Bioorganic & Medicinal Chemistry 17 (2009) 1456–1463

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological testing of thioalkane- and thioarene-spaced bis-β-D-glucopyranosides

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ARTICLE INFO

Article history:
Received 4 July 2008
Revised 8 January 2009
Accepted 10 January 2009
Available online 15 January 2009

Keywords:
Apoptosis
Biological tests
Sulfenic acids
Thio-bis-glucopyranosides

ABSTRACT

A three-step synthesis of bis-β-D-glucopyranosides containing thioalkane or thioarene spacers of different length and flexibility is described. The key-step reaction allows an easy modulation of final saccharidic products so that a library of molecules with different glycosidic residues and spacers can be obtained. Two of the new thioarene-spaced bis-β-D-glucopyranosides endow with a specific cytotoxic potential. A more detailed investigation of one of the two compounds ascertains that this effect is attributable to induction of cell death by apoptosis.

1. Introduction

A number of biological processes are controlled by protein-carbohydrate interactions that function as transfer of information between cell surface and cell substrata or in cell–cell recognition events.[1] In particular, carbohydrates have been implicated in fundamental processes that regulate cellular growth and death, or cause the onset of infection, for two fundamental reasons: (i) most cells are covered by carbohydrates and (ii) carbohydrates are capable of forming many different combinatorial structures, each one carrying a specific biological message, from relatively small numbers of sugar units. This fundamental and eclectic role of carbohydrates has inspired the synthesis of glycoconjugates to be used as potential therapeutics or useful tools for the understanding of binding specificity and other properties of carbohydrate recognition events. Tailored small molecules have been synthesized with the aim of developing libraries of glycoconjugate inhibitors. A calix[4]arene has been used as scaffold for the introduction of sugar units with the idea of mimicking, to some extent, a small portion of the cell surface presenting glycosylated residues on the exterior of the lipophilic region.[2] A significant investigation has been conducted on the binding and cross-linking interactions of Con A and *Dioclea grandiflora* lectin (DGL) with a series of divalent carbohydrates that possess spacer groups with increasing flexibility and length between terminal mannopyranoside residues.[3] Recently, the synthesis of multivalent arrays of mannose mono- and disaccharides was described as potential anti-bacteria infective agents.[4] Finally, a series of β-D-manno- and β-D-glucopyranosides have been synthesized that show inhibitory activity for the HIV-1 protease, providing a basis for the development of more potent carbohydrate based peptidomimetic inhibitors.[5] These investigations on small molecule inhibitors highlight how the control of molecular architecture and saccharide spacing can play a significant role on the enhancement of the glycoconjugate-model receptor binding activity.

In this work we describe a three-step synthesis of bis-β-D-glucopyranosides containing thioalkane or thioarene spacers of different lengths and flexibility and the results of biological tests conducted on some of them. The adopted synthetic procedure allows an easy modulation of final saccharidic products so that a library of molecules with different glycosidic residues and spacers is achievable in principle. The presence of sulfur atoms in the skeleton of glucodisaccharides offers the opportunity to vary the oxidation state of sulfur atoms and to study the effect that this variation can play on the biological profile of the corresponding thioglycoconjugates. In order to investigate the relationship between molecular structure and biological response, we performed a preliminary screening of the effects of the newly synthesized thioalkane- and thioarene-spaced bis-β-D-glucopyranosides on cell viability. Moreover we focused our attention on the ability of the synthesized compounds to induce apoptotic cell death, taking into account

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doi:10.1016/j.bmc.2009.01.010

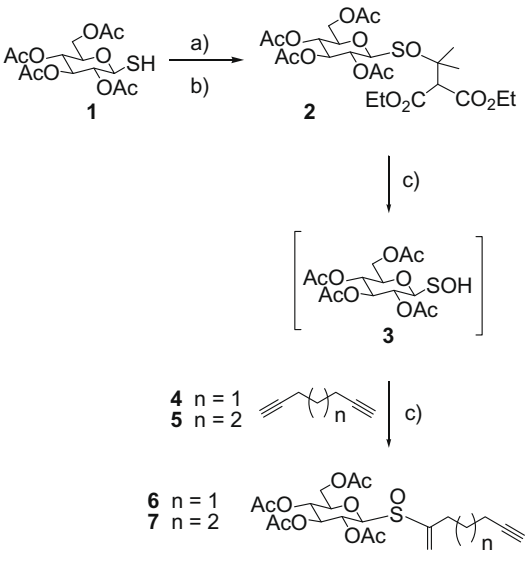
the pivotal role of apoptosis regulation in both the controlled expansion and removal of immune cells and cancer progression and therapy.[6]

2. Chemistry

As part of a programme centred on the study of the *syn*-addition of sulfenic acids on the triple bond of suitable unsaturated molecules, we forecast that the reaction of glycosulfenic acids, such as **3**, with terminal diynes, such as **4** and **5** (Scheme 1), could represent an efficient procedure to obtain thioglycoconjugates with two sugar moieties separated by alkane or arene spacers. The possibility of obtaining the desired products in three steps, starting from various monosaccharide derivatives and commercially available, structurally different, unsaturated linkers, would give easy access to a library of molecules to submit for biological testing.

2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (**1**) was used as starting material for the preparation of epimeric sulfoxide precursors **2** (R_S/S_S 1:2) of sulfenic acid **3**, following a well-assessed synthetic pathway (Scheme 1).[7] In a series of attempts, where reagent ratios and reaction times were changed, the epimeric mixture of sulfoxides **2** was thermolyzed in dichloromethane (DCM) at reflux in the presence of 1,6-heptadiyne (**4**) or 1,7-octadiyne (**5**). The access to thioalkane- and thioarene-spaced bis-β-D-glucopyranosides *via* this method proved problematic, and this approach was successful only in the preparation of monosaccharides **6** and **7**, possessing an unsaturation qualified for a further attack by glucosulfenic acid **3**. Compounds **6** and **7** were obtained in low yield (see experimental section) as 2:1 mixtures each of sulfur epimers. In both cases the column chromatography allowed the isolation of both epimers, whose configuration was assigned taking into account the role of the *exo*-anomeric effect.[8] Although the methodology did not offer a direct entry to thioalkane- and thioarene-spaced bis-β-D-glucopyranosides as desired, monosaccharides **6** and **7** were subjected to a second thermolysis in the presence of sulfoxides **2** but, even in this case, no traces of the corresponding thioalkane- and thioarene-spaced bis-β-D-glucopyranosides were detected.

