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Synthesis of Antimitotic Thioglycosides: In Vitro and in Vivo Evaluation of Their Anticancer Activity

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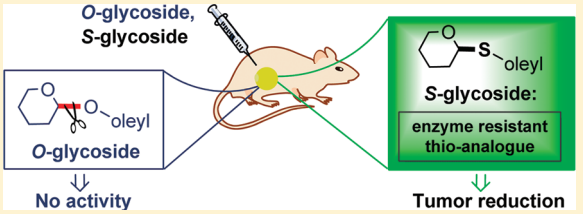
Supporting Information

ABSTRACT: The synthesis and biological activity of oleyl*N*-acetyl-α- and β-D-glucosaminides (**1** and **2**, respectively) and their thioglycosyl analogues (**3** and **4**, respectively) are reported. The compounds exhibited antimitotic activity on rat glioma (C6) and human lung carcinoma (A549) cell cultures in the micromolar range. Analysis of cell extracts using ultra performance liquid chromatography−mass spectrometry showed that the synthetic glycosides produce alterations in glycosphingolipid metabolism, with variable effect on the level of glucosylceramide depending on the configuration of the antimitotic used. In vivo experiments in nude mice bearing an implanted C6 glioma showed that the α-thioglycoside **3** reduced tumor volume, while the *O*-glycosyl derivative was inactive, highlighting the importance of using enzyme resistant glycosides.

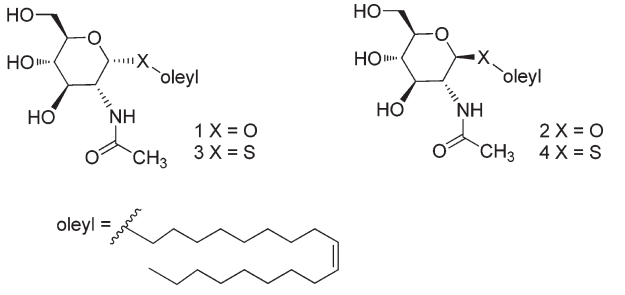


1. INTRODUCTION

We have previously reported the synthesis of a family of glycosides derived from *N*-acyl-glucosamine and their evaluation as inhibitors of glioma and melanoma cell proliferation.[1] The results indicated that the activity of the compounds increased with a long hydrocarbon chain at position C-1 of the glucosamine scaffold. Among the compounds tested, oleyl glycoside **1** was the most effective (Chart 1). A favorable effect of an oleyl moiety on the antitumor activity was also observed in a family of alkylated iminosugars.[2] These findings suggest that the conjugation of a sugar with an oleyl chain is beneficial for obtaining compounds with good antitumor activity. Preliminary studies on the mechanism of growth inhibition by **1** using proton magic angle spining nuclear magnetic resonance (^{1}H MAS NMR) suggested that the glycoside may be altering the metabolism of lipids.[3] Further studies of its effect on the sphingolipidome of the A549 human lung carcinoma cell line showed that **1** caused important changes in glycosphingolipid and ganglioside content.[4] These biomolecules have important regulatory roles in tumor formation and progression[5,6] and are involved in processes of cell death and cell proliferation.[7,8]

The convenient synthesis of **1**, together with its antimitotic activity on different cancer cell lines, makes this compound a potential anticancer drug. With the aim to gain further insight in this direction, recently, we performed preliminary in vivo experiments in nude mice bearing an implanted C6 glioma (unpublished work). Animals were treated with two doses of **1** (1 and 10 mg/kg per day) by intratumor administration. After 8 days of treatment, the glycoside neither slowed tumor growth nor caused any reduction of tumor volume as compared to the controls. Nevertheless, in the case of the treatment with the highest dose of **1**, we observed the formation of a small bag or cyst, attached to the surface of the tumor. The analysis of the intact cyst by ^{1}H MAS NMR spectroscopy showed that it was formed mainly by oleyl alcohol, probably derived from the cleavage of **1**. Because hydrolysis of **1** was not observed during experiments in cell culture,[4] the cleavage of **1** could be the result of an inflammatory process

