*epi*-(+)-goniofufurone (compounds **7** and **8**). However, treatment of **19** with titanium(IV) iodide in dry dichloromethane (Scheme 2) did not afford the expected 7-iodo derivatives, but resulted in 5-*O*-debenzylation with the formation of alcohol **20** (Scheme 2). Hence we were forced to look for an alternative procedure based on the Garegg-Samuelsson reaction [14].

<Insert Scheme 2>

The synthesis started from the known benzylic alcohols **21** and **22** (Scheme 2) that were previously prepared in our laboratory starting from D-xylose [15]. Treatment of lactone **21** with iodine, imidazole and triphenyl phosphine in dry toluene afforded 7-iodo derivate **23** (41%) as the main reaction product, accompanied with a minor amounts of C-7 epimer **24** (11%) and olefin **25** (10%). Under the similar reaction conditions, compound **22** gave the expected 7-iodo derivate **24** in a 44% yield, along with traces of **23** (2%), and a minor amount of olefin **25** (11%). It should be underlined that the major reaction products in both reactions have inverted configuration at the C-7 position. Further treatment of compounds **23** or **24** with titanium(IV) iodide in dry dichloromethane gave a mixture of **7** (17% from **23**, 31% from **24**,) and **8** (17% from **23**, 11% from **24**). Obviously, titanium(IV) iodide apart from debenzylation partly causes the epimerization at the C-7 position. Stereochemistry of iodo-lactones **7** and **24** were confirmed by single crystal X-ray diffraction analysis (for the crystal structures of **7** and **24**, see the Supplementary data).

Since our recent study showed that treatment of **19** (Scheme 2) with titanium(IV) fluoride does not give the corresponding 7-fluoro derivatives [16], we decided to apply a different methodology for

the introduction of fluoro groups at the C-7 position. Again, the synthesis of target molecules **9** and **10** starts from known [15] styryl lactones **21** and **22** (Scheme 3).

<Insert Scheme 3>

Treatment of compound **21** with diethylaminosulfur trifluoride (DAST) in dichloromethane gave 7-deoxy-7-fluoro derivative **26** (63%), as the major product, accompanied with a minor amount of the C-7 epimer **27** (12%). Under the same reaction conditions, compound **22** gave the expected 7-deoxy-7-fluoro derivative **27** as a major product (76%), along with a minor amount of C-7 epimer **26** (24%). It appears that the replacement of hydroxyl group at C-7 in both **21** and **22** preferentially occurs with retention of configuration at the electrophilic position. 5-*O*-Benzyl protective groups were removed under the standard reaction conditions (H_2, Pd/C, cat. HCl, EtOAc) whereby the desired 7-fluoro derivatives **9** and **10** were obtained in 70 and 79% yield, respectively. Stereochemistry of fluoro-lactones **10** and **27** has been unambiguously confirmed by single crystal X-ray diffraction analysis (for the crystal structures of **10** and **27**, see the Supplementary data).

After obtaining the halogen isosteres of natural products **1** and **2**, our further activities were focused on the synthesis of the corresponding 7-azido derivatives. Apart from being an isostere, the azido group has been extensively used in joining two entirely different building blocks ("click reaction"), thus enabling an easy access to highly complex molecular architectures of interest for both medicinal chemistry and chemical biology [17,18]. In order to prepare azido isosteres **11** and **12** we have adopted a literature procedure that was previously applied for the epoxide ring opening by using trimethylsilyl azide/boron trifluoride diethyl etherate reagent system [19,20]. The synthesis of azido derivatives **11** and **12** started from the known oxetane **28** (Scheme 4) that is readily available from 7-*epi*-(+)-goniofufurone in one step [15].

<Insert Scheme 4>

Treatment of **28** with trimethylsilyl azide in dry dichloromethane, in presence of boron trifluoride diethyl etherate gave the expected analogue **12** as a major product (24%) and the corresponding C-7 epimer **11** (10%) as a minor reaction product. Stereochemistries of these 7-azido-lactones were unequivocally confirmed by single crystal X-ray diffraction analysis (for the crystal structures of **11** and **12** see the Supplementary data). An unexpected product **29** with unusual pyrano-furone skeleton was also isolated in 12% yield. ^{1}H NMR data of **29** ($J_{5,6}$ = 12.4 Hz and H-6/H-3 NOE contact) indicated that the six-member ring occupies a twist-boat conformation with all substituents being pseudo-equatorially oriented. The single crystal X-ray analysis of **29**, confirmed the twist-

boat geometry of the six-member ring (Fig. 1), as well as the *anti* (*trans*-diaxial) arrangement of H-5 and H-6 (τ = 173.1°), which is in accordance with the observed vicinal coupling of 12.4 Hz. Finally, the distance between H-3 and H-6 (2.87 Å) in the molecular structure of **29** is consistent with NOE results.

<Insert Figure 1>

A possible mechanism of this reaction (Scheme 4) involves an initial formation of the stabilized carbocation **28a**. The attack of azide nucleophile from the less hindered *re*-face gave **12** as the major product, while the minor stereoisomer **11** is formed as a result of attack to the electrophilic C-7 position from the more hindered *si*-face. The formation of pyrano-furone **29** started with the rearrangement of benzylic carbocation **28a** to the intermediate **28b**, followed by the carboxonium ion capture preferentially from the less hindered β-side.

The synthesis of 7-dephenylated goniofufurone analogues bearing a halogen or azido group at the C-7 position was carried out to examine the role of the phenyl group in the biological activities of lactones of type **1**. Preparation of these analogues (compounds **14**–**18**) is presented in Scheme 5.

<Insert Scheme 5>

The known furano-lactone **13** [13,21–23], which might be considered as a dephenylated analogue of goniofufurone (**1**), was used as a convenient starting material for this part of the work. Apart from being a starting material in the synthesis, compound **13** will be also used as a positive control in biological testing of analogues **14**–**18**. Lactone **13** readily reacted with carbon tetrachloride and triphenyl phosphine in dry dichloromethane, in the presence of pyridine to afford 7-chloro derivative **14** in 38% yield. The reaction of **13** with carbon tetrabromide and triphenyl phosphine gave 7-bromo derivative **15** in 28% yield. Absolute value of optical rotation of our sample **15** {$[\alpha]^{20}_{D}$ +10.9 (*c* 1.8, $CHCl_3$)} is somewhat smaller, but of an opposite sign, than the reported value for the enantiomer *ent*-**15** {$[\alpha]^{20}_{D}$ −22.0 (*c* 1.8, $CHCl_3$)} [24]. However, the melting point {lit. [24] mp = 88–90 °C, found 87–89 °C} and NMR data of synthesized sample **15** were in full agreement with those reported for *ent*-**15** [24]. Moreover, lactone **13** readily reacted with iodine, imidazole and triphenyl phosphine, under the standard conditions of the Garegg-Samuelsson reaction [14], to afford the known [25] 7-iodo derivative **16** in 63% yield. Physical constants (mp and optical rotation) and NMR spectral data (^{1}H and ^{13}C) of thus obtained sample **16** are in reasonable agreement with previously reported data [25]. Displacement of 7-iodo function in **16** with azido group was achieved by treatment of compound **16** with sodium azide in DMF, in the presence of

ammonium chloride, to give the desired compound **18** in 66% yield. The molecular structure of 7-azido-lactone **18** was confirmed by single crystal X-ray diffraction analysis (for the crystal structure of **18**, see the Supplementary data). The synthesis of dephenylated 7-fluoro derivative **17** was completed by using the methodology similar to that already used for the preparation of 7-fluoro derivatives **9** and **10** (Scheme 3). Thus, treatment of known lactone **30** [13] with DAST in dichloromethane gave a moderate yield of the corresponding 7-fluoro derivative **31** (35%). Catalytic hydrogenation of **31** over 10% Pd/C, in the presence of traces of concentrated hydrochloric acid, gave the desired debenzylated derivative **17** in 57% yield.

2.2. In vitro antitumour activities and SAR

After completion of the synthesis, all synthesized compounds were evaluated for their in vitro cytotoxic activity against a panel of seven human malignant cell lines, including human myelogenous leukaemia (K562), human promyelocytic leukaemia (HL-60), T cell leukaemia (Jurkat), Burkitt′s lymphoma (Raji), ER^+ breast adenocarcinoma (MCF-7), cervix carcinoma (HeLa), lung adenocarcinoma epithelial cells (A549) and a single normal cell line – foetal lung fibroblasts (MRC-5). Cell growth inhibition was evaluated by using the standard MTT colorimetric assay after exposure of cells to the test compounds for 72 h. (+)-Goniofufurone (**1**), 7-*epi*-(+)-goniofufurone (**2**), dephenyl goniofufurone (**13**) and the commercial antitumour agent doxorubicin (DOX) were used as positive controls.

<Insert Table 1>

According to the resulting IC_{50} values of the cytotoxic assay (Table 1), the K562, HL-60, Jurkat, Raji, HeLa and A549 cell lines were sensitive to all of the synthesized analogues. The highest potency in the culture of K562 was recorded after treatment with 7-chloro derivative **4** (IC_{50} 0.11 μM), which demonstrated about 2-fold higher potency than the commercial antitumour agent DOX. A potent submicromolar antiproliferative activity was recorded after treatment of K562 cells with bromo derivative **6** (IC_{50} 0.98 μM). The highest potency in the HL-60 cell line was recorded after treatment with 7-bromo isostere **5** (IC_{50} 0.11 μM), which was approximately 8-fold more active than DOX. All goniofufurone mimics (**3**, **5**, **7**, **9** and **11**) demonstrated powerful to moderate antiproliferative effects toward HL-60 cells, in contrast to lead **1**, which was completely inactive against this cell line. With the exception of analogue **8**, the remaining four of 7-*epi*-(+)-goniofufurone mimics (**4**, **6**, **10** and **12**) were more potent than lead **2**. The most active compound in the culture of Jurkat cells was analogue **4** (IC_{50} 0.03 μM), which demonstrated the same potency as

the commercial antitumour drug DOX. The most active molecules against Raji cell line were the following isosteres: 7-fluoro derivative **10** (IC_{50} 0.03 μM), 7-chloro derivative **3** (IC_{50} 0.04 μM) and 7-iodo derivative **7** (IC_{50} 0.04 μM), which exhibited 75- and 99-fold higher potency than the commercial antitumour agent DOX (IC_{50} 2.98 μM), as well as 42- and 461-fold stronger activity with respect to leads **2** and **1**, respectively. Several compounds exhibited submicromolar activities toward the HeLa cells. These are: 7-azido derivative **12** (IC_{50} 0.12 μM), 7-fluoro derivative **17** (IC_{50} 0.52 μM), 7-bromo derivative **5** (IC_{50} 0.68 μM) and 7-chloro derivative **3** (IC_{50} 0.81 μM). However, the potencies of these analogues were significantly lower than that recorded for DOX in the same cell line (IC_{50} 0.07 μM). The highest potency in the culture of A549 was recorded after treatment with dephenyl 7-chloro derivative **14** (IC_{50} 4.02 μM), which demonstrated the similar potency as DOX in the same cell line. The most potent compound against MCF-7 cells (IC_{50} 0.85 μM) is dephenyl azido derivative (molecule **18**), but the potency of this compound was 4-fold lower than that recorded for commercial drug doxorubicin. Remarkably, all of the synthesized styryl lactones including natural products **1** and **2** were completely inactive toward normal MRC-5 cells, in contrast to the commercial antitumour drug DOX, which exhibited a potent cytotoxic activity (IC_{50} 0.10 μM) against this cell line. These results do suggest that the synthesized styryl lactones represent selective antitumour agents, but this assumption should be verified by additional in vitro experiments with additional normal cell lines.