The failure of this approach to provide the desired molecules was overcome by applying the sulfenic acid chemistry to commercially available bis-thiols.[9] The later compounds were considered as suitable starting products for the synthesis of precursors of bis-sulfenic acids, such as **20**–**23** in Scheme 2, to be generated in situ in the presence of 2-propynyl tetra-*O*-acetyl-β-D-glucopyranoside (**24**).[10] For instance the double *syn*-addition of the bis-sulfenic acid **20** onto the triple bond of the monosaccharide **24** should provide the thioarene-spaced bis-β-D-glucopyranoside **25**. This approach was considered attractive for entry to bis-β-D-glucopyranosides also because the starting products are less expensive with respect to diynes such as **6** or **7**.



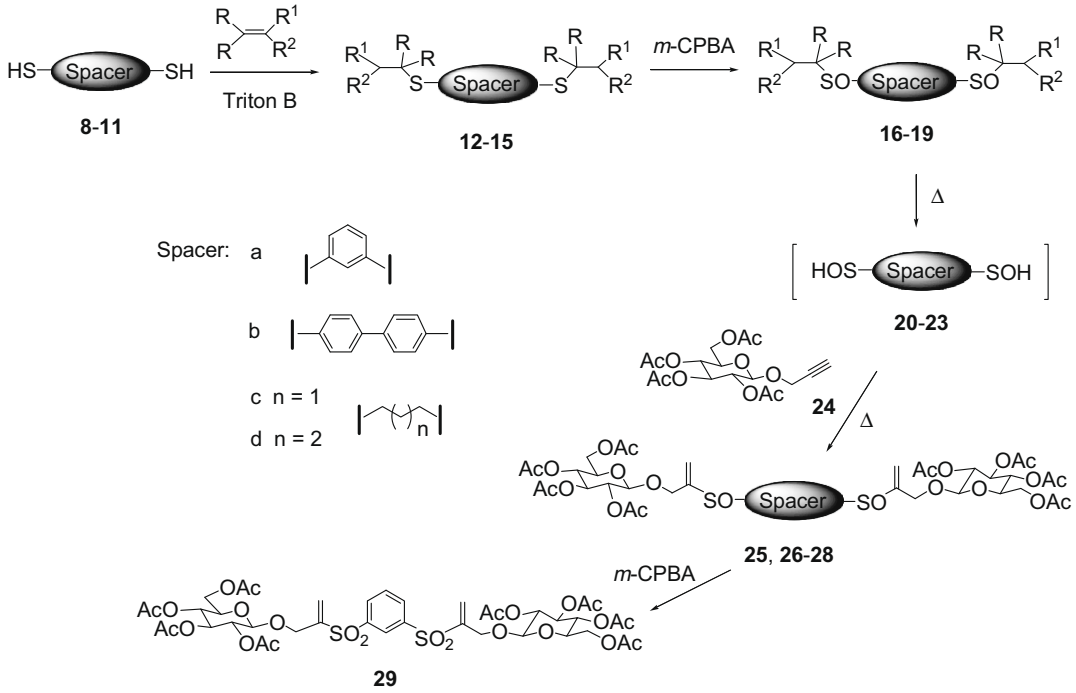
Scheme 1. Reagents and conditions: (a) Diethyl isopropylidenemalonate, Triton B, THF, −78 °C; (b) *m*-CPBA, DCM, −78 °C; (c) DCM, reflux.

1,3-Benzenedithiol (**8**), 1,3-propanedithiol (**10**), and 1,5-pentanedithiol (**11**) were involved in the nucleophilic addition of the two sulfur functions to acrylonitrile in the presence of a catalytic quantity of Triton B, whereas diethyl isopropylidenemalonate was used as electrophile acceptor towards (1,1′-biphenyl)-4,4′-dithiol (**9**) after having experienced that the double addition of **9** to acrylonitrile leads to intractable sulfenic acid precursors. Subsequent controlled oxidation of disulfides **12**–**15** allowed the formation of bis-sulfoxides **16**–**19** that represent the precursors of the corresponding sulfenic acids **20**–**23**. Thermolysis of **16** in 1,2-dichloroethane (95 °C), of **18** and **19** in toluene (110 °C), and of **17** in DCM, in the presence of monosaccharide **24**, provided sulfurated bis-β-D-glucopyranosides **25**–**28**. The diastereomeric mixture of glucosulfoxides **25** was further oxidized with *m*-CPBA to the unique bis-sulfone **29** in order to investigate by comparison if the oxidation state of sulfur atom plays any significant role in the biological behavior of the compounds under study.

3. Biological results and discussion

The relationship between molecular structure and biological activity of sulfurated bis-β-D-glucopyranosides **25**–**29** was investigated firstly by comparing the toxic effect exerted by some of the synthesized compounds using a conventional viability assay. Sugars **25**–**29** were dissolved in DMSO (10 mM). These mother solutions were diluted in RPMI-1640 medium supplemented with 5% FBS, 2 mM L-glutamine and penicillin–streptomycin to reach 10-fold increasing concentrations (1, 10 and 100 μM). The obtained samples were added to 2×10^4 U937 monocytoid cells in 100 μl total volume and incubated at 37 °C in a CO_2 incubator. As a control, the same cells were exposed to the vehicle alone (DMSO), in amounts corresponding to those employed for dissolving the compounds. The percentage of dead cells was evaluated after 24 h incubation by microscopy analysis of stained cells using a standard trypan blue exclusion test. No significant change of dead cell percentage was detected with any compound at the lower concentrations (1 and 10 μM) assayed. At 100 μM concentration, disaccharides **26** and **28** induced low levels of toxicity (Fig. 1B and D), without modifying the absolute number of cells. Compound **27** was slightly more cytotoxic than compounds **26** and **28** at the same concentration (Fig. 1C). The obtained results allowed us to associate the highest, specific cytotoxic potential to compound **29** (Fig. 1E) and compound **25** (Fig. 1A), both characterized by a single central ring linking the two disaccharide units. Moreover, the cytotoxic potential of compounds **25**, **27** and **29**, sharing a similar three carbon linker length, was associated with a decrease in the absolute number of cells at 100 μM concentration. The observed biological activity appears not significantly affected by the oxidation state of the sulfur atoms.