Chart 1. Chemical Structures of Glycosides 1 and 2 and Thio-glycosides 3 and 4



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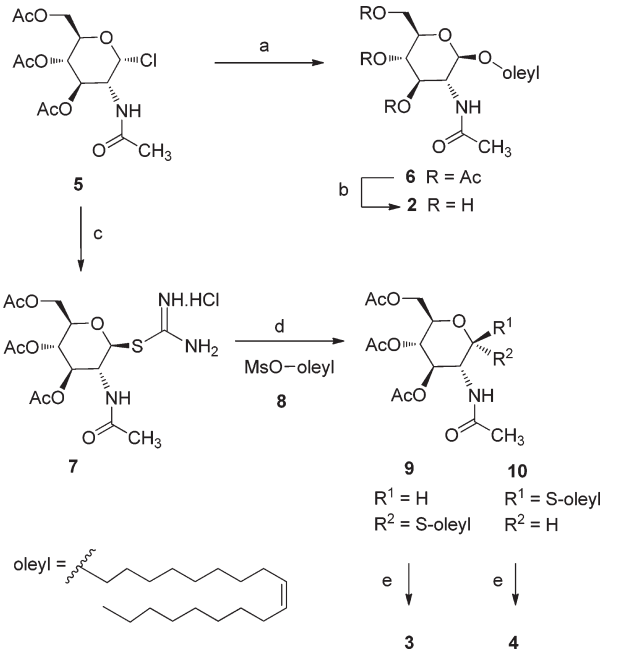
Scheme 1. Synthesis of Compounds 2−4[a]



[a] Reagents and conditions: (a) Oleyl alcohol, $SnCl_4$, CH_3CN, 55 °C, 24 h (48%). (b) 0.1 M NaOMe/MeOH, room temperature, 2 h (quantitative). (c) Thiourea, acetone, 56 °C, 30 min (73%). (d) Et_3N, DMF, 60 °C, 8 h (60%). (e) 0.1 M NaOMe/MeOH, room temperature, 1 h (73% for **3**, 77% for **4**).

taking place in vivo after tumor implantation or compound injection. Activated macrophages can generate lysosomal enzymes, including hexosaminidases with the capacity to cleave the glycosidic bond of *N*-acetyl-glucosaminides.[9,10]

Thioglycosides are known to be resistant to glycosidase-catalyzed hydrolysis.[11] In view of the results obtained in vivo, we decided to synthesize the corresponding thioanalogues of **1** and its β-configured glycoside **2** (compounds **3** and **4**, respectively, Chart 1). In the present work, we describe the synthesis of **2**−**4** and their inhibitory activity against C6 and A549 tumor cell lines. The effect of the change of the oxygen by a sulfur atom and the influence of the configuration at the anomeric carbon on the ability to alter glycosphingolipid levels in A549 cells have also been evaluated.

The stability of the thioanalogues to enzymatic hydrolysis has been tested with the β-thioglycoside **4** against a commercially available β-*N*-acetylhexosaminidase. Finally, we report the results of testing the in vivo activity of the α-glycosides **1** and its thioanalogue **3**.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Inhibitory Activity. The synthesis of **2** was carried out in two steps from α-D-glucosaminyl chloride peracetate **5** (Scheme 1). Stereoselective glycosylation of **5** with oleic alcohol using $SnCl_4$ as an acid promotor gave the β-glycopyranoside **6**, which was submitted to *O*-deacetylation to afford **2**. For the synthesis of thioglycosides **3** and **4**, we employed the approach involving the reaction of alkyl halides or mesylates with *S*-glycosyl isothiouronium salts.[12] Thus, isothiouronium derivative **7** was prepared by treatment of chloride **5** with thiourea. Only the β-anomer was obtained, indicating that the nucleophilic substitution at the anomeric position proceeded through an SN_2-like mechanism. Oleyl mesylate **8** was treated with the isothiouronium salt **7** to give a mixture of α- and β-thioglycosides **9** and **10** in a 1:1 ratio. This result is in contrast with the general observation that glycosyl thiolates are configurationally stable;[11] nevertheless, this isomerization allowed us to obtain both anomeric thioglycosides through the same synthetic route. Compounds **9** and **10** were separated by silica gel column chromatography and subsequently treated with a solution of sodium methoxide in methanol to give **3** and **4** (Scheme 1).