In order to correlate the structure of synthesized goniofufurone mimics with their cytotoxic activities, we first considered the effect of halogen and azido groups at C-7 position. The natural products **1**, **2** and synthetic dephenyl goniofufurone **13** have been used as controls in this SAR analysis. As the data in Table 1 reveal the introduction of halogen or azido group caused the increased antitumour potency originally displayed by lead **1**, but decreased the cytotoxicity originally demonstrated by lead **2**. However, almost each of dephenyl analogues (**14**–**18**) displayed the increased antitumour potency with respect to that originally displayed by the control molecule **13**, against the majority of tumour cell lines. However, several interesting exceptions deserve to be commented: replacement of C-7 hydroxyl group by a bromine in the lead **1** (compound **5**) has the smallest impact on antitumour potency, but produces the most active analogue which exhibited antitumour activity in a submicromolar range against HL-60 cell line (IC_{50} 0.11 μM). The same structural modification of lead **2** increased potency of resulting analogue **6** toward HL-60 and Jurkat cells. Replacement of the hydroxyl group by a fluorine atom in the structure of lead **2** has a small impact on antitumour potency of analogues against the majority of tumour cells. However it produces the most active analogue (**10**) in this study which exhibited antitumour activity in a low

submicromolar range (IC_{50} 0.03 μM) against Raji cell line (for more details see the Supplementary data, Table S6 and Fig. S8 a–c).

The next round of modifications undertaken to improve the antiproliferative activities have been performed by a formalremoval of phenyl group from the C-7 position. The relationships between these structural changes and antiproliferative potencies were established by comparing the IC_{50} values of dephenyl derivatives (analogues **14**–**18**) with those recorded for the corresponding halogen and azido derivatives with phenyl group that are arbitrarily used as control molecules (analogues **3**–**12**). As the data in Table 1 indicate the removal of phenyl group decreased antiproliferative activity of the chloro, bromo and iodo isosteres, against majority of tumour cell lines under evaluation. Remarkably, the same structural modification of mimics bearing fluoro and azido groups increased antitumour potency of resulting analogues (**17** and **18**), against almost the all malignant cell lines under evaluation. We also compared the activity of dephenyl derivative **13** with that of natural products **1** and **2**, and found that compound **13** was more active against four cell lines when compared to goniofufurone (**1**), but against three cell lines when compared to 7-*epi*-goniofufurone (**2**). (For more details see the Supplementary data, Table S6 and Fig. S9).

Finally, we wanted to explore the effects of absolute stereochemistry at C-7 to antiproliferative activity of analogues. Our previous studies [10,26] indicated that the styryl lactones having the (7*S*)-stereochemistry represent more potent cytotoxic agents with respect to the corresponding (7*R*)-epimers. To further verify this assumption, we compared the IC_{50} values of six pairs of C-7 epimers (**1** and **2**, **3** and **4**, **5** and **6**, **7** and **8**, **9** and **10**, **11** and **12**), each of which contains exactly the same substituents and differs only in their absolute stereochemistry at C-7. Epimers with (7*R*)-absolute configuration (**1**, **3**, **5**, **7**, **9** and **11**) are considered as controls in this part of SAR analysis. Similarly to natural products **1** and **2**, the (7*S*)-stereoisomers bearing an azido group (analogue **12**) showed a more potent cytotoxicity than the corresponding (7*R*)-epimer **11**. These results are consistent with our previous findings [10,26]. In contrast to that, all stereoisomers of (7*R*)-series with chloro, bromo, iodo and fluoro groups at the C-7 position (**3**, **5**, 7 and **9**) showed increased antitumour potency then (7*S*)-epimers (**4**, **6**, **8** and **10**) against a majority of malignant cell lines under evaluation (for details see the Supplementary data, Table S6 and Fig. S8 d). Unfortunately, these findings disagree with the results of our previous studies [10,26].

2.3. Cell cycle analysis

The cell passes through a series of events (cell cycle) leading to cell division and duplication. Cells that actively pass through the cell cycle represent the targets in cancer chemotherapy. We studied effects of (+)-goniofufurone (**1**) and analogues (**3**, **5**, **7**, **9** and **11**), as well as 7-*epi*-(+)-goniofufurone (**2**) and its analogues (**4**, **6**, **8**, **10** and **12**), and finally dephenyl goniofufurone (**13**) and analogues (**14**, **15**, **16**, **17** and **18**) to the cell cycle perturbations. The cell cycle profile of exponentially growing K562 cells treated with synthesized compounds for 72 h was analyzed by flow cytometry in cells stained with propidium iodide. Untreated cells served as a control. The results are presented in Table 2A (for graphic presentation see Figs. S13, S14 and S15 in the Supplementary data). As the data in Table 2A indicate a 72-h treatment of K562 cells with goniofufurone (**1**) and the corresponding analogues (**3**, **5**, **7**, **9** and **11**) slightly change the percentage of cells in G0/G1, S and G2/M phase of cell cycle compared to control. But all of these compounds increased the percentage of cells in the sub-G1 phase (which is suggestive of apoptosis) from cca. 2- to 10-fold compared to the control. 7-Azido mimetic of goniofufurone (compound **11**) induced the most prominent sub-G1 peak in K562 cells after 72-h treatment.

<Insert Table 2>

Table 2A further reveals that 7-*epi*-(+)-goniofufurone (**2**) and the corresponding analogues (**4**, **6**, **8**, **10** and **12**), slightly change the percentage of cells in G0/G1, S and G2/M phase of cell cycle compared to control. However, all analogues increased 2- to 10-fold the percentage of cells in the sub-G1 phase when compared to untreated control. The exception is 7-*epi*-(+)-goniofufurone which decreased the percentage of cells in the sub-G1 phase with respect to control. 7-Chloro analogue of 7-*epi*-(+)-goniofufurone (compound **4**) induced the most prominent sub-G1 peak in K562 cells after 72-h treatment.

The data in Table 2A also indicate that 72-h treatment of K562 cells with dephenyl goniofufurone (**13**) and the corresponding analogues (**14**–**18**) slightly change the percentage of cells in G0/G1, S and G2/M phase of cell cycle. But all of these compounds significantly increased the percentage of cells in the sub-G1 phase (from 1.5- to 30-fold compared to the control). Bromo, fluoro and azido derivatives of dephenyl goniofufurone (compound **15**, **17** and **18**) induced the most prominent sub-G1 peaks in K562 cells after 72-h treatment.

The obtained results showed that synthesized halogen isosteres of **1** (compounds **3**, **5**, **7**, **9**, **11**), **2** (compounds **4**, **6**, **8**, **10**, **12**) and of **13** (compounds **14**–**18**) induced changes in cell cycle distribution of K562 cells.

2.4. Detection of apoptosis

Cell cycle analysis indicated the pro-apoptotic effect of synthesized analogues through the formation of sub-G1 peak. Therefore, we further analyzed apoptotic cell death using double staining with Annexin V-FITC and propidium iodide. Double staining enables detection of cells in the early phase of apoptosis and clearly discriminates truly necrotic cells from the Annexin V positive cells. The type of cell death induced by goniofufurone (**1**), 7-*epi*-goniofufurone (**2**), dephenyl goniofufurone (**13**) and analogues (**3**–**12**, **14**–**18**) in K562 human leukaemia cells, was determined by flow cytometry after staining, and the results are shown in Table 2B (for a graphic presentation see Figs. S10, S11 and S12 in the Supplementary data).

Apoptotic response, which was presented as a percentage of specific apoptosis, shows that all analogues increased the percentage of Annexin V-positive cells compared to parent compound **1**. Compound **3** causes the greatest specific apoptosis (58.36%). As it can be seen, a few analogues induced several-fold more Annexin V positive cells compared to parent compound **2**. Exceptions are compounds **6** and **12**. However, the 7-chloro mimic of 7-*epi*-goniofufurone (compound **4**) causes the greatest specific apoptosis (38.77%). As it can be further seen, all 7-dephenyl analogues induced several-fold more Annexin V positive cells when compared to the parent compound **13**. Compounds **17**, **18** and **15** cause the greatest specific apoptosis (11.33, 18.10 and 19.59%, respectively). These results confirmed that 7-chloro analogues **3** and **4**, and dephenyl 7-fluoro, 7-azido and 7-bromo isosteres **17**, **18** and **15** increased the percentage of Annexin V positive cells 47-, 8-, 226-, 362- and 391-fold respectively, compared to parent compounds **1**, **2** and **13**. These findings agree well with the results of cell cycle analysis (sub-G1 peak). The highest percentage of specific necrosis caused analogues bearing 7-bromo, 7-iodo and 7-azido groups, while the analogues with 7-chloro and 7-fluoro groups showed slightly lower values. But all 7-dephenyl analogues increase the percentage of specific necrosis to 13.4-fold.

To evaluate the mechanisms underlying the apoptosis induced with synthesized analogues (**3**–**18**) we have studied the ability of these compounds to modulate expression of selected apoptosis markers, such as Bcl-2, Bax, caspase-3 and Poly (ADP-ribose) polymerase (PARP). The results are presented in Table 3 (for more details see the Supplementary data, Figs. S16, S17 and S18).

<Insert Table 3>

Semi-quantitative Western blot analysis revealed that the majority of examined compounds (**1**–**5**, **7**–**12** with the exception of **6**, **13**–**18**) increased expression of anti-apoptotic Bcl-2 protein in K562

cells when compared to control. The majority of tested compounds (with the exception of **1**, **15**, **16** and **17**) reduced expression of pro-apoptotic protein Bax (Bcl-2 associated protein X) which, when activated, forms the oligomers that disrupt outer mitochondrial membrane. Cytochrome c released from damaged mitochondria promotes caspase-9 activation on the scaffold protein APAF1 [27,28]. Caspases, a family of aspartate-specific cysteine proteases, play central role in the mechanism of apoptosis as they both initiate and execute the apoptotic process. The expression level of the caspase-3 precursor and active subunit of caspase-3 effector, as well as catalytic fragment of PARP in the cells exposed to synthesized compounds were measured in order to determine whether apoptosis was associated with the activation of caspases. Caspase-3 activation is followed by cleavage of different downstream targets including poly ADP-ribose polymerase (PARP), which is specifically cleaved on a DNA-binding domain and a catalytic fragment [29]. Western blot analysis showed over-expression of both caspase-3 active subunit and cleaved catalytic PARP fragment (85 kD) in K562 cells after treatment with lactones **9**, **11**, **12** and **18** while all other synthesized compounds (with exception of bromo derivative **6**) influenced only PARP cleavage. During the execution phase of apoptosis, PARP-1 is specifically proteolyzed by caspases and *C*-terminal apoptotic fragment of PARP-1 loses its DNA-dependent catalytic activity upon cleavage with caspase-3 while *N*-terminal apoptotic fragment, retains DNA-binding activity and inhibits the catalytic activity of uncleaved PARP-1 [29]. These results suggest that all the investigated compounds (with exception of **6**) induce apoptosis of K562 cells in caspase-dependent way.