We then focused our attention on the effects on cell growth and apoptosis of sugar **25** as a model for this family of compounds. To this purpose, the biological activity of **25** was investigated deeper by performing further experiments at a narrow range of concentrations (from 10 to 75 μM). The effects of **25** on cell metabolic activity



Thiol	Spacer	R	R^1	R^2	Sulfide	Sulfoxide	Sulfenic acid	Glucodisaccharide (yield %)
8	a	H	H	CN	**12**	**16**	**20**	**25** (40)
9	b	Me	CO_2Et	CO_2Et	**13**	**17**	**21**	**26** (30)
10	c	H	H	CN	**14**	**18**	**22**	**27** (20)
11	d	H	H	CN	**15**	**19**	**23**	**28** (40)

Scheme 2.

were first measured by determining oxygen burst using the MTS assay in the monocytic cell line U937 and in the lymphocytic cell line Molt-3. The results, shown in Figure 2A and B, respectively, represent the mean values of three determinations for each concentration tested. A highly significant inhibitory effect of **25** on cell metabolic activity was observed in both cell lines at the highest concentration assayed, in agreement with preliminary experiments indicating a cytotoxic potential for this compound. Surprisingly, lower concentrations of **25** did not inhibit the cell metabolic activity, but rather stimulated it both in U937 and Molt-3 cells, although in the latter ones the differences between control and treated cells were not significant. The absolute number of viable cells were, then, evaluated at 24 h incubation in cultures undergoing treatment with different concentrations of **25**, in order to ascertain whether this effect could be associated to the thioarene-spaced bis-β-D-glucopyranoside influence on cell growth. Results indicate that the treatment with **25** significantly stimulated the growth of both U937 (Fig. 3A) and Molt-3 (Fig. 3B) cells at concentrations ranging from 25 to 50 μM, while the concentration of 75 μM caused a dramatic decrease of total viable cell number. These results paralleled those obtained in the MTS assay enforcing their reliability, due to the comprehensible association between the effects of **25** on the conversion of MTS into the formazan product, accomplished by dehydrogenase enzymes in metabolically active cells and the actual quantity of viable cells.

We then investigated whether cell toxicity exerted by **25** could be attributable to its capability of inducing apoptotic death. For detection of apoptosis, U937 and Molt-3 cells were treated either with the vehicle alone or with **25** at concentrations ranging from 10 to 75 μM. Moreover the interest in evaluating the influence that different significant moieties of **25** could exert on its cytotoxic potential prompted us to treat U937 cells also with compounds **8**, **16**, and 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (PAGP). 1,3-Benzenedithiol (**8**) corresponds to the aromatic central ring, 3,3′-(*m*-phenylenedisulfinyl)dipropionitriles **16** mimic the rigid core with flexible arms, both constituting with the sugar moiety the molecule of **25** in its whole. After 24 h incubation, apoptosis was evaluated by microscopy analysis followed by staining with acridine orange. Except at the lower concentration of 10 μM, compound **25** was found to induce a significantly higher level of apoptosis, in a dose-response fashion, in comparison with the vehicle alone in U937 cells as well as in Molt-3 cells (Fig. 4A and B). Actually, the latter cells appeared to be much more sensitive than the former ones to apoptosis induction at the highest concentration of **25** assayed. No significant difference was observed between apoptosis values detected in samples treated with **8**, **16** or PAGP, all of which representing the significant features of the **25** skeleton, and vehicle treated samples (Fig. 4A). These results suggest that apoptosis is the main form of cell death associated with the cytotoxic potential of compound **25** and that the specific induction of apoptosis is a consequence of the interactions of the whole molecular structure of the thiglucodisaccharide under study with the cells rather than the effect of one of the significant moieties that constitute compound **25**.

4. Conclusion

In conclusion, we have described an easy synthetic approach to a new class of thioalkane- and thioarene-spaced bis-β-D-glucopyr-

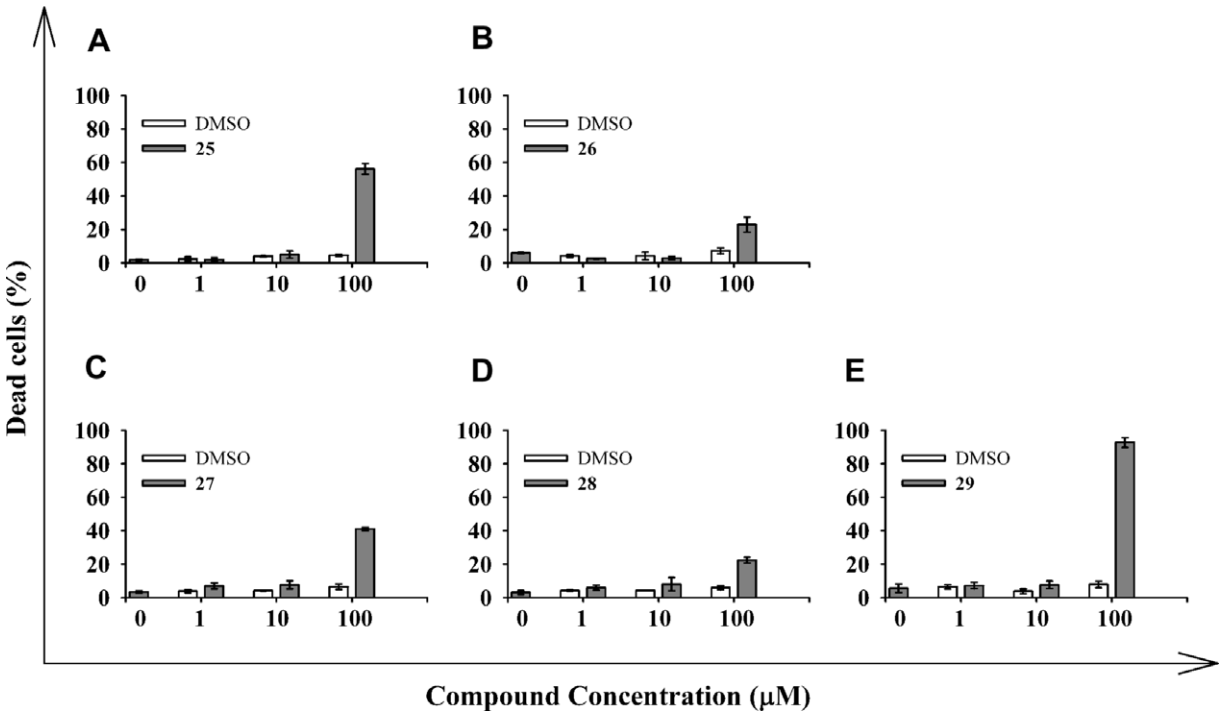


Figure 1. Effects of sugars **25-29** on cell death in U937 cells. U937 cells were treated with control vehicle (DMSO) or with increasing concentrations of the different sugars. (A) compound **25**. (B) compound **26**. (C) compound **27**. (D) compound **28**. (E) compound **29**. The percentage of dead cells was evaluated after 24 h incubation and determined by the trypan blue exclusion test. Each data point represents the mean and standard deviation from three different determinations. Comparison of the means between treated samples and corresponding controls, by Student's t-test for independent samples: 100 μM, $p < 0.001$ for all compounds; all other comparisons, NS.