Table 1. ID_{50} Values (μM)[a] for Compounds 1−4 on A549 and C6 Tumor Cells

	1	2	3	4
A549	25 ± 4[4]	50 ± 4	35 ± 5	30 ± 4
C6	16 ± 4	38 ± 3	14 ± 1	26 ± 2

[a] Results represent the mean ± SEM of two experiments in triplicate. Cells were treated with serial dilutions of compounds (**1**−**4**) for 48 h. Cell proliferation was measured by the MTT assay.

Scheme 2. Schematic Representation of Glycosphingolipid Biosynthesis

Compounds **2**−**4** together with **1** as a reference were evaluated for their ability to inhibit A549 (human adenocarcinoma) and C6 (rat glioma) cell growth. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of the water-soluble MTT into an insoluble formazan. The results, summarized in Table 1, show that the new compounds **2**−**4** were as active as **1** against tumor cell lines with 50% inhibitory dose (ID_{50}) values in the same range than that of **1**. Therefore, the change of the anomeric oxygen by a sulfur atom or the configuration at the anomeric position have only a moderate influence on the inhibitory activity.

2.2. Effect on Glycosphingolipid Content. Glucosylceramide (GlcCer) and lactosylceramide (LacCer) are the precursors of glycosphingolipids, including gangliosides GM3 and GM2 (Scheme 2). Glycosphingolipids are involved in multiple cellular processes[5−8] and also in the formation of membrane lipid rafts. Alterations in the metabolism of glycosphingolipids may result in tumor growth inhibition.[13,14] To compare the effect of compounds **1**−**4** on the content of GlcCer, LacCer, and gangliosides GM3 and GM2 of A549 cells, **1**−**4** (15 μM) were incubated with the cells for 48 h, and the glycosphingolipids were then extracted and analyzed by ultra performance liquid chromatography−mass spectrometry (UPLC-MS), as described in the Experimental Section. The results are shown in Figure 1.

All compounds caused a similar effect on LacCer and ganglioside content, namely, a rise (40−60%) in LacCer and a marked decrease in the levels of gangliosides GM3 and GM2. Considering the biosynthetic pathway of gangliosides (Scheme 2), these results suggest that compounds **1**−**4** partially inhibited their formation at the step of GM3 (the first ganglioside in the