Biochemical changes in apoptosis, DNA fragmentation and caspase activation, may explain in part some of the morphological changes in apoptosis. Therefore, it is important to note that apoptosis may occur without oligonucleosomal DNA fragmentation and can be caspase-independent [30].

3. Conclusions

In conclusion, fifteen new analogues of goniofufurone (**1**) and 7-*epi*-goniofufurone (**2**), bearing a halogen or azido functionality at the C-7 position, were designed and synthesized in this work. All the synthesized compounds were tested in vitro toward a panel of human tumour cell lines, and the structure–activity relationships are briefly discussed. The ability of synthesized compound to induce apoptosis and to change the distribution of K562 cell cycle was also studied. Some of the synthesized compounds showed potent antitumour activity, especially analogues **3**, **7**, **10** (0.03–0.04 μM against Raji), and **4** (0.03 μM against Jurkat), which displayed the highest activity of all compounds under evaluation.

The preliminary SAR analysis suggested the following structural requirements for the antiproliferative action of synthesized analogues: (A) the presence of halogen or azido group at C-7 increases the antitumour potency originally displayed by lead **1**, but decreases the cytotoxicity originally demonstrated by lead **2**; (B) removal of the phenyl group may increase or decrease antiproliferative activity depending on the nature of substituent at the C-7 position; (C) generally, styryl lactones having the (7*S*)-stereochemistry represent more potent cytotoxic agents than the corresponding (7*R*)-isomers, but the activity also depends on the nature of substituent present at the C-7 position.

The cell cycle analysis reveals that treatment of cells with synthesized compounds increases percentage of K562 cell in sub G1 phase indicating that the mechanism of action of these compounds probably involves apoptosis. The flow-cytometry further confirmed a significant percentage of specific apoptotic cells that was detected after treatment with majority of analogues. Compound **3** causes the greatest specific apoptosis (58.36%). Western blot analysis of apoptosis markers (Bcl-2, Bax, caspase-3, PARP) suggested that all of the synthesized compounds (with exception of **6**) induced apoptosis in K562 cells in caspase-dependent way.

4. Experimental section

4.1. Chemistry

4.1.1. General experimental procedures

Melting points were determined on a Hot Stage Microscope Nagema PHMK 05 and were not corrected. Optical rotations were measured on an Autopol IV (Rudolph Research) polarimeter at room temperature. NMR spectra were recorded on a Bruker AC 250 E or a Bruker Avance III 400 MHz instrument and chemical shifts are expressed in ppm downfield from TMS. IR spectra were recorded with an FTIR Nexus 670 spectrophotometer (Thermo-Nicolet). High resolution mass spectra (ESI) of synthesized compounds were acquired on a Agilent Technologies 1200 series instrument equipped with Zorbax Eclipse Plus C18 (100 mm × 2.1 mm i.d. 1.8 μm) column and DAD detector (190–450 nm) in combination with a 6210 time-of-flight LC/MS instrument (ESI) in the positive ion mode. Flash column chromatography was performed using Kieselgel 60 (0.040–0.063, E. Merck). Preparative TLC was performed on hand-made plates, 20 × 20 cm size with ~1 mm layer thickness. Kieselgel 60 G (E. Merck) with fluorescent indicator F_{254} as additive was used as stationary phase. The corresponding bands were scraped and eluted with EtOAc. All organic extracts were dried with anhydrous Na_2SO_4. Organic solutions were concentrated in a rotary

evaporator under diminished pressure at a bath temperature below 35 °C. The purities of the tested compounds were established by HPLC (compounds **3**–**13** and **15**–**18**) or by combustion analysis (compound **14**) and were found to be >95% pure. HPLC analysis was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with DAD detector and Zorbax SB-C 18 column (4.6 × 150 mm, 5 μm). Eluent: acetonitrile-water (HPLC grade); gradient elution: 60–100% acetonitrile; flow rate: 0.5 mL/min. Temperature: 25 °C. Detection: 254 nm (compounds **3**–**10**, **12**, **13** and **16**) and 210 nm (compounds **11**, **15**, **17** and **18**). For details see the Supplementary data.

The procedures for preparation of 7-chloro (**3** and **4**) and 7-bromo derivatives (**5** and **6**) are described in reference 10.

*4.1.2. 3,6-Anhydro-7-O-benzoyl-2-deoxy-7-C-phenyl-D-glycero-D-ido-heptono-1,4-lactone (**20**)*

To a cooled (0 °C) and stirred solution of **19** (0.103 g, 0.23 mmol) in anhydrous CH_2Cl_2 (6 mL) was added TiI_4 (0.419 g, 0.75 mmol). The mixture was stirred at 0 °C for 0.5 h and then at room temperature for 3.5 h. The mixture was poured onto ice and water, and extracted with CH_2Cl_2. The combined extracts were washed with 10% aq $NaHCO_3$, dried and evaporated. The residue was first purified by flash column chromatography (19:1 CH_2Cl_2/EtOAc) and then by preparative TLC (1:1 toluene/EtOAc) to give pure **20** (0.042 g, 55%) as a colourless syrup, $[\alpha]^{20}_D = +50.0$ (*c* 1.0, $CHCl_3$), $R_f = 0.25$ (19:1 CH_2Cl_2/EtOAc). IR (film): ν_{max} 3454 (OH), 1787 (C=O, lactone), 1722 (PhC=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.30 (br s, 1H, OH), 2.55 (d, 1H, $J_{2a,2b}$ = 18.8 Hz, H-2a), 2.67 (dd, 1H, $J_{2a,2b}$ = 18.8, $J_{2b,3}$ = 5.1 Hz, H-2b), 4.32 (dd, 1H, $J_{5,6}$ = 2.1, $J_{6,7}$ = 9.2 Hz, H-6), 4.45 (d, 1H, $J_{5,6}$ = 2.1 Hz, H-5), 4.95–5.04 (m, 2H, H-3 and H-4), 6.12 (d, 1H, $J_{6,7}$ = 9.3 Hz, H-7), 7.35–8.14 (m, 10H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 35.8 (C-2), 73. 2 (C-5), 73.2 (C-7), 77.1 (C-3), 82.5 (C-6), 87.0 (C-4), 127.6, 128.6, 128.7, 128.9, 129.9, 133.9, 136.8 (Ph), 166.9 (Ph*C*=O), 175.40 (C-1). HRMS (ESI): *m/e* 377.0984 (M^+ + Na), calcd for $C_{20}H_{18}NaO_6$: 377.0996; *m/e* 393.0723 (M^+ + K), calcd for $C_{20}H_{18}KO_6$: 393.0735.

*4.1.3. Preparation of 7-iodo derivatives **23** and **24***

Procedure A. To a solution of **21** (0.081 g, 0.24 mmol) in dry toluene (5.5 mL) was added iodine (0.118 g, 0.47 mmol), imidazole (0.043 g, 0.64 mmol) and Ph_3P (0.162 g, 0.62 mmol). The mixture was stirred at 70 °C for 3 h, then evaporated. The mixture of **23**, **24** and **25** was first purified by flash column chromatography (3:2 light petroleum/Et_2O) and then by preparative TLC (3:2 CH_2Cl_2/light petroleum, 5 successive developments). Eluted first was the minor product **25** (0.008

g, 10%) that was isolated in the form of colourless needles, mp 165 °C, $[\alpha]^{20}_D$ = +7.5 (*c* 0.5, $CHCl_3$), R_f = 0.52 (CH_2Cl_2). Further elution gave pure compounds **23** (41%) and **24** (11%).

Procedure B. To a cooled (0 °C) and stirred solution of **22** (0.099 g, 0.29 mmol) in anhydrous CH_2Cl_2 (1.5 mL) was added 2,6-lutidine (0.11 mL, 0.94 mmol), Ph_3P (0.244 g, 0.93 mmol) and iodine (0.246 g, 0.97 mmol). The mixture was stirred at 0 °C for 0.5 h and then at room temperature for 28 h, under nitrogen atmosphere. The mixture was poured in 1 M HCl (pH 1), and extracted with CH_2Cl_2. The combined extracts were washed with 10% aq NaCl (pH 7), dried and evaporated. The mixture of **23**, **24** and **25** was first purified by flash column chromatography (3:2 light petroleum/Et_2O) and then by preparative TLC (3:2 CH_2Cl_2/light petroleum, 5 successive developments). Eluted first was the minor product **25** (0.011 g, 11%). Further elution gave pure compounds **23** (2%) and **24** (44%).

*3,6-Anhydro-5-O-benzyl-2,7-dideoxy-7-iodo-7-C-phenyl-L-glycero-D-ido-heptono-1,4-lactone (**23**)*

Colourless needles, mp 136–138 °C, $[\alpha]^{20}_D$ = +35.0 (c 0.5, $CHCl_3$), R_f = 0.30 (CH_2Cl_2). IR (film): ν_{max} 1789 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.76 (dd, 1H, $J_{2a,2b}$ = 18.9, $J_{2a,3}$ = 5.8 Hz, H-2a), 2.88 (bd, 1H, $J_{2a,2b}$ = 18.6 Hz, H-2b), 3.78 (d, 1H, $J_{5,6}$ = 3.2 Hz, H-5), 4.08 and 4.24 (2×d, 2H, J_{gem} = 11.3 Hz, C**H**$_2$Ph), 4.69 (dd, 1H, $J_{5,6}$ = 3.2, $J_{6,7}$ = 10.2 Hz, H-6), 4.92 (d, 1H, $J_{3,4}$ = 4.5 Hz, H-4), 5.07 (bt, 1H, $J_{3,4}$ = 4.6 Hz, H-3), 5.29 (d, 1H, $J_{6,7}$ = 10.2 Hz, H-7), 7.08–7.48 (m, 10H, 2×Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 27.8 (C-7), 35.9 (C-2), 72.9 (**C**H_2Ph), 77.7 (C-3), 79.3 (C-5), 85.1 (C-4), 85.5 (C-6), 127.5, 127.8, 128.2, 128.4, 128.5, 128.8, 136.4, 140.5 (2×Ph), 175.1 (C-1). HRMS (ESI): *m/e* 468.0658 (M^++NH_4), calcd for $C_{20}H_{23}INO_4$: 468.0666.

*3,6-Anhydro-5-O-benzyl-2,7-dideoxy-7-iodo-7-C-phenyl-D-glycero-D-ido-heptono-1,4-lactone (**24**)*

Colourless needles, mp 149–150 °C, $[\alpha]^{20}_D$ = −9.8 (*c* 0.5, $CHCl_3$), R_f = 0.38 (CH_2Cl_2). IR (film): ν_{max} 1789 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.54 (d, 1H, $J_{2a,2b}$ = 18.9 Hz, H-2a), 2.64 (dd, 1H, $J_{2b,3}$ = 4.8, $J_{2a,2b}$ = 18.9 Hz, H-2b), 4.63 (d, 1H, $J_{5,6}$ = 2.9 Hz, H-5), 4.75 (d, 1H, J_{gem} = 10.9 Hz, C**H**$_2$Ph), 4.81–5.03 (m, 4H, C**H**$_2$Ph, H-3, H-4 and H-6), 5.31 (d, 1H, $J_{6,7}$ = 10.7 Hz, H-7), 7.28–7.54 (m, 10H, 2×Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 26.2 (C-7), 36.4 (C-2), 74.2 (**C**H_2Ph), 78.8 (C-3), 82.7 (C-5), 84.1 and 84.2 (C-4 and C-6), 127.8, 128.3, 128.4, 128.43, 128.7, 128.8, 136.9, 140.8 (2×Ph), 175.1 (C-1). HRMS (ESI): *m/e* 468.0653 (M^++NH_4), calcd for $C_{20}H_{23}INO_4$: 468.0666.