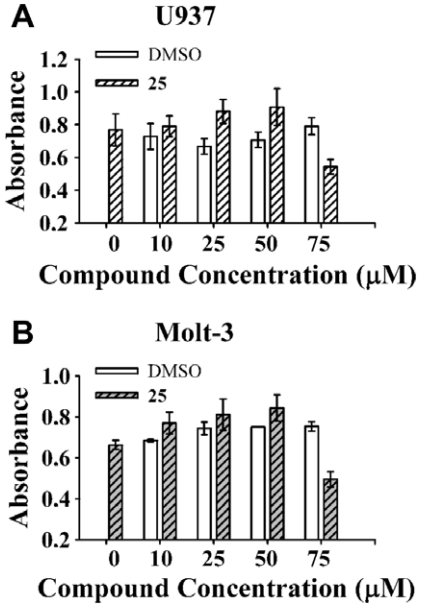


Figure 2. Effect of sugar **25** on metabolic activity. (A) U937 cells were grown in 96-well microtiter plates and exposed to different concentrations of control vehicle (DMSO) or compound **25** for 24 h, before cell metabolic activity was quantified by the MTS assay. Results are expressed as absorbance values measured at 490 nm. Each data point represents the mean and standard deviation from three different determinations. Comparison of the means between treated samples and corresponding controls, by Student's t-test for independent samples: 10 μM, NS; 25 μM, $p = 0.013$; 50 μM, $p = 0.047$; 75 μM, $p = 0.003$. (B) Molt-3 cells were processed as described in (A) for assaying the effect of **25** on metabolic activity. Each data point represents the mean and standard deviation from three different determinations. Comparisons of the means between treated samples and corresponding controls, by Student's t-test for independent samples: 75 μM, $p = 0.001$; all other comparisons, NS.

anosides. From a chemical point of view the key-step of the process offers wide chances of modulating the synthesis due to the possibility of changing both the sugar nature of the unsaturated acceptors and the sulfenic acid skeleton. The obtained data on the biological activity of this new class of compounds indicate that their effects greatly vary according to their molecular structure. While most of the compounds showed very low levels of toxicity even at high concentrations, sulfurated bis-β-D-glucopyranosides with a central aromatic ring endowed with a specific cytotoxic potential. In particular, a more detailed investigation performed on compound **25,** demonstrated that it was a good inductor of cell death by apoptosis. Moreover, the unexpected observation that in the range of 10–50 μM compound **25** stimulated the metabolic activity and the cell growth, suggests that the induction of cell death might underline an activation-induced cell death.[11] The obtained results open new perspectives in future investigations on the synthesis of new compounds of this family and on the comprehension of their biological activities.

5. Experimental

5.1. Chemistry

5.1.1. Materials and methods

Solvents were purified according to standard procedures. All reactions were monitored by TLC on commercially available precoated plates (Aldrich silica gel 60 F 254) and the products were visualized with vanillin [1 g of 4-hydroxy-3-methoxybenzaldehyde dissolved in MeOH (60 mL) and conc. H_2SO_4 (0.6 mL)]. Silica gel used for column chromatography was Aldrich 60, 230–400 mesh ASTM, 0,040–0,063 mm. Melting points were recorded on a microscopic apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 spectrometer at 300 and 75 MHz respectively in $CDCl_3$ solutions. *J* values are given in Hz;

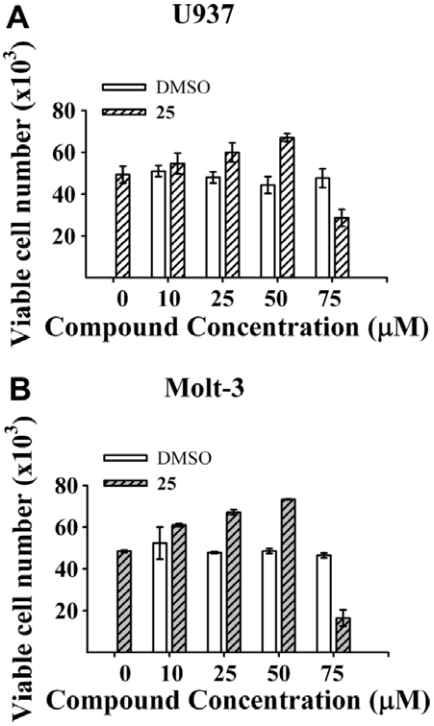


Figure 3. Effect of sugar **25** on cell growth. (A) U937 cells were grown in 96-well microtiter plates and exposed to different concentrations of control vehicle (DMSO) or compound **25** for 24 h, before cell growth was assayed by evaluating the total number of viable cells per well using the trypan blue exclusion test. Each data point represents the mean and standard deviation from three different determinations. Comparisons of the means between treated samples and corresponding controls, by Student's t-test for independent samples: 10 μM, NS; 25 μM, $p = 0.026$; 50 μM, $p = 0.004$; 75 μM, $p = 0.006$. (B) Molt-3 cells were processed as described in (A) for assaying the effect of **25** on cell growth. Each data point represents the mean and standard deviation from three different determinations. Comparisons of the means between treated samples and corresponding controls, by Student's t-test for independent samples: 10 μM, NS; 25 μM, $p < 0.001$; 50 μM, $p = 0.001$; 75 μM, $p = 0.003$.

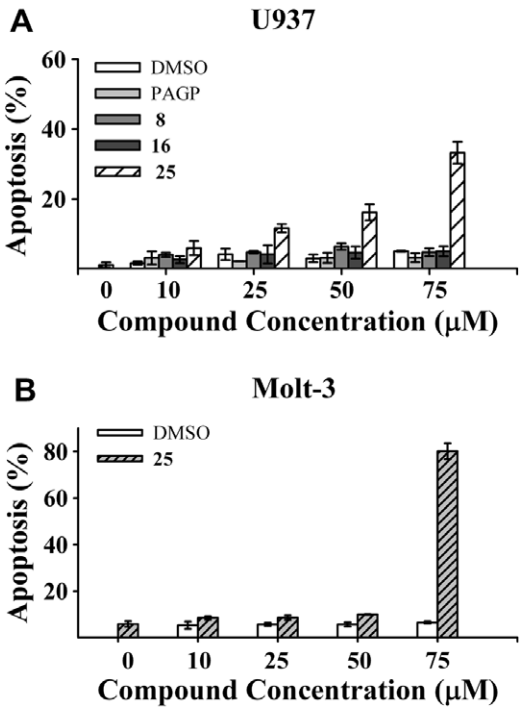


Figure 4. Effect of compound **25** on apoptosis. (A) U937 cells were exposed for 24 h to different concentrations of control vehicle (DMSO), 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (PAGP), compound **8**, compound **16** and compound **25**. Apoptosis was evaluated by fluorescence microscopy after staining of the cells with acridine orange. Percentage apoptosis = number of apoptotic cells / total cells counted × 100%. Each data point represents the mean and standard deviation from three different determinations. Multiple comparisons, by Tukey's honestly significant difference (HSD) test: 10 μM, NS among all groups; all other concentrations, NS among groups except for **25** versus all corresponding groups, $p < 0.001$. (B) Molt-3 cells were exposed for 24 h to different concentrations of control vehicle (DMSO) and compound **25**. Apoptosis was evaluated as described in (A). Each data point represents the mean and standard deviation from three different determinations. Comparisons of the means between treated samples and corresponding controls, by Student's t-test for independent samples: 10 μM, NS; 25 μM, $p = 0.018$; 50 μM, $p = 0.013$; 75 μM, $p = < 0.001$.