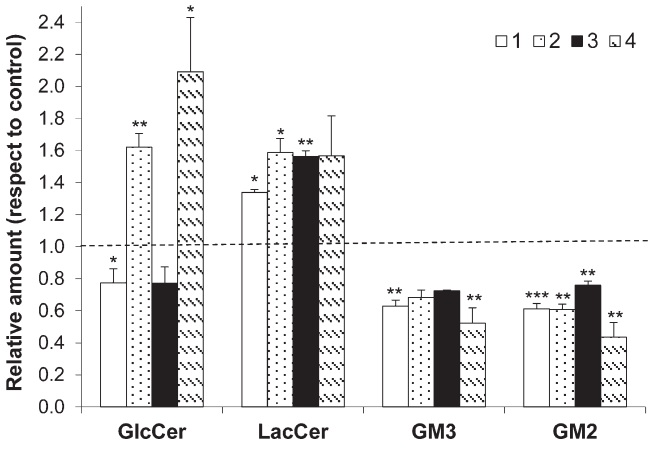


Figure 1. Effect of compounds **1**−**4** (15 μM) on glycosphingolipid content of A549 carcinoma cells. Glycosphingolipids: glucosilceramide (GlcCer), lactosylceramide (LacCer), and gangliosides GM3 and GM2. Data were normalized to the same number of cells (1×10^6 cells). The content in pmol per 1×10^6 cells for the control was 150 ± 2, 13 ± 1, 608 ± 65, and 705 ± 7 for GlcCer, LacCer, GM3, and GM2, respectively. The data are the means of two experiments performed by triplicate. The diagram includes the ANOVA results for each treatment with respect to control at a significance level of $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Table 2. Detected Compounds by UPLC-MS in A549 Cells after Treatment with Compounds 1−4 (15 μM)

compd	amount[a]	rt (min)[b]	exact mass[b] (error in ppm)
		1-treated cells	
1	2916 ± 417	3.19	472.3638 (−0.4)
Gly-**1**[c]	3.5 ± 0.5	3.01	634.4166 (1.3)
		2-treated cells	
2	952 ± 69	3.11	472.3638 (−1.9)
Gly-**2**[c]	320 ± 9	3.01	634.4166 (−1.4)
		3-treated cells	
3	3675 ± 409	3.34	488.3410 (1.0)
Gly-**3**[c]	ND[d]	ND[d]	ND[d]
		4-treated cells	
4	3333 ± 814	3.24	488.3410 (1.3)
Gly-**4**[c]	812 ± 178	3.13	650.3938 (1.2)

[a] Amount in pmol/1×10^6 cells. [b] As indicated by UPLC-MS. [c] Compounds Gly-**1**, Gly-**2**, Gly-**3**, and Gly-**4** have been putatively identified as disaccharides derived from glycosylation of **1**, **2**, **3**, and **4**, respectively (see the text). [d] ND, not detected.

biosynthetic route), leading to lower ganglioside levels and the accumulation of LacCer.

On the other hand, a different behavior between the glycosides with α- and β-anomeric configuration was observed when analyzing GlcCer content. While the α-compounds (**1** and **3**) reduced the GlcCer content, the β-compounds (**2** and **4**) caused a rise in the level of this metabolite (Figure 1). Differences between anomers were also observed when the extracts were analyzed for the presence of compounds **1**−**4** (Table 2). All of the compounds were detected in cell extracts, which indicated that they are efficiently taken up by cells. However, an unnatural metabolite not present in control samples was detected in much higher

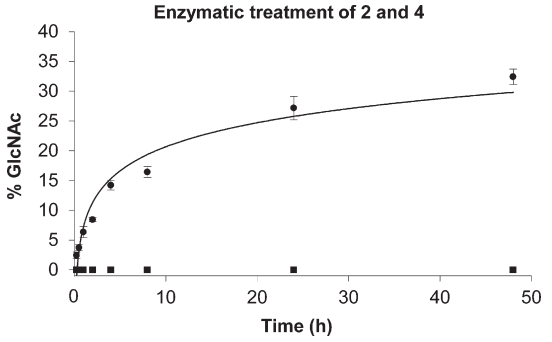


Figure 2. Enzymatic hydrolysis of compound **2** (circle) and **4** (square). All experiments were performed in triplicate. Product formation, this is GlcNAc formation, was calculated as % percentage and expressed as the mean ± SEM.