*Olefin **25***

Colourless needles, mp 165 °C, $[\alpha]^{20}_D$ = +7.5 (*c* 0.5, $CHCl_3$), R_f = 0.52 (CH_2Cl_2). IR (film): ν_{max} 1793 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.83 (dd, 1H, $J_{2a,2b}$ = 18.6, $J_{2a,3}$ = 4.6 Hz, H-2a), 2.94 (d, 1H, $J_{2a,2b}$ = 18.6 Hz, H-2b), 4.60 (s, 1H, H-5), 4.61 and 4.79 (2×d, 2H, J_{gem} = 11.5 Hz, C**H**$_2$Ph), 4.94 (d, 1H, $J_{3,4}$ = 3.8 Hz, H-4), 5.34 (t, 1H, $J_{2a,3}$ = 4.2, $J_{3,4}$ = 4.0 Hz, H-3), 5.55 (s, 1H, H-7), 7.16–7.62 (m, 10H, 2×Ph); NOE contact: H-5 and H-7. ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 35.8 (C-2), 70.6 (**C**H_2Ph), 81.0 (C-3), 81.5 (C-5), 83.5 (C-4), 105.7 (C-7), 126.5, 127.9, 128.2, 128.3, 128.6, 128.65, 134.6, 136.8 (2×Ph), 152.0 (C-6), 173.8 (C-1). HRMS (ESI): *m/e* 323.1270 (M^++H), calcd for $C_{20}H_{19}O_4$: 323.1278; *m/e* 345.1089 (M^++Na), calcd for $C_{20}H_{18}NaO_4$: 345.1097; *m/e* 361.0838 (M^++K), calcd for $C_{20}H_{18}KO_4$: 361.0837.

*4.1.4. General procedure for the preparation of 7-iodo analogues 7 and **8***

To a cooled (0 °C) and stirred solution of **23** or **24** (1 equiv) in dry CH_2Cl_2 (0.04 M) was added TiI_4 (2.5–2.7 equiv). The mixture was first stirred at 0 °C for 0.5 h and then at room temperature until the starting materials were consumed (TLC, 3 h for **23**, 2.5 h for **24**). The mixture was poured onto ice and water and the resulting suspension was extracted with CH_2Cl_2. The combined extracts were washed with saturated aq $NaHCO_3$, dried and evaporated. The residue was first purified by flash column chromatography (19:1 CH_2Cl_2/EtOAc), and then by preparative TLC (9:1 CH_2Cl_2/EtOAc, 3 successive developments), to afford pure **7** and **8**.

3,6-Anhydro-2,7-dideoxy-7-iodo-7-C-phenyl-D-glycero-D-ido-heptono-1,4-lactone (7). Yield: 31% (from **24**); 17% (from **23**). Colourless needles, mp 123–125 °C, $[\alpha]^{20}_D$ = −71.2 (*c* 0.5, $CHCl_3$), R_f = 0.49 (4:1 CH_2Cl_2/EtOAc). IR (KBr): ν_{max} 3396 (OH), 1752 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.62 (d, 2H, $J_{2a,2b}$ = 17.4 Hz, H-2a and OH), 2.66 (dd, 1H, $J_{2b,3}$ = 5.0, $J_{2a,2b}$ = 18.8 Hz, H-2b), 4.84–4.91 (m, 2H, $J_{5,6}$ = 2.7 Hz, H-5 and H-6), 4.96 (d, 1H, $J_{3,4}$ = 4.2 Hz, H-4), 5.04 (m, 1H, H-3), 5.21 (d, 1H, $J_{6,7}$ = 10.5 Hz, H-7), 7.28–7.54 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 26.8 (C-7), 36.4 (C-2), 75.8 (C-5), 78.7 (C-3), 84.1 (C-6), 86.9 (C-4), 127.8, 128.5, 128.9, 140.5 (Ph), 175.2 (C-1). HRMS (ESI): *m/e* 378.0196 (M^++NH_4), calcd for $C_{13}H_{17}INO_4$: 378.0197.

*3,6-Anhydro-2,7-dideoxy-7-iodo-7-C-phenyl-L-glycero-D-ido-heptono-1,4-lactone (**8**).* Yield: 17% (from **23**); 11% (from **24**). Colourless needles, mp 204–206 °C, $[\alpha]_D$ = +38.6 (*c* 0.5, $CHCl_3$), R_f = 0.44 (4:1 CH_2Cl_2/EtOAc). IR ($CHCl_3$): ν_{max} 3440 (OH), 1739 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.77 (dd, 1H, $J_{2a,2b}$ = 18.9, $J_{2a,3}$ = 5.7 Hz, H-2a), 2.88 (d, 1H, $J_{2a,2b}$ = 18.8 Hz, H-2b), 4.09 (br s, 1H, H-5), 4.61 (d, 1H, $J_{5,6}$ = 2.7, $J_{6,7}$ = 10.1 Hz, H-6), 4.95 (d, 1H, $J_{3,4}$ = 4.3 Hz, H-4), 5.10 (m, 1H, H-3), 5.24 (d, 1H, $J_{6,7}$ = 10.1 Hz, H-7), 7.28–7.51 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ

27.2 (C-7), 35.9 (C-2), 72.2 (C-5), 76.4 (C-3), 85.7 (C-6), 88.0 (C-4), 127.4, 128.5, 129.1. 140.2 (Ph), 175.1 (C-1). HRMS (ESI): *m/e* 378.0194 (M^++NH_4), calcd for $C_{13}H_{17}INO_4$: 378.0197.

*4.1.5. General procedure for the preparation of 7-fluoro derivatives **26** and **27***

To a cooled (0 °C) and stirred solution of **21** or **22** (1 equiv) in dry CH_2Cl_2 (0.17 M) was added DAST (3.0–3.1 equiv). The mixture was first stirred at 0 °C for 0.5 h and then at room temperature until the starting materials were consumed (TLC, 3.5 h for **21**, 2.5 h for **22**). The mixture was suspended in cold 10% aq $NaHCO_3$, and the resulting suspension was extracted with CH_2Cl_2, dried and evaporated. The residue was then purified by flash column chromatography (4:1 CH_2Cl_2/light petroleum), to give pure products **26** and **27**.

*3,6-Anhydro-5-O-benzyl-2,7-dideoxy-7-fluoro-7-C-phenyl-D-glycero-D-ido-heptono-1,4-lactone (**26**)*. Yield: 63% (from **21**); 24% (from **22**). Colourless needles, mp 89 °C, $[\alpha]_D$ = +29.4 (*c* 0.5, $CHCl_3$), R_f = 0.30 (4:1 CH_2Cl_2/light petroleum). IR (KBr): ν_{max} 1785 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.54 (d, 1H, $J_{2a,2b}$ = 18.5 Hz, H-2a), 2.66 (dd, 1H, $J_{2a,2b}$ = 18.5 Hz, H-2b), 4.34 (m, 1H, $J_{5,6}$ = 3.1, $J_{6,7}$ = 8.8, $J_{F,6}$ = 4.6 Hz, H-6), 4.46 (d, 1H, $J_{5,6}$ = 3.1 Hz, H-5), 4.75 and 4.82 (2×d, 2H, J_{gem} = 11.6 Hz, C**H**$_2$Ph), 4.95 (m, 2H, H-3 and H-4), 5.70 (dd, 1H, $J_{6,7}$ = 8.7, $J_{F,7}$ = 44.8 Hz, H-7), 7.20–7.49 (m, 10H, 2×Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 35.8 (C-2), 72.6 (**C**H_2Ph), 77.5 (C-3), 80.8 (C-5), 81.8 (d, $J_{F,6}$=36.2 Hz, C-6), 85.1 (C-4), 89.8 (d, $J_{F,7}$=167.8 Hz, C-7), 126.7, 127.9, 128.3, 128.4, 128.6, 128.8, 137.0, 137.2 (2×Ph), 175.0 (C-1). HRMS (ESI): *m/e* 360.1608 (M^++NH_4), calcd for $C_{20}H_{23}FNO_4$: 360.1606.

*3,6-Anhydro-5-O-benzyl-2,7-dideoxy-7-fluoro-7-C-phenyl-L-glycero-D-ido-heptono-1,4-lactone (**27**)*. Yield: 12% (from **21**); 76% (from **22**). Colourless plates, mp 123–124 °C, $[\alpha]^{20}_D$ = +71.8 (*c* 0.5, $CHCl_3$), R_f = 0.19 (4:1 CH_2Cl_2/light petroleum). IR (KBr): ν_{max} 1793 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.63–2.87 (m, 2H, 2×H-2), 3.85 (d, 1H, $J_{5,6}$ = 3.9 Hz, H-5), 4.32 and 4.47 (2×d, 2H, J_{gem} = 11.5 Hz, C**H**$_2$Ph), 4.49 (ddd, 1H, $J_{5,6}$ = 4.1, $J_{6,7}$ = 7.1, $J_{F,6}$ = 12.0 Hz, H-6), 4.89 (t, 1H, $J_{3,4}$ = 3.1, $J_{3,F}$ = 3.6 Hz, H-4), 5.14 (m, 1H, H-3), 5.70 (dd, 1H, $J_{6,7}$ = 7.1, $J_{F,7}$ = 48.2 Hz, H-7), 7.23–7.46 (m, 10H, 2×Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 36.0 (C-2), 72.7 (**C**H_2Ph), 77.8 (C-3), 82.0 (d, $J_{F,5}$=6.7 Hz, C-5), 83.0 (d, $J_{F,6}$=21.9 Hz, C-6), 84.6 (C-4), 93.3 (d, $J_{F,7}$=172.6 Hz, C-7), 126.9, 127.2, 128.2, 128.55, 128.58, 128.9, 135.9, 136.6 (2×Ph), 175.0 (C-1). HRMS (ESI): *m/e* 360.1604 (M^++NH_4), calcd for $C_{20}H_{23}FNO_4$: 360.1606.

*4.1.6. General procedure for the preparation of fluoride analogues **9** and **10***

A solution of **26** or **27** (1 equiv) in EtOAc (0.06 M + 2% conc. HCl) was hydrogenated over 10% Pd/C (0.02 g; the catalyst contained 50% of water) for 30 h (for **26**) or 21 h (for **27**) at room temperature. The mixture was filtered through a Celite pad and the catalyst washed with EtOAc. The combined organic solutions were evaporated and the residues were purified by flash chromatography (7:3 toluene/EtOAc for **9**; 3:2 toluene/EtOAc for **10**), to afford pure **9** or **10**.