the attributions are supported by Attached Proton Test (APT). Proton and carbon nuclei, marked with (′) pertain to the monosaccharide residues when the sugar function is stated as substituent in the compound nomenclature. IR spectra were recorded in $CHCl_3$ solution on a Perkin-Elmer FT-IR Spectrum BX.

5.1.2. General procedure A for the synthesis of 2-[(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)sulfinyl]-1-alkenynes 6 and 7

A solution of sulfoxides **2**[9] (1,16 g, 2,00 mmol) and commercial ethynyl acceptor **4** or **5** (11.00 mmol) in DCM (3 mL) was maintained at reflux. When the reaction appeared complete by TLC (disappearance of starting sulfoxides required about one night), the solvent was removed under reduced pressure and the reaction crude was purified by flash column chromatography on silica gel (petrol/EtOAc 8:2).

5.1.2.1. 2-[(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)sulfinyl]-1-hepten-6-ynes 6. Epimeric mixture **6** was prepared from sulfoxides **2** and 1,6-heptadiyne (**4**), following procedure A. A subsequent column chromatography on silica gel (petrol/EtOAc 5:5) allowed the separation of the two sulfinyl sulfur epimers **6**,[10] as colorless oils (24% total yield). The more mobile **(S_S)-6** was obtained in 2:1 ratio with respect to the less mobile **(R_S)-6**. ^{1}H NMR of **(S_S)-6**: δ 5.85 (d, $J_{1A,1B}$ 0.9, H_A-1), 5.73 (d, H_B-1), 5.34 (dd, $J_{1',2'}$ 9.1, $J_{2',3'}$ 8.8, H-2′), 5.28 (dd, $J_{3',4'}$ 9.2, H-3′), 5.09 (dd, $J_{4',5'}$ 9.8, H-4′), 4.45 (d, H-1′), 4.26 (AB dd, $J_{5',6'A}$ 4.6, $J_{6'A,6'B}$ 12.6, H_A-6′), 4.17 (AB dd, $J_{5',6'B}$ 2.4, H_B-6′), 3.77 (ddd, H-5′), 2.6–1.8 (m, H_2-3,4,5, H-7), 2.09, 2.03, 2.02 and 2.01 [4 s, 4 × C(O)Me]. Elemental analysis calcd (%) for $C_{21}H_{28}O_{10}S$ (472.50): C 53.38, H 5.97; found: C 53.29, H 5.84.^{1}H NMR of **(R_S)-6**: δ 5.90 (d, $J_{1A,1B}$ 1.2, H_A-1), 5.78 (d, H_B-1), 5.4–5.0 (m, H-2′,3′,4′), 4.35 (d, $J_{1',2'}$ 9.7, H-1′), 4.3–4.1 (split AB system, H_2-6′), 3.8–3.7 (m, H-5′), 2.6–1.8 (m, H_2-3,4,5, H-7), 2.10, 2.09, 2.04 and 2.01 [4 s, 4 × C(O)Me]. Elemental analysis calcd (%) for $C_{21}H_{18}O_{10}S$ (472.50): C 53.38, H 5.97; found: C 53.31, H 5.80.

5.1.2.2. 2-[(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)sulfinyl]-1-octen-7-ynes 7. Epimeric mixture **7** was prepared from sulfoxides **2** and 1,7-octadiyne (**5**), following procedure A. A subsequent column chromatography on silica gel (petrol/EtOAc 2:8) allowed the separation of the two sulfinyl sulfur epimers **7**,[10] as colorless oils (25% total yield). The more mobile **(S_S)-7** was obtained in 2:1 ratio with respect to the less mobile **(R_S)-7**. ^{1}H NMR of **(S_S)-7**: δ 5.83 (d, $J_{1A,1B}$ 0.9, H_A-1), 5.72 (d, H_B-1), 5.33 (dd, $J_{1',2'}$ 9.3, $J_{2',3'}$ 9.1, H-2′), 5.28 (dd, $J_{3',4'}$ 9.4, H-3′), 5.08 (dd, $J_{4',5'}$ 9.8, H-4′), 4.44 (d, H-1′), 4.26 (AB dd, $J_{5',6'A}$ 4.7, $J_{6'A,6'B}$ 12.6, H_A-6′), 4.17 (AB dd, $J_{5',6'B}$ 2.4, H_B-6′), 3.76 (ddd, H-5′), 2.5–1.5 (m, H_2-3,4,5,6, H-8), 2.09, 2.03, 2.01 and 2.00 [4 s, 4 × C(O)Me]. Elemental analysis calcd (%) for $C_{22}H_{30}O_{10}S$ (486.53): C 54.31, H 6.22; found: C 54.26, H 6.12. ^{1}H NMR of **(R_S)-7**: δ 5.87 (broad s, H_A-1), 5.77 (broad s, H_B-1), 5.40 (dd, $J_{1',2'}$ 9.3, $J_{2',3'}$ 9.2, H-2′), 5.33 (dd, $J_{3',4'}$ 9.4, H-3′),

5.10 (dd, $J_{4',5'}$ 9.5, H-4′), 4.31 (d, H-1′), 4.3–4.1 (split AB system, H_2-6′), 3.8–3.7 (m, H-5′), 2.6–1.6 (m, H_2-3,4,5, H-7), 2.08, 2.07, 2.05 and 2.03 [4 s, 4 × C(O)Me]. Elemental analysis calcd (%) for $C_{22}H_{30}O_{10}S$ (486.53): C 54.31, H 6.22; found: C 54.15, H 6.19. ^{13}C NMR of **(S_S)-7**: 170.4, 170.2, 169.2 and 169.1 [4 × *C*(O)Me], 149.9 (C-2), 118.8 (C-1), 91.3 (C-1′), 83.8 (C-7), 76.6, 73.7, 67.4 and 67.1 (C-2′,3′,4′,5′), 68.9 (C-8), 61.5 (C-6′), 27.7, 27.6 and 26.8 (C-3,4,5), 20.71, 20.68, 20.54 and 20.51 [4 × C(O)*Me*], 18.1 (C-6). ^{13}C NMR of **(R_S)-7**: 170.43, 170.40, 169.1 and 168.8 [4 × *C*(O)Me], 148.5 (C-2), 118.6 (C-1), 88.3 (C-1′), 83.7 (C-7), 76.8, 73.8, 67.7 and 67.3 (C-2′,3′,4′,5′), 68.9 (C-8), 61.9 (C-6′), 28.0, 27.6 and 26.7 (C-3,4,5), 20.7, 20.60, 20.57 and 20.5 [4 × C(O)*Me*], 18.0 (C-6).