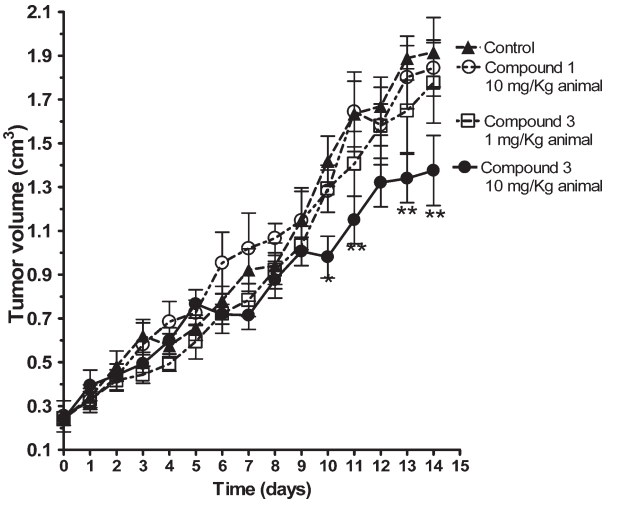


Figure 3. Evaluation of the antitumoral activity of compounds **1** and **3** in an orthotopical transplantation of the rat C6 glioma cells in the flank of nude mice. The results are expressed as the mean ± SEM of at least six animals per group. Intergroup differences for repeated measures were evaluated statistically using two-way analyses of variance (ANOVA) followed by posthoc Tukey test. Statistical significance of the treatments related to control was assigned as $^*p < 0.05$ and $^{**}p < 0.01$.

amounts in cells incubated with β-anomers **2** and **4** than with α-anomer **1** (not detected in **3**-treated cells, Table 2). On the basis of their accurate mass measurement and their relative retention times, these compounds have been putatively identified as oleyl disaccharides derived from the glycosylation of **1**, **2**, and **4** with a six-carbon glycosyl moiety ($C_6H_{10}O_5$) (Gly-**1**, Gly-**2**, and Gly-**4**, respectively, Table 2). The chemical resemblance between the β-configured compounds **2** and **4** and GlcCer, which is also a β-glycoside, makes us think that **2** and **4** have been galactosylated by the enzyme that transformed GlcCer into LacCer (LacCer synthase). If this were the case, the β-compounds would act as "decoy acceptors" of LacCer synthase and would cause an accumulation of GlcCer, as it is actually observed in the analysis of this metabolite (Figure 1).

2.3. Hexosaminidase Hydrolysis Resistance. The stability of the *O*- and *S*-glycosyl derivatives to glycosidases was assayed with the β-configured compounds, taking advantage that *N*-acetyl-β-D-glucosaminidase from Jack bean is commercially available. The

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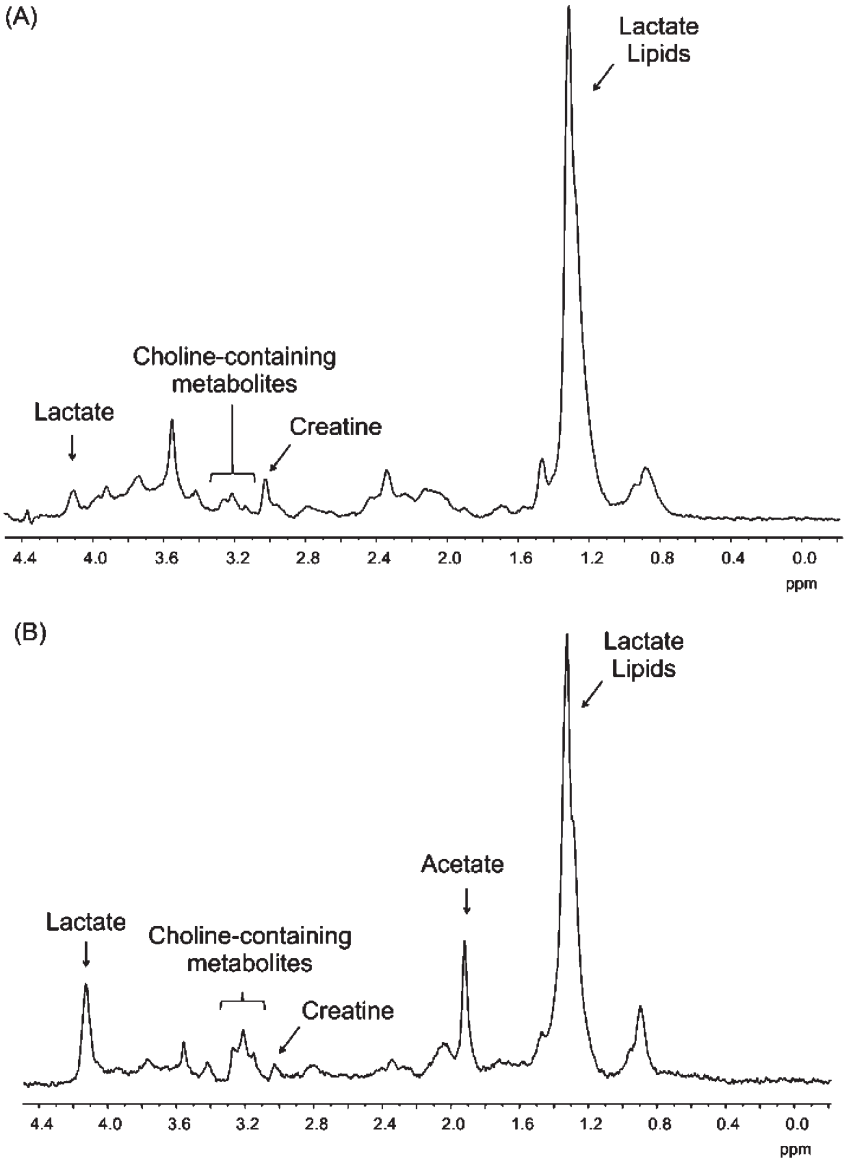