*3,6-Anhydro-2,7-dideoxy-7-fluoro-7-C-phenyl-D-glycero-D-ido-heptono-1,4-lactone (**9**)*. Yield: 70%. Colourless needles, mp 124–125 °C, $[\alpha]^{20}_{D}$ = +18.2 (*c* 0.5, $CHCl_3$), R_f = 0.32 (3:2 toluene/EtOAc). IR (KBr): ν_{max} 3456 (OH), 1771 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.62 (d, 1H, $J_{2a,2b}$ = 18.4 Hz, H-2a), 2.74 (dd, 1H, $J_{2a,2b}$ = 18.8, $J_{2b,3}$ = 5.4 Hz, H-2b), 4.26 (td, 1H, $J_{5,6}$ = 2.9, $J_{6,7}$ = $J_{F,6}$ = 7.5 Hz, H-6), 4.70 (br s, 1H, H-5), 4.95 (d, 1H, $J_{3,4}$ = 4.2 Hz, H-4), 5.05 (t, 1H, J = 4.5 Hz, H-3), 5.66 (dd, 1H, $J_{6,7}$ = 7.9, $J_{F,7}$ = 45.5 Hz, H-7), 7.32–7.59 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 35.9 (C-2), 74.0 (C-5), 77.5 (C-3), 82.1 (d, $J_{F,6}$ = 31.9 Hz, C-6), 87.4 (C-4), 91.0 (d, $J_{F,7}$ = 167.8 Hz, C-7), 126.5, 126.6, 129.0, 129.3, 136.3, 136.6 (Ph), 175.2 (C-1). HRMS (ESI): *m/e* 270.1138 (M^++NH_4), calcd for $C_{13}H_{17}FNO_4$: 270.1136.

*3,6-Anhydro-2,7-dideoxy-7-fluoro-7-C-phenyl-L-glycero-D-ido-heptono-1,4-lactone (**10**)*. Yield: 79%. Colourless prisms, mp 152–154 °C, $[\alpha]^{20}_{D}$ = +132.9 (*c* 0.5, $CHCl_3$), R_f = 0.24 (3:2 toluene/EtOAc). IR (KBr): ν_{max} 3497 (OH), 1763 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.49 (d, 1H, $J_{5,OH}$ = 5.7 Hz, OH), 2.77 (m, 2H, 2×H-2), 4.12 (br s, 1H, H-5), 4.37 (ddd, 1H, $J_{5,6}$ = 4.4, $J_{6,7}$ = 7.1, $J_{F,6}$ = 16.8 Hz, H-6), 4.85 (t, 1H, $J_{3,4}$ = 3.2, $J_{3,F}$ = 3.3 Hz, H-4), 5.14 (m, 1H, H-3), 5.69 (dd, 1H, $J_{6,7}$ = 7.1, $J_{F,7}$ = 48.1 Hz, H-7), 7.33–7.56 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 36.0 (C-2), 74.5 ($J_{F,5}$ = 6.7 Hz, C-5), 77.0 (C-3), 83.1 (d, $J_{F,6}$ = 21.0 Hz, C-6), 87.6 (C-4), 93.3 (d, $J_{F,7}$ = 172.6 Hz, C-7), 126.7, 126.8, 129.0, 129.5, 135.5, 135.8 (Ph), 175.3 (C-1). HRMS (ESI): *m/e* 270.1135 (M^++NH_4), calcd for $C_{13}H_{17}FNO_4$: 270.1136.

*4.1.7. Preparation of azido derivatives **11**, **12** and **29***

To a cooled (0 °C) and stirred solution of **28** (0.042 g, 0.18 mmol) in dry CH_2Cl_2 (1 mL) was added Me_3SiN_3 (0.12 mL, 0.90 mmol) and $BF_3{\cdot}Et_2O$ (0.03 mL, 0.24 mmol). The mixture was stirred at 0 °C for 4 h, then Et_3N (0.05 mL, 0.33 mmol) was added, and the mixture was stirred at 0 °C for additional 0.5 h. The reaction mixture was poured in water and extracted with CH_2Cl_2. The combined organic solutions were dried and evaporated. The remaining crude residue was first purified by flash column chromatography (19:1 CH_2Cl_2/EtOAc) and then by preparative TLC (Et_2O, 2 successive developments).

*(3aR,5S,6S,7S,7aR)-5-Azido-7-hydroxy-6-phenyl-hexahydrofuro[3,2-b]piran-2-on (**29**)*. Yield: 12%. Colourless needles, mp 185−186 °C, $[\alpha]^{20}_D$ = +225.8 (*c* 0.4, $CHCl_3$), R_f = 0.25 (9:1 CH_2Cl_2/EtOAc). IR (film): ν_{max} 3439 (OH), 2105 (N_3), 1774 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.83 (br s, 1H, OH), 2.70 (dd, 1H, $J_{5,6}$ = 12.4, $J_{6,7}$ = 8.3 Hz, H-6), 2.73 (d, 1H, $J_{2a,2b}$ = 18.7 Hz, H-2a), 2.98 (dd, 1H, $J_{2a,2b}$ = 18.7, $J_{2b,3}$ = 6.9 Hz, H-2b), 4.23 (dd, 1H, $J_{4,5}$ = 5.9, $J_{5,6}$ = 12.4 Hz, H-5), 4.58 (dd, 1H, $J_{3,4}$ = 4.9, $J_{4,5}$ = 5.7 Hz, H-4), 4.93 (dd, 1H, $J_{2b,3}$ = 6.4, $J_{3,4}$ = 5.0, H-3), 5.37 (d, 1H, $J_{6,7}$ = 8.3 Hz, H-7), 7.20–7.48 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 35.1 (C-2), 50.7 (C-6), 68.5 (C-3), 71.6 (C-5), 85.3 (C-4), 93.1 (C-7), 128.5, 128.6, 129.5, 135.7 (Ph), 174.3 (C-1). HRMS (ESI): *m/e* 293.1243 (M^++NH_4), calcd for $C_{13}H_{17}N_4O_4$: 293.1244.

*3,6-Anhydro-7-azido-2,7-dideoxy-7-C-phenyl-D-glycero-D-ido-heptono-1,4-lactone (**11**)*. Yield: 10%. Colourless needles, mp 137−138 °C, $[\alpha]^{20}_D$ = −75.2 (*c* 0.25, $CHCl_3$), R_f = 0.66 (Et_2O). IR (film): ν_{max} 3448 (OH), 2106 (N_3), 1785 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.43 (br s, 1H, OH), 2.61 (d, 1H, $J_{2a,2b}$ = 18.5 Hz, H-2a), 2.71 (dd, 1H, $J_{2a,2b}$ = 18.7, $J_{2b,3}$ = 5.4 Hz, H-2b), 4.18 (dd, 1H, $J_{5,6}$ = 2.8, $J_{6,7}$ = 8.0 Hz, H-6), 4.57 (d, 1H, $J_{5,6}$ = 2.7 Hz, H-5), 4.85 (d, 1H, $J_{6,7}$ = 8.0 Hz, H-7), 4.92 (d, 1H, $J_{3,4}$ = 4.1 Hz, H-4), 5.04 (dd, 1H, $J_{3,4}$ = 4.3, $J_{2,3}$ = 5.2 Hz, H-3), 7.32–7.52 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 35.9 (C-2), 64.1 (C-7), 74.1 (C-5), 77.4 (C-3), 82.4 (C-6), 87.2 (C-4), 127.6, 129.07, 129.09, 135.59 (Ph), 175.1 (C-1). HRMS (ESI): *m/e* 298.0797 (M^++Na), calcd for $C_{13}H_{13}N_3NaO_4$: 298.0798.

*3,6-Anhydro-7-azido-2,7-dideoxy-7-C-phenyl-L-glicero-D-ido-heptono-1,4-lactone (**12**)*. Yield: 24%. Colourless plates, mp 133−134 °C, $[\alpha]^{20}_D$ = +149.2 (*c* 0.25, $CHCl_3$), R_f = 0.59 (Et_2O). IR (film): ν_{max} 3442 (OH), 2104 (N_3), 1785 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.40 (br s, 1H, OH), 2.79 (d, 2H, $J_{2,3}$ = 3.2 Hz, 2×H-2), 4.04 (d, 1H, $J_{5,6}$ = 2.6 Hz, H-5), 4.24 (dd, 1H, $J_{5,6}$ = 2.9, $J_{6,7}$ = 8.3 Hz, H-6), 4.73–4.95 (m, 2H, H-4 and H-7), 5.13 (m, 1H, H-3), 7.32–7.55 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 35.6 (C-2), 64.8 (C-7), 74.0 (C-5), 77.4 (C-3), 83.6 (C-6), 87.3 (C-4), 127.7, 129.1, 135.7 (Ph), 175.2 (C-1). HRMS (ESI): *m/e* 298.0803 (M^++Na), calcd for $C_{13}H_{13}N_3NaO_4$: 298.0798.

*4.1.8. 3,6-Anhydro-2-deoxy-D-ido-heptono-1,4-lactone (**13**)*

Prepared according to the literature procedure [21]. Colourless crystals, mp 72–74 °C, $[\alpha]^{20}_D$ = +26.8 (*c* 1.8, H_2O), R_f = 0.72 (9:1 CH_2Cl_2/MeOH). lit. [22] mp 72–74 °C, $[\alpha]^{20}_D$ = +28.4 (*c* 1.97, H_2O), lit. [21] $[\alpha]^{20}_D$ = +23.0 (*c* 0.5, H_2O). IR (film): ν_{max} 3419 (OH), 1770 (C=O). ^{1}H NMR (250 MHz, acetone-d_6): δ 2.47 (d, 1H, $J_{2a,2b}$ = 18.8 Hz, H-2a), 2.85 (dd, 1H, $J_{2b,3}$ = 6.1 Hz, H-2b), 3.66

(dd, 1H, $J_{7a,7b}$ = 11.6, $J_{7a,6}$ = 5.2 Hz, H-7a), 3.75 (d, 1H, $J_{6,7b}$ = 5.1 Hz, H-7b), 3.96 (m, 1H, $J_{5,6}$ = 1.8 Hz, H-6), 4.25 and 5.01 (br s, 2H, exchangeable with D_2O, 2×OH), 4.34 (d, 1H, H-5), 4.88 (d, 1H, $J_{3,4}$ = 4.4 Hz, H-4), 4.94 (t, 1H, H-3). ^{13}C NMR (62.9 MHz, acetone-d_6): δ 36.5 (C-2), 60.9 (C-7), 75.1 (C-5), 77.5 (C-3), 82. 1 (C-6), 89.1 (C-4), 176.3 (C-1). HRMS (ESI): *m/e* 175.0597 (M^++H), calcd for $C_7H_{11}O_5$: 175.0601.

*4.1.9. 3,6-Anhydro-7-chloro-2,7-dideoxy-D-ido-heptono-1,4-lactone (**14**)*

To a cooled (0 °C) and stirred solution of **13** (0.149 g, 0.86 mmol) in dry CH_2Cl_2 (15 mL) and Py (0.74 mL) was added Ph_3P (1.011 g, 3.85 mmol) and CCl_4 (0.75 mL, 7.71 mmol). The mixture was stirred at 0 °C for 0.5 h and then at room temperature for 51 h. The mixture was poured in 6 M HCl, and extracted first with CH_2Cl_2 and then with EtOAc. The combined extracts were washed with 10% aq NaCl, dried and evaporated. The residue was purified by flash column chromatography (1:1 light petroleum/Et_2O), to afford pure **14** (0.063 g, 38%) as colourless plates, mp 92–94 °C, $[\alpha]^{20}_D$ = +21.0 (*c* 0.5, $CHCl_3$), R_f = 0.49 (1:1 light petroleum/EtOAc). IR (film): ν_{max} 1786 (C=O), 3391 (OH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.61 (br s, 1 H, OH), 2.67 (br d, 1H, $J_{2a,2b}$ = 18.8 Hz, H-2a), 2.78 (dd, 1H, $J_{2a,2b}$ = 18.8, $J_{2b,3}$ = 5.2 Hz, H-2b), 3.68 (dd, 1H, $J_{6,7}$ = 6.3, $J_{7a,7b}$ = 10.8 Hz, H-7a), 3.72 (dd, 1H, $J_{6,7}$ = 7.8, $J_{7a,7b}$ = 10.8 Hz, H-7b), 4.27 (m, 1H, H-6), 4.59 (br s, 1H, H-5), 4.93 (d, 1H, $J_{3,4}$ = 4.2 Hz, H-4), 5.01 (m, 1H, H-3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 36.0 (C-2), 39.8 (C-7), 73.6 (C-5), 77.3 (C-3), 80.9 (C-6), 87.6 (C-4), 175.4 (C-1). HRMS (ESI): *m/e* 210.0526 (M^++NH_4), calcd for $C_7H_{13}ClNO_4$: 210.0528. Anal. Found: C, 43.44; H, 4.78. Calcd for $C_7H_9ClO_4$: C, 43.65; H, 4.71.