5.1.3. General procedure B for the synthesis of 3,3′-dithiodipropionitriles 12, 14, and 15

To a stirred solution of the bis-thiol (2.29 mmol) in anhyd THF (6 mL) at −78 °C, in an inert atmosphere, Triton B (40 wt. % solution in MeOH, 0.15 mL, 0.33 mmol) and, after 10 min, acrylonitrile (0.30 mL, 4.58 mmol) were added. The mixture was allowed to reach rt spontaneously, and when the reaction appeared complete by TLC (petrol/EtOAc 5:5), it was quenched by water addition. The crude product was extracted twice with Et_2O (10 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. After filtration of the inorganic solid, the solvent was removed under reduced pressure.

5.1.3.1. 3,3′-(m-Phenylenedithio)dipropionitrile (12). Commercial dithiol **8** was subjected to general procedure B. The reaction crude was purified by flash column chromatography on silica gel (petrol/EtOAc 9:1). Compound **12** was isolated as a pale yellow oil (0.51 g, 2.05 mmol, 90% yield). ^1H NMR: δ 7.5–7.3 (m, ArH), 3.16 (t, $J_{2,3}$ 7.2, 2 × H_2-3), 2.64 (t, 2 × H_2-2). Elemental analysis calcd (%) for $C_{12}H_{12}N_2S_2$ (248.36): C 58.03, H 4.87; found: C 57.89, H 4.72.

5.1.3.2. 3,3′-(Propane-1,3-diyldithio)dipropionitrile (14). Commercial dithiol **10** was subjected to general procedure B. Compound **14** was obtained, as a yellow oil (0.48 g, 2.24 mmol), in quantitative yield, not needing of further purification. ^1H NMR: δ 2.8–2.6 (m, 2 × $CH_2SCH_2CH_2CN$), 1.91 (quintuplet, J_{vic} 7,0, $CH_2CH_2CH_2$). Elemental analysis calcd (%) for $C_9H_{14}N_2S_2$ (214.35): C 54.31, H 6.22; found: C 54.26, H 6.12.

5.1.3.3. 3,3′-(Pentane-1,5-diyldithio)dipropionitrile (15). Commercial dithiol **11** was subjected to general procedure B. Compound **15** was obtained, as a yellow oil (0.49 g, 2.03 mmol), in 91% yield, not needing of further purification. ^1H NMR: δ 2.8–2.6 (m, 2 × $CH_2SCH_2CH_2CN$), 1.7–1.5 (m, $CH_2CH_2CH_2CH_2CH_2$). ^{13}C NMR: 118.3 (2 × C-1), 32.0 [$SCH_2(CH_2)_3CH_2S$], 28.9, 27.7 and 27.6 [2 × C-3 e $CH_2(CH_2)_3CH_2$], 18.9 (2 × C-2). Elemental analysis calcd (%) for $C_{11}H_{18}N_2S_2$ (242.40): C 54.50, H 7.48; found: C 54.32, H 7.21.

5.1.4. Tetraethyl [1,1′-(1,1′-biphenyl-4,4′-diyldithio)]di-[(1-methyl)ethyl]propanedioate (13)

To a stirred solution of the commercial thiol **9** (0.5 g, 2.29 mmol) in anhyd THF (10 mL) at −78 °C, in an inert atmosphere, Triton B (40 wt. % solution in MeOH, 0.16 mL, 0.35 mmol)) and, after 10 min, diethyl isopropylidenemalonate (1.79 mL, 9.13 mmol) were added. The mixture was allowed to reach rt spontaneously, and when the reaction appeared complete by TLC (petrol/EtOAc 5:5; disappearance of starting sulfoxides required about one night), the solvent was removed under reduced pressure. The reaction crude was purified by column chromatography (petrol/EtOAc 9:1). Compound **13** was obtained as a yellow oil (0.64 g, 1.03 mmol) in 45% yield. ^1H NMR: δ 7.70 (half A_2B_2 system, J_{ortho} 8.3, ArH-2,2′,6,6′), 7.60 (half A_2B_2 system, ArH-3,3′,5,5′), 4.25 (q, J_{vic} 7.2, 4 × OCH_2), 3.59 (s, 2 × H-2), 1.54 (s, 2 × CMe_2), 1.31 (t, 4 × CH_2Me). Elemental analysis calcd (%) for $C_{32}H_{42}O_8S_2$ (618.80): C 62.11, H 6.84; found: C 61.96, H 6.82.

5.1.5. General procedure C for the synthesis of disulfoxides 16 and 17, and disulfone 29

m-CPBA (80%) was dissolved in DCM (10 mL/*m*-CPBA mmol) and added dropwise to a solution of the disulfide or disulfoxide in the same volume of DCM at −78 °C (1 mol of *m*-CPBA for every molar site to be oxidized in the substrate). When the reaction appeared complete by TLC (EtOAc/petrol 8:2), a 10% water solution of $Na_2S_2O_3$ was added. Almost all experiments performed were concluded just after finishing the addition of the oxidant. The separated organic layers was washed twice with a saturated solution of $NaHCO_3$ and then twice with brine. Evaporation of the solvent gave the expected sulfoxide or sulfone.

5.1.5.1. 3,3′-(m-Phenylenedisulfinyl)dipropionitriles 16. Disulfide **12** was oxidized following the general procedure C. Disulfoxides **16** (*meso*/racemate 1:1) were obtained as a yellow oil (0.53 g, 1.89 mmol, 95% yield) not needing any purification before its involvement in the next reaction steps. ^1H NMR of 1:1 *meso*/racemate mixture: δ 8.0–7.8 (m, ArH), 3.4–2.6 (m, 2 × H_2-2,3). Elemental analysis calcd (%) for $C_{12}H_{12}N_2O_2S_2$ (280.53): C 51.41, H 4.31; found: C 51.37, H 4.25.