Figure 4. Representative ^{1}H MAS NMR spectra of C6 tumor tissue (T2 = 60 ms, ns = 256). (A) Tumor of control mice and (B) tumor of **3**-treated mice (10 mg/kg).

enzyme activity was determined using *p*-nitrophenyl β-D-glucopyranoside as a substrate, by measuring the liberation of *p*-nitrophenol. The assay was performed in the presence of 20% of isopropanol to dissolve the glycosides. This cosolvent did not alter severely the enzyme activity,[15] as evidenced by the control reaction. Moreover, no glycoside product derived from transglycosidation of isopropanol was observed. Compounds (**2** or **4**) were incubated in the presence of the enzyme at pH 5 and 37 °C for different time periods. After 48 h of incubation, high-performance liquid chromatography (HPLC) analysis showed that thioglycoside **4** remained unchanged, while 32% of **2** was hydrolyzed to *N*-acetyl-D-glucosamine (GlcNAc) (Figure 2). This result confirmed the higher resistance to enzymatic hydrolysis of thioglycosides as compared to *O*-glycosyl derivatives, which makes these compounds good candidates for in vivo assays.

2.4. In Vivo Effect on Tumor Growth. The activity of compound **1** and **3** on tumor growth was evaluated using an orthotopic model in female nude mice (Figure 3). Tumors treated with compound **3** with the higher dose (10 mg/kg animal) reduce significantly their size as compared to the controls. Conversely, there is no significant effect in the size of the tumors treated with compound **1** or compound **3** with the lower concentration (1 mg/kg animal).

Moreover, ^{1}H MAS NMR analysis of intact tumor tissues showed higher cell death in the samples from treated animals than in the controls (Figure 4). MAS NMR spectroscopy allows the simultaneous detection of many metabolites present in the sample of interest and requires minimal or no sample preparation.[16−18] Thus, in the case of mice treated with the higher dose (10 mg/kg) of thioglycoside **3**, the spectra showed a low intensity in the peak centered at 3.0 ppm, which was assigned to creatine, as compared to the control (Figure 4). Creatine is commonly used as an internal concentration reference for in vivo ^{1}H NMR because its concentration correlates with the number of metabolically active cells and is used as a measure of viable cell number.[19] An increase of choline-containing metabolites observed in the spectra of treated mice may also be indicative of cell death processes.[20] On

the other hand, the tumor samples of animals treated with **3** showed pronounced peaks corresponding to lactate and acetate, metabolites that have been related to the anaerobic energy metabolism of cancer cells.[21] All together, the results from ^{1}H MAS NMR analysis suggest that a higher decrease in tumor volume of treated mice could be obtained in treated mice if the animals were kept alive longer, allowing macrophages to engulf dead tissue.