*4.1.10. 3,6-Anhydro-7-bromo-2,7-dideoxy-D-ido-heptono-1,4-lactone (**15**)*

To a cooled (0 °C) and stirred solution of **13** (0.306 g, 1.76 mmol) in dry CH_2Cl_2 (50 mL) and Py (1.5 mL) was added Ph_3P (1.152 g, 4.39 mmol) and CBr_4 (1.457 g, 4.40 mmol). The mixture was stirred at 0 °C for 0.5 h and then at room temperature for 72 h. The mixture was poured in 6 M HCl, and extracted with EtOAc. The combined extracts were washed with 10% aq NaCl, dried and evaporated. The residue was purified by three flash column chromatography (first Et_2O; second and third $CHCl_3$), gave pure **15** (0.116 g, 28%) as colourless needles, mp 87–89 °C, $[\alpha]^{20}_D$ = +10.9 (*c* 1.8, $CHCl_3$), R_f = 0.26 (1:1 light petroleum/EtOAc); lit. [24] mp (for enantiomer) 88–90 °C, $[\alpha]^{20}_D$ = −22.0 (*c* 1.8, $CHCl_3$) (for enantiomer). IR (film): ν_{max} 1782 (C=O), 3448 (OH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.66 (d, 1H, , $J_{2a,2b}$ = 18.7 Hz, H-2a), 2.70 (dd, 1H, $J_{2a,2b}$ = 18.7, $J_{2b,3}$ = 5.2 Hz , H-2b), 3.12 (br s, 1H, OH), 3.40–3.60 (m, 2H, $J_{6,7}$ = 6.4 Hz, H-7), 4.30 (m, 1H, $J_{5,6}$ = 3.0, $J_{5,6}$ = 6.3 Hz, H-6), 4.58 (d, 1H, $J_{5,6}$ = 2.9 Hz, H-5), 4.93 (d, 1H, $J_{3,4}$ = 4.2 Hz, H-4), 5.02 (m, 1H, H-3). ^{13}C NMR

(100 MHz, D_2O): δ 28.4 (C-7), 36.4 (C-2), 73.8 (C-5), 78.1 (C-3), 81.7 (C-6), 89.2 (C-4), 180.0 (C-1). HRMS (ESI): *m/e* 254.0020 (M^++NH_4), calcd for $C_7H_{13}NBrO_4$: 254.0022.

*4.1.11. 3,6-Anhydro-2,7-dideoxy-7-iodo-D-ido-heptono-1,4-lactone (**16**)*

To a solution of **13** (0.281 g, 1.61 mmol) in dry THF (2.5 mL) was added imidazole (0.219 g, 3.22 mmol) and Ph_3P (0.845 g, 3.22 mmol). In the refluxing mixture was added dropwise a solution of iodine (0.491 g, 1.93 mmol) in dry THF (1.4 mL). The mixture was stirred for 3 h under reflux, and then evaporated. The residue was purified by flash column chromatography (2:1 light petroleum/EtOAc), to afford pure **16** (0.290 g, 63%) as a colourless powder, mp 131 °C, $[\alpha]^{20}_D$ = +10.7 (*c* 1, $CHCl_3$), R_f = 0.32 (1:1 light petroleum/EtOAc). lit. [25] mp 131–133 °C, $[\alpha]^{20}_D$ = +17.2 (*c* 1.0, $CHCl_3$). IR (film): ν_{max} 1778 (C=O), 3436 (OH). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.64 (d, 1H, $J_{2a,2b}$ = 18.7 Hz, H-2a), 2.76 (dd, 1H, $J_{2a,2b}$ = 18.7, $J_{2b,3}$ = 5.3 Hz, H-2b), 3.10 (br s, 1H, OH), 3.18–3.34 (m, 2H, H-7a and H-7b), 4.30 (m, 1H, $J_{5,6}$ = 2.6, $J_{6,7}$ = 7.2 Hz, H-6), 4.59 (br s, 1H, H-5), 4.93 (d, 1H, $J_{3,4}$ = 4.2 Hz, H-4), 5.04 (m, 1H, H-3). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ −1.1 (C-7), 36.2 (C-2), 73.9 (C-5), 77.6 (C-3), 81.4 (C-6), 87.7 (C-4), 175.6 (C-1). HRMS (ESI): *m/e* 301.9887 (M^++NH_4), calcd for $C_7H_{13}NIO_4$: 301.9884.

*4.1.12. 3,6-Anhydro-7-azido-2,7-dideoxy-D-ido-heptono-1,4-lactone (**18**)*

To a solution of **16** (0.208 g, 0.73 mmol) in anhydrous DMF (17 mL) was added NH_4Cl (0.055 g, 1.03 mmol) and NaN_3 (0.477 g, 7.33 mmol). The mixture was stirred for 7.5 h at 50 °C, then for 24 h at room temperature and evaporated. The residue was purified by flash column chromatography (1:1 light petroleum/EtOAc), to give pure **18** (0.096 g, 66%) as colourless plates, mp 85–87 °C, $[\alpha]^{20}_D$ = +11.0 (*c* 0.2, $CHCl_3$), R_f = 0.31 (1:1 light petroleum/EtOAc). IR (KBr): ν_{max} 1755 (C=O), 2097 (N_3), 3412 (OH). ^{1}H NMR (250 MHz, $CDCl_3$:) δ 2.68 (dd, 1H, $J_{2a,2b}$ = 18.7, $J_{2a,3}$ = 1.2 Hz, H-2a), 2.79 (dd, 1H, $J_{2a,2b}$ = 18.8, $J_{2b,3}$ = 5.2 Hz, H-2b), 3.09 (br s, 1H, OH), 3.58 (d, 1H, $J_{6,7}$ = 5.8 Hz, H-7), 4.17 (td, 1H, $J_{6,7}$ = 5.8, $J_{5,6}$ = 3.4 Hz, H-6), 4.50 (br s, 1H, H-5), 4.90 (d, 1H, $J_{3,4}$ = 4.2 Hz, H-4), 5.02 (m, 1H, H-3). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 36.0 (C-2), 49.5 (C-7), 71.3 (C-5), 76.9 (C-3), 79.2 (C-6), 88.0 (C-4), 175.6 (C-1). HRMS (ESI): *m/e* 217.0927 (M^++NH_4), calcd for $C_7H_{13}NO_4$: 217.0931.

*4.1.13. 3,6-Anhydro-3-O-benzyl-2,7-dideoxy-7-fluoro-D-ido-heptono-1,4-lactone (**31**)*

To a cooled (0 °C) and stirred solution of **30** (0.074 g, 0.28 mmol) in dry CH_2Cl_2 (1.5 mL) was added DAST (0.11 mL g, 0.84 mmol). The mixture was stirred at 0 °C for 0.5 h and then at room

temperature for 3 h. The mixture was suspended in 10% aq $NaHCO_3$, and the resulting suspension was extracted first with CH_2Cl_2 and then with EtOAc, dried and evaporated. The residue was purified by flash column chromatography (4:1 CH_2Cl_2/light petroleum), to give pure product **31** (0.026 g, 35%) as a colourless syrup, $[\alpha]^{20}_D$ = +16.2 (*c* 0.2, $CHCl_3$), R_f = 0.80 (3:2 light petroleum/acetone). IR (film): ν_{max} 1782 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.73 (m, 2H, H-2), 4.29 (d, 1H, $J_{5,6}$ = 4.7 Hz, H-5), 4.31–4.45 (m, 1H, H-6), 4.48–4.79 (m, 3H, H-7 and C**H$_2$**Ph), 4.95 (d, 1H, $J_{3,4}$ = 4.7 Hz, H-4), 5.01 (m, 5H, H-3), 7.47–7.29 (m, 5H, Ph). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 36.0 (C-2), 72.8 (**C**H_2Ph), 77.2 (C-3), 78.9 and 79.2 ($J_{F,6}$ = 21.4 Hz, C-6), 80.4 and 83.1 ($J_{F,7}$ = 168.6 Hz,C-7), 81.4 and 81.5 ($J_{F,5}$ = 5.0 Hz, C-5), 85.4 (C-4), 127.8, 128.3, 128.7, 136.7 (Ph), 174.9 (C-1). HRMS (ESI): *m/e* 284.1285 (M^++NH_4), calcd for $C_{14}H_{15}FNO_4$: 284.1293.

*4.1.14. 3,6-Anhydro-2,7-dideoxy-7-fluoro-D-ido-heptono-1,4-lactone (**17**)*

A solution of **31** (0.047 g, 0.18 mmol) in EtOAc (3 mL + 1% conc. HCl) was hydrogenated over 10% Pd/C (0.047 g; the catalyst contained 50% of water) for 19 days at room temperature. The mixture was filtered through a Celite pad and the catalyst washed with EtOAc. The combined organic solutions were evaporated and the residue was purified by flash column chromatography (7:3 light petroleum/acetone) to afford pure **17** (0.017 g, 57%) as a colourless syrup, $[\alpha]^{20}_D$ = +30.3 (*c* 0.3, $CHCl_3$), R_f = 0.42 (7:3 light petroleum/acetone). IR (film): ν_{max} 1783 (C=O), 3431 (OH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.20 (br s, 1H, OH), 2.70 (bd, 1H, $J_{2a,2b}$ = 18.8 Hz, H-2a), 2.80 (dd, 1H, $J_{2a,2b}$ = 18.8, $J_{2b,3}$ = 5.7 Hz, H-2b), 4.31 (m, 1H, $J_{6,7}$ = 4.5, $J_{F,7}$ = 13.6 Hz, H-6), 4.61 (d, 1H, $J_{5,6}$ = 3.7 Hz, H-5), 4.70 (m, 2H, $J_{6,7}$ = 4.5, $J_{7a,7b}$ = 10.1, $J_{F,7}$ = 46.8 Hz, C**H$_2$**-7), 4.91 (d, 1H, $J_{3,4}$ = 4.2 Hz, H-4), 5.10 (m, 1H, H-3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 36.0 (C-2), 74.6 and 74.7 ($J_{F,5}$ = 4.9 Hz, C-5), 77.0 (C-3), 79.2 and 81.0 ($J_{F,6}$ = 20.9 Hz, C-6), 80.9 and 82.5 ($J_{F,7}$ = 167.3 Hz, C-7), 88.2 (C-4), 175.5 (C-1). HRMS (ESI): *m/e* 194.0821 (M^++NH_4), calcd for $C_7H_{13}FNO_4$: 194.0821.