5.1.5.2. Tetraethyl 1,1′-[1,1′-biphenyl-4,4′-diyldi(sulfinyl)]di-[(1-methyl)ethyl]propanedioates 17. Disulfide **13** was oxidized following the general procedure C. Disulfoxides **17** (*meso*/racemate 1:1) were obtained in a quantitative yield as an oil not needing any purification before its prompt involvement in the next reaction step. ^1H NMR of diastereomeric mixture: δ 7.77 (m, ArH), 4.3–4.2 (m, 4 × OCH_2), 3.78 (s, 2 × H-2), 1.4–1.3 (m, 2 × CMe_2 e 4 × CH_2Me).

5.1.5.3. 2,2′-(m-Phenylendisulfonyl)di-2,2′-propenyl bis-β-D-glucopyranoside bis-2,3,4,6-tetraacetate (29). Bis-sulfoxides **25** were oxidized following the general procedure C. Bis-sulfone **29** was purified by flash column chromatography (petrol/EtOAc 6:4 up to petrol/EtOAc 4:6) and obtained as a white solid (m.p. 65–70 °C, 0.10 g, 0.10 mmol, 77% yield).^1H NMR: δ 8.36 (t, J_{meta} 1.7, H-2″), 8.13 (dd, J_{ortho} 7.7, H-4″,6″), 7.79 (t, H-5″), 6.53 (d, J_{gem} 0.4, 2 × H_A-3′), 6.16 (d, 2 × H_B-3′), 5.19 (dd, $J_{2,3}$ 9.2, $J_{3,4}$ 9.5, 2 × H-3), 5.06 (dd, $J_{4,5}$ 9.9, 2 × H-4), 4.93 (dd, $J_{1,2}$ 8.2, 2 × H-2), 4.53 (d, 2 × H-1), 4.47 (d AB, $J_{1'A,1'B}$ 14.1, 2 × H_A-1′), 4.32 (d AB, 2 × H_B-1′), 4.3–4.1 (m, 2 × H_2-6), 3.69 (ddd, $J_{5,6A}$ 4.7, $J_{5,6B}$ 2.5, 2 × H-5), 2.09, 2.02 and 2.00 [3 s, 8 × C(O)*Me*]. ^{13}C NMR: 170.6, 170.1, 169.4 and 169.3 [8 × *C*(O)Me], 145.6 (C-1″,3″), 141.0 (2 × C-2′), 133.1 (C-4″,6″), 130.7 (C-5″), 127.9 (2 × C-3′), 127.7 (C-2″), 100.0 (2 × C-1), 72.5, 72.0, 70.8 and 68.0 (2 × C-2,3,4,5), 65.1 (2 × C-1′), 61.6 (2 × C-6), 20.7, 20.6 and 20.5 [8 × C(O)*Me*]. IR: ν_{max} 1756 (CO) cm^{-1}. Elemental analysis calcd (%) for $C_0H_{50}O_{24}S_2$ (978.94): C 49.08, H 5.15; found: C 48.97, H 5.05.

5.1.6. General procedure D for the synthesis of disulfoxides 18 and 19

m-CPBA carefully dried over P_2O_5 (90%, 1.17 g, 6.10 mmol) was dissolved in DCM (40 mL) and slowly added to a solution of an equimolar amount of the disulfide (3.05 mmol) in DCM (20 mL) at −78 °C. The reaction mixture was allowed to reach room temperature and stirred for 2 h. Anhydrous KF (1.5 g) was added and the mixture stirred overnight. After filtration, the solvent was evaporated under reduced pressure.

5.1.6.1. 3,3′-(Propane-1,3-diyldisulfinyl)dipropionitriles 18. Disulfide **14** was oxidized following the general procedure D. Disulfoxides **18** (*meso*/racemate 1:1) were obtained as a rose-colored

solid (0.45 g, 1.83 mmol, 60% yield) not very soluble in $CHCl_3$. ^1H NMR of 1:1 *meso*/racemate mixture: δ *3.1–2.9* (m, 2 × $CH_2SCH_2CH_2CN$), 2.5–2.4 (m, $CH_2CH_2CH_2$). Elemental analysis calcd (%) for $C_9H_{14}N_2O_2S_2$ (246.35): C 43.88, H 5.73; found: C 43.62, H 5.71.

5.1.6.2. 3,3′-(Pentane-1,5-diyldisulfinyl)dipropionitriles 19. Disulfide **15** was oxidized following the general procedure D. Disulfoxides **19** (*meso*/racemate 1:1) were obtained as a white solid (0.81 g, 2.95 mmol, 97% yield) not very soluble in $CHCl_3$. ^1H NMR of 1:1 *meso*/racemate mixture: δ 3.1–2.7 (m, 2 × $CH_2SCH_2CH_2CN$), 2.0–1.7 (m, $CH_2CH_2CH_2CH_2CH_2$). ^{13}C NMR of 1:1 *meso*/racemate mixture: 117.4 (2 × C-1), 51.9 [$SCH_2(CH_2)_3CH_2S$], 46.5 (2 × C-3), 27.6 [$CH_2CH_2CH_2CH_2CH_2$], 22.3 [$CH_2CH_2CH_2CH_2CH_2$], 11.0 (2 × C-2). Elemental analysis calcd (%) for $C_{11}H_{18}N_2O_2S_2$ (274.40): C 48.15, H 6.61; found: C 48.09, H 6.54.

5.1.7. 2,2′-(*m*-Phenylendisulfinyl)di-2,2′-propenyl bis-β-D-glucopyranoside bis-2,3,4,6-tetraacetates 25

A solution of bis-sulfoxides **16** (0.11 g, 0.39 mmol) and 2-propynyl β-D-glucopyranoside 2,3,4,6-tetraacetate (**24**) (0.61 g, 1.57 mmol) in 1,2-dichloroethane (4 mL) was maintained at reflux. When the reaction appeared complete by TLC (disappearance of starting sulfoxides required about one night), the solvent was removed under pressure and the reaction crude was purified by flash column chromatography on silica gel (petrol/EtOAc 6:4 up to EtOAc 100%). The mixture of the three sulfinyl diastereomers **25** was obtained as a white solid (m.p. 65–68 °C, 0.14 g, 0.15 mmol, 40% yield).^1H NMR of diastereomeric mixture: δ 7.9–7.3 (m, ArH), 6.3–5.6 (m, 2 × $\rangle CH_2$), 5.3–3.6 (m, GluH and 2 × $CH_2\rangle CCH_2$), 2.1–2.0 [m, 8 × C(O)Me]. Elemental analysis calcd (%) for $C_{40}H_{50}O_{22}S_2$ (946.94): C 50.73, H 5.32; found: C 50.66, H 5.21.