4.2. X-ray Crystal Structure Analysis

Diffraction experiments were performed on an Oxford Diffraction Gemini S diffractometer. Crystal structures were solved by SHELXT [31] or SIR92 [32] and refined with SHELXL [33]. See the Supplementary data for details. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre. CCDC Numbers: 1514910 (**7**), 1514911 (**10**), 1514912 (**11**), 1514913 (**12**) 1514914 (**18**), 1514915 (**24**), 1514916 (**27**), and 1514917 (**29**). These data are available free of charge via www.ccdc.cam.ac.uk/data_request/cif.

4.3. Biological materials

Rhodamine B, RPMI 1640 medium, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide, foetal calf serum, propidium iodide and RNase A, were purchased from Sigma (St. Louis, MO, USA). Penicillin and streptomycin were purchased from ICN Galenika (Belgrade, Serbia). Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences Pharmingen (Belgium). Proteins were detected by Western blotting using the following monoclonal antibodies against: Bax, Bcl-2 and Caspase-3 (obtained from R&D Systems, Minneapolis, MN) and anti-Poly (ADP-ribose) polymerase (PARP) (purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence (ECL Plus) kit and Hyperfilm were purchased from Amersham Biosciences (Arlington Heights, IL). All other chemicals used in the experiments were commercial products of reagent grade. Stock solution (10 mM) was prepared in DMSO and diluted to various concentrations with serum-free culture medium.

4.4. Cell lines

Human chronic myelogenous leukaemia (K562), promyelocytic leukaemia (HL-60), human T cell leukaemia (Jurkat) and Burkitt's lymphoma (Raji) were grown in RPMI 1640 while ER^+ breast adenocarcinoma (MCF-7), cervix carcinoma (HeLa), lung adenocarcinoma epithelial (A549) malignant cells, and normal foetal lung fibroblasts (MRC-5) were grown in DMEM medium. Both media were supplemented with 10% of foetal calf serum (FTS, NIVNS) and antibiotics (100 IU/mL of penicillin and 100 mg/mg of streptomycin). Cell lines were cultured in flasks (Costar, 25 mL) at 37°C in the atmosphere of 100% humidity and 5% of CO_2 (Heraeus). Exponentially growing viable cells were used throughout the assays.

4.5. Cells treatment

The cells were seeded in six-well plates at a concentration of 5×10^5 cells/well. Cells were treated for 72 h with goniofufurone (**1**), 7-epi-goniofufurone (**2**), dephenyl goniofufurone (**13**) and the corresponding analogues (**3**–**12**, **14**–**18**) at their IC_{50}/72 h concentrations. Untreated cells were used as control. Viable cells of treated and control samples were used for cell cycle and apoptosis detection and Western blot analysis. Viability was determined using trypan blue dye-exclusion assay.

4.6. MTT assay

Cells were harvested, counted by trypan blue and plated into 96-well microtitar plates (Costar) at optimal seeding density of 5×10^3 cells per well to assure logarithmic growth rate throughout the

assay period. Viable cells were placed in a volume of 90 mL per well, and pre-incubated in complete medium at 37 °C for 24 h to allow cell stabilization prior to the addition of substances. Tested substances, at 10-fold the required final concentration, were added (10 mL/well) to all wells except to the control ones and microplates were incubated for 72 h. The wells containing cells without tested substances were used as control. Three hours before the end of incubation period MTT solution (10 mL) was added to each well. MTT was dissolved in medium at 5 mg/mL and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. Acidified 2-propanol (100 mL of 0.04 M HCl in 2-propanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals of formazan. After a few minutes at room temperature, to ensure that all crystals were dissolved, the plates were read on a spectrophotometer plate reader (Multiscan MCC340, Labsystems) at 540 and 690 nm. The wells without cells containing complete medium and MTT acted as blank.

4.7. Cell cycle analysis

After treatment K562 cells were washed in cold PBS, fixed and incubated for 30 min in 70% ethanol on ice, centrifuged and incubated with 500 µL RNase A (100 units/mL) and 500 µL propidium iodide (400 µL/mL) for 30 min at 37 °C. Cell cycle was analyzed by FACS Calibur E440 (Becton Dickinson) flow cytometer and the Cell Quest software. Results were presented as percentage of cell cycle phases.

4.8. Detection of apoptosis

Apoptosis of K562 cells was evaluated with an Annexin V-FITC detection kit. Cells from each sample were collected (800 rpm/5 min, Megafuge 1.0 R, Heraeus, Thermo Fisher Scientific) and pellet was re-suspended in 1 mL of phosphate buffer (PBS, pH 7.2). K562 cells were washed twice with cold PBS and then re-suspended in binding buffer to reach the concentration of 1×10^6 cells/mL. The cell suspension (100 mL) was transferred to 5 mL culture tubes and mixed with Annexin V (5 mL) and propidium iodide (5 mL). The cells were gently vortexed and incubated for 15 min at 25 °C. After incubation, 400 mL of binding buffer was added to each tube and suspension was analyzed after 1 h on FACS Calibur E440 (Becton Dickinson) flow cytometer. Results were presented as percent of Annexin V positive gated cells. Percentage of specific apoptosis was calculated according to Bender et al [34].

4.9. Western blot analysis

For the Western blot, 50 mg of proteins per sample were separated by electrophoresis and electro-transferred to a PVDF membrane Hybond-P and then blotted with primary antibodies (Bcl-2, Caspase-3 and PARP). Proteins were detected by an enhanced chemiluminescence (ECL Plus) kit that includes peroxidase-labelled donkey anti-rabbit and sheep anti-mouse secondary antibodies. Blots were developed with an ECL Plus detection system and recorded on the Amersham Hyperfilm.

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Supplementary data

Contains the results of: X-ray crystal structure determination, SAR analysis, flow cytometry and Western blot analysis. Copies of ^{1}H, and ^{13}C NMR spectra of final products, purity grade of final compounds by HPLC.

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Captions

Figure 1. ORTEP presentation of compound **29**.

Scheme 1. Design of styryl lactone analogues: (a) isosterism (OH → X); (b) ring removal (Ph → H).

Scheme 2. Reagents and conditions: (a) TiI_4, CH_2Cl_2, 0 °C, 0.5 h, then rt, 3.5 h for **19**, 55% of **20**, 2.5 h for **24**, 31% of **7**, 11% of **8**, 3 h for **23**, 17% of **7** and **8**; (b) I_2, Ph_3P, imidazole, toluene, 70 °C, 3 h, 41% of **23**, 11% of **24**, 10% of **25**; (c) I_2, Ph_3P, 2,6-lutidine, CH_2Cl_2, 0 °C, 0.5 h, then rt, 28 h, 44% of **24**, 2% of **23**, 11% of **25**.

Scheme 3. Reagents and conditions: (a) DAST, CH_2Cl_2, 0 °C, 0.5 h, then rt, 3.5 h for **21**, 63% of **26**, 2.5 h for **22**, 76% of **27**; (b) H_2-Pd/C, EtOAc, conc. HCl, rt, 30 h for **26**, 70% of **9**, 21 h for **27**, 79% of **10**.

Scheme 4. Reagents and conditions: (a) Me_3SiN_3, $BF_3 \cdot OEt_2$, CH_2Cl_2, 0 °C, 4 h, then Et_3N, 0 °C, 0.5 h, 12% of **29**, 10% of **11**, 24% of **12**.

Scheme 5. Reagents and conditions: (a) CCl_4, Ph_3P, Py, CH_2Cl_2, 0 °C, 0.5 h, then rt, 51 h, 38%; (b) CBr_4, Ph_3P, Py, CH_2Cl_2, 0 °C, 0.5 h, then rt, 72 h, 28%; (c) I_2, imidazole, Ph_3P, THF, reflux, 3 h, 63%; (d) NaN_3, NH_4Cl, DMF, 50 °C, 7.5 h, then rt, 24 h, 66%; (e) DAST, CH_2Cl_2, 0 °C, 0.5 h, then rt 3 h, 35%; (f) H_2-Pd/C, EtOAc, conc. HCl, rt, 19 days, 57%.

Table 1. In vitro cytotoxicity of natural lactones **1** and **2**, their analogues (**3**–**12**, **13**–**18**) and DOX.

Compd	$IC_{50}(\mu M)$ [a]							
	K562	HL-60	Jurkat	Raji	MCF-7	HeLa	A549	MRC-5
1	0.41	>100	32.45	18.45	16.59	8.32	35.21	>100
3	2.28	7.37	13.17	0.04	>100	0.81	15.45	>100
5	1.12	0.11	3.07	18.87	13.51	0.68	48.85	>100
7	5.95	13.64	11.77	0.04	21.01	21.85	11.75	>100
9	10.98	15.87	5.41	1.85	21.39	2.31	30.45	>100
11	11.54	27.14	5.96	18.64	8.85	19.56	33.78	>100
2	0.03	22.02	18.64	1.25	9.24	0.89	21.02	>100
4	0.11	0.15	0.03	1.85	>100	1.11	47.35	>100
6	0.98	0.32	5.44	15.34	23.32	1.64	51.24	>100
8	11.08	24.36	9.56	2.85	1.28	5.69	25.61	>100
10	11.68	14.58	1.55	0.03	31.15	23.64	34.25	>100
12	3.09	15.47	46.32	2.12	44.88	0.12	18.34	>100
13	8.45	15.96	11.05	13.31	24.25	10.05	16.23	>100
14	35.47	4.65	32.01	21.04	3.25	4.62	4.02	>100
15	32.14	8.62	14.97	3.88	6.21	54.13	11.85	>100
16	18.64	11.32	16.01	4.97	2.64	44.25	16.55	>100
17	2.02	0.99	1.02	8.32	3.52	0.52	11.22	>100
18	1.23	0.89	1.21	1.02	0.85	2.69	21.01	>100
DOX	0.25	0.92	0.03	2.98	0.20	0.07	4.91	0.10

[a] IC_{50} is the concentration of compound required to inhibit the cell growth by 50% compared to an untreated control. Values are means of three independent experiments. Coefficients of variation were less than 10%.

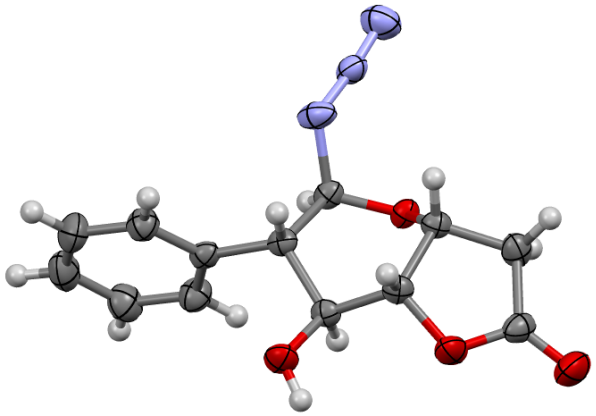
Table 2. (A) Influence of synthesized compound on the K562 cell cycle; (B) percentage of specific apoptosis and necrosis induced with synthesized compounds in the K562 cell culture.

Compd	(A) distribution of K562 cells in cell cycle phases (%)				(B) type of cell death	
	Sub G1	G0/G1	S	G2/M	Specific apoptosis (%)	Specific necrosis (%)
control	0.55	44.16	40.15	15.14	–	–
1	0.97	45.45	43.35	10.23	1.23	0.95
3	2.25	42.17	41.11	14.47	58.36	0.81
5	4.17	40.01	39.43	16.39	2.27	4.35
7	1.77	39.61	48.01	10.61	2.61	2.20
9	1.91	41.06	49.02	8.01	7.63	0.31
11	5.56	34.06	49.03	11.35	31.42	1.89
control	0.55	44.16	40.15	15.14	–	–
2	0.24	44.11	39.56	16.09	4.39	1.28
4	5.46	36.13	49.06	9.35	38.77	0.51
6	0.75	39.12	43.53	16.60	1.70	4.56
8	2.26	39.93	50.75	7.06	5.81	1.45
10	1.68	34.26	51.98	12.08	6.43	0.58
12	1.20	46.29	42.82	9.69	0.16	1.74
control	0.44	55.41	22.92	21.28	–	–
13	1.00	52.55	23.77	22.68	0.05	0.68
14	0.67	47.97	24.55	26.82	0.30	9.13
15	10.99	51.35	21.01	16.65	19.59	0.87
16	2.01	41.16	27.17	29.67	0.97	2.67
17	9.33	39.03	26.31	25.54	11.33	0.77
18	13.01	58.67	17.23	11.11	18.10	1.23

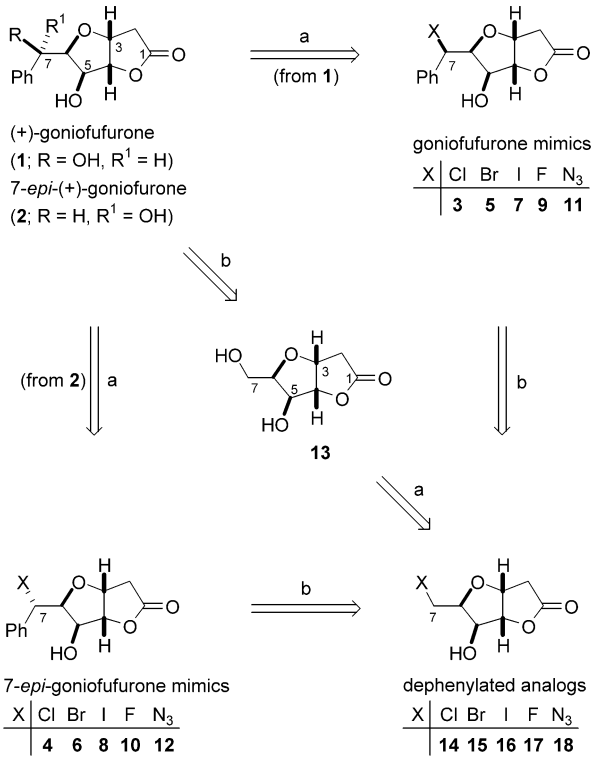
Table 3. Results of Western blot analysis.

Compd	% of control					
	Bcl-2	BAX	Caspase-3 precursor (32 kD)	Caspase-3 active subunit (18 kD)	PARP (112 kD)	PARP (85 kD)
1	195.8	103.7	128.9	37.6	124.3	107.9
3	421.7	92.5	175.8	99.6	118.7	132.0
5	346.4	94.9	149.7	87.1	154.5	142.2
7	388.0	59.3	157.5	48.8	148.2	128.5
9	317.6	40.1	157.0	112.9	161.8	161.5
11	601.5	59.5	242.5	166.9	179.3	195.5
2	224.8	99.6	117.5	53.6	116.8	108.4
4	138.1	83.4	82.5	70.0	134.9	102.4
6	75.0	46.7	115.5	69.9	130.6	94.7
8	164.1	47.3	143.7	95.7	118.2	106.0
10	151.2	21.0	177.1	87.5	155.8	103.5
12	132.5	16.9	145.1	113.5	164.5	127.1
13	50.6	97.7	114.8	80.6	132.6	201.7
14	72.6	94.4	111.0	70.9	230.7	241.3
15	72.3	102.5	93.7	59.2	305.9	354.5
16	70.6	103.8	68.0	50.7	235.9	324.2
17	75.3	119.1	148.2	77.8	199.3	191.8
18	73.9	88.8	163.7	108.8	343.6	425.4

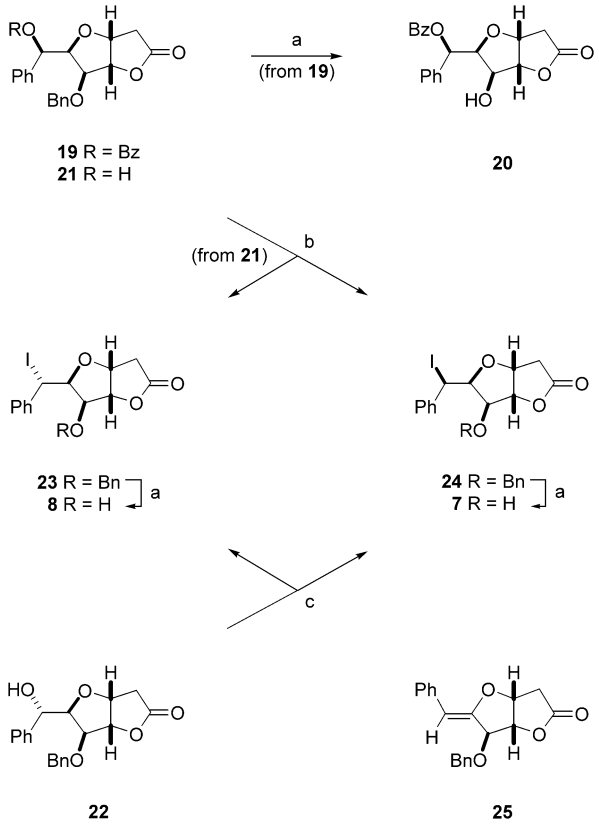
Figure 1.



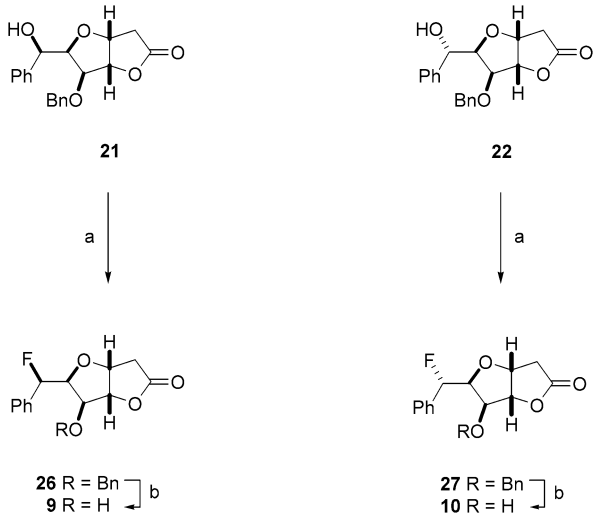
Scheme 1.


(+)-goniofufurone
(1; R = OH, R1 = H)
7-epi-(+)-goniofurone
(2; R = H, R1 = OH)
a
(from 1)
goniofufurone mimics
X Cl Br I F N3
3 5 7 9 11
b
(from 2)
a
13
b
a
b
7-epi-goniofufurone mimics
X Cl Br I F N3
4 6 8 10 12
dephenylated analogs
X Cl Br I F N3
14 15 16 17 18

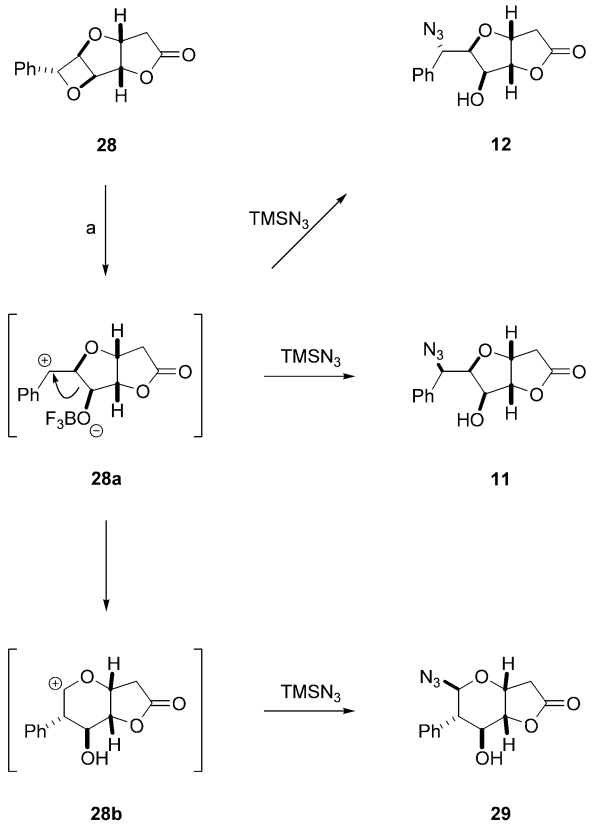
Scheme 2.


RO
Ph
BnO
a
(from 19)
BzO
HO
19 R = Bz
21 R = H
20
b
(from 21)
I
23 R = Bn
8 R = H
24 R = Bn
7 R = H
c
HO
22
Ph
25

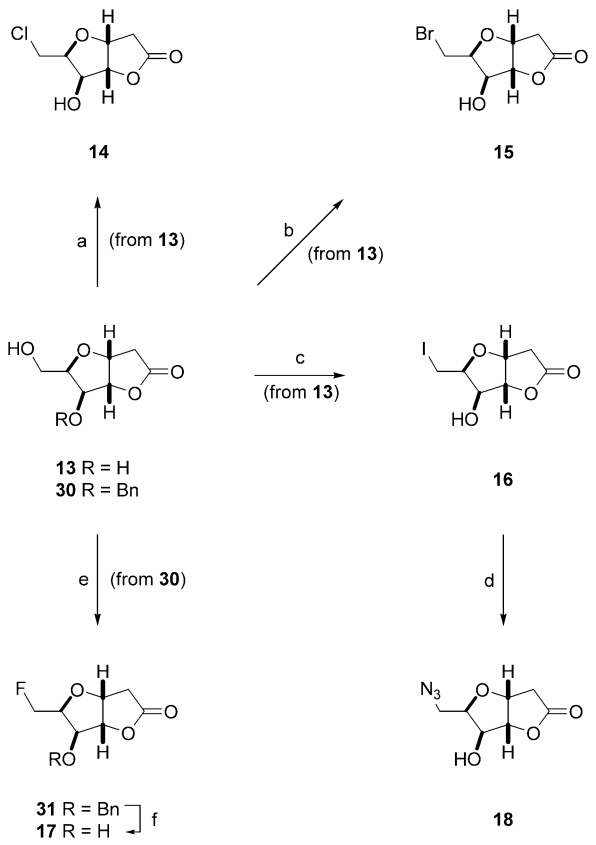
Scheme 3.



Scheme 4.



Scheme 5.


Cl
Br
HO
RO
F
N_3
I
14
15
16
17
18
30
31
13 R = H
30 R = Bn
31 R = Bn
17 R = H
a (from 13)
b (from 13)
c (from 13)
d
e (from 30)
f