5.1.8. 2,2′-[1,1′-Biphenyl-4,4′-diyldi(sulfinyl)]di-2,2′-propenyl bis-β-D-glucopyranoside bis-2,3,4,6-tetraacetates 26

A solution of bis-sulfoxides **17** (0.62 g, 0.95 mmol) and the ethynyl acceptor **24** (1.48 g, 3.81 mmol) in DCM (6 mL) was maintained at reflux. When the reaction appeared complete by TLC (disappearance of starting sulfoxides required about one night), the solvent was removed under pressure and the reaction crude was purified by flash column chromatography on silica gel (petrol/EtOAc 6:4 up to petrol/EtOAc 2:8). The mixture of the three sulfinyl diastereomers **26** was obtained as a pale yellow oil (0.30 g, 0.28 mmol, 30% yield).^1H NMR of diastereomeric mixture: δ 7.8–7.6 (m, ArH), 6.2–5.9 (m, 2 × $\rangle CH_2$), 5.2–4.9 (m, 2 × H-2,3,4), 4.5–3.9 (m, 2 × H-1, 2 × H_2-1′,6), 3.7–3.6 (m, 2 × H-5), 2.1–1.0 [m, 8 × C(O)Me]. IR: n_{max} 1756 (CO), 1042 (SO) cm^{-1}. Elemental analysis calcd (%) for $C_{46}H_{54}O_{22}S_2$ (1023.03): C 54.01, H 5.32; found: C 53.96, H 5.28.

5.1.9. 2,2′-[Propane-1,3-diyldi(sulfinyl)]di-2,2′-propenyl bis-β-D-glucopyranoside bis-2,3,4,6-tetraacetates 27

A solution of bis-sulfoxides **18** (0.30 g, 1.24 mmol) and the ethynyl acceptor **24** (1.45 g, 3.73 mmol) in toluene (6 mL) was maintained at reflux. When the reaction appeared complete by TLC (disappearance of starting sulfoxides required about 4 h), the solvent was removed under pressure and the reaction crude was purified by flash column chromatography on silica gel (petrol/EtOAc 5:5 up to EtOAc/MeOH 9:1). The mixture of the three sulfinyl diastereomers **27** was obtained as a pale yellow oil (0.23 g, 0.25 mmol, 20%).^1H NMR of diastereomeric mixture: δ 6.0–5.9 (m, 2 × $\rangle CH_2$), 5.2–5.0 (m, 2 × H-2,3,4), 4.6–4.1 (m, 2 × H-1, H_2-6, $CH_2\rangle CCH_2$), 3.7 (m, 2 × H-5), 3.0–2.7 (m, 2 × CH_2S), 2.0 (m, $CH_2CH_2CH_2$), 2.1–2.0 [m, 8 × C(O)Me]. IR: n_{max} 1756 (CO), 1040 (SO) cm^{-1}. Elemental analysis calcd (%) for $C_{38}H_{54}O_{22}S_2$ (926.95): C 49.24, H 5.87; found: C 49.10, H 5.65.

5.1.10. 2,2′-[Pentane-1,5-diyldi(sulfinyl)]di-2,2′-propenyl bis-β-D-glucopyranoside bis-2,3,4,6-tetraacetates 28

A solution of bis-sulfoxides **19** (0.34 g, 1.24 mmol) and the ethynyl acceptor **24** (1.45 g, 3.73 mmol) in toluene (6 mL) was maintained at reflux. When the reaction appeared complete by TLC (disappearance of starting sulfoxides required about 4 h), the solvent was removed under pressure and the reaction crude was purified by flash column chromatography on silica gel (petrol/EtOAc 5:5 up to EtOAc/MeOH 9:1). The mixture of the three sulfinyl diastereomers **28** was obtained as a pale yellow oil (0.47 g, 0.50 mmol, 40% yield).^1H NMR of diastereomeric mixture: δ 6.0–5.9 (m, 2 × $\rangle CH_2$), 5.2–5.0 (m, 2 × H-2,3,4), 4.6–4.1 (m, 2 × H-1, H_2-6, $CH_2\rangle CCH_2$), 3.7 (m, 2 × H-5), 2.9–2.6 (m, 2 × CH_2S), 2.1–2.0 [m, 8 × C(O)Me], 1.9–1.5 (m, $CH_2(CH_2)_3CH_2$). IR: n_{max} 1756 (CO), 1056 (SO) cm^{-1}. Elemental analysis calcd (%) for $C_{39}H_{56}O_{22}S_2$ (940.97): C 49.78, H 6.00; found: C 49.56, H 5.92.

5.2. Biological tests

5.2.1. Cells

The biological tests were performed in U937 and Molt-3 cells. The U937 cell line was established from the pleural effusion of a patient with histiocytic lymphoma and has been characterized as a monocytic cell line. The Molt-3 cell line was established from the peripheral blood of a patient with acute lymphoblastic leukemia and has been characterized as a T-lymphocytic cell line. The utilized cell lines share characteristics suitable for this study, such as rapid growth in suspension and sensitivity to cytotoxic agents.

5.2.2. Evaluation of cytotoxicity and apoptosis

Cytotoxicity was evaluated by microscopy analysis, using the trypan blue exclusion test as a standard viability assay. Apoptosis was evaluated in the cells by their morphological analysis followed by staining with acridine orange, as previously described.[12] Over 600 cells, including those showing typical apoptotic characteristics, were analyzed using a fluorescence microscope. The identification of apoptotic cells was based on the presence of uniformly stained nuclei showing chromatin condensation and nuclear fragmentation.

5.2.3. Evaluation of metabolic activity and cell growth

The effects on the metabolic activity were examined by the MTS colorimetric method, using a commercial kit (MTS, Cell Titer 96 Aqueous One Solution, Promega). The assay was performed by seeding 2×10^4 U937 cells in 100 μL in the presence of different concentrations of the thioarene-spaced bis-β-D-glucopyranoside or the vehicle, in RPMI-1640 medium supplemented with 5% FBS, 2 mM L-glutamine and penicillin-streptomycin, for 24 h. Twenty microliters of "CellTiter 96 Aqueous One Solution Reagent" were added directly to the culture wells at the end of the culture and incubated for 4 h, after which, absorbance was read at 490 nm.

5.2.4. Statistical analysis

Data analysis was performed using the SPSS statistical software system (version 12.0 for Windows, Chicago, IL). Comparison of means were carried out using the Student's t-test for independent samples and the Tukey's honestly significance test (HSD), as a multiple comparison test, where appropriate.

Acknowledgement

The authors thank the *Università degli Studi di Messina* for financial support (*PRA*).

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