3. CONCLUSION

On the basis of our previous studies on the antitumor activity of glycoside **1**, in the present work, we report on the synthesis and biological activity of its β-anomer **2** and thioanalogues **3** and **4**. The new compounds also showed antiproliferative activity in cancer cells and caused alterations in the levels of glycosphingolipids of A549 cells. The anomeric configuration of the glycosides was important for the effect on glucosylceramide content, which was increased after treatment with the β-anomers, while with the α-anomers a decrease in GlcCer content was observed as compared to control. The different effect could be related to a glycosylation of the β-anomers by glycosyltransferases involved in GlcCer metabolism. In vivo experiments with thioglycoside **3** led to a reduction of tumor volume, in contrast to the *O*-glycosyl derivative **1** that did not cause any reduction of tumor volume. The results show that α-thioglycoside **3** is an enzymatically stable product that maintains antitumor activity, making it a good candidate for further studies in cancer therapy.

4. EXPERIMENTAL SECTION

4.1. Chemistry. General Synthetic Methods. All chemicals were of reagent grade or higher and were purchased from commercial suppliers or purified by standard techniques. Compounds **5** and **7** were prepared following described procedures.[22,23] Thin-layer chromatography (TLC) was performed on aluminum sheets 60 F254 Merck silica gel, and compounds were visualized by irradiation with UV light and/or by treatment with a solution of Ce_2MoO_4 or 5% H_2SO_4 in EtOH, followed by heating. Flash column chromatography was performed using thick-walled columns, employing silica gel (Merck 60: 0.040−0.063 mm). The eluent used is indicated, and solvent ratios refer to volume. Melting points are not corrected and were measured with a Reicher Jung Thermovar micromelting apparatus. Optical rotations were recorded on a Perkin-Elmer 241 Polarimeter (λ = 589 nm, 1 dm cell). ^{1}H NMR spectra were registered at 400 or 300 MHz, and ^{13}C NMR spectra were obtained at 100 MHz on a Varian INOVA spectrometers, using $CDCl_3$, CD_3OD, or D_2O as the solvent at room temperature. Chemical shift values are reported in parts per million (δ). Coupling constant values (J) are reported in hertz (Hz), and spin multiplicities are indicated by the following symbols: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). High-resolution mass spectra (HRMS) were recorded on an Agilent 6520 Accurate Mass Q-TOF spectrometer with an ESI source. The purity of all compounds was ≥95% as determined by elemental analyses using a Heraus CHN-O analyzer.

*4.1.1. Oleyl 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (**6**).* A solution of **5**[22] (500 mg, 1.37 mmol) and oleic alcohol (85%) (1.53 mL, 4.11 mmol) in anhydrous CH_3CN (55 mL) containing 4 Å molecular sieves was stirred at room temperature for 10 min. Then, $SnCl_4$ (0.3 mL, 2.4 mmol) was added, and the reaction mixture was stirred at 55 °C for 24 h. After this time, the mixture was heated at 80 °C (under reflux) and stirred for 1 h. The reaction mixture was cooled at room temperature, treated with Et_3N (0.4 mL), filtered under Celite, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane−EtOAc, 1:1) to give **6** (396 mg, 48%). $[\alpha]_D$ −9.6° (*c* 1.1, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 5.5−5.3 (m, 2H), 5.22 (dd, 1H, J = 10.5, 9.3 Hz), 4.98 (dd, 1H, J = 10.1, 9.5 Hz), 4.63 (d, 1H, J = 8.5 Hz), 4.28 (dd, 1H, J = 12.4, 4.8 Hz), 4.11 (dd, 1H, J = 12.3, 2.4 Hz), 3.9−3.7 (m, 2H), 3.6−3.4 (m, 2H), 2.1−2.0 (m, 13H), 1.90 (s, 3-H), 1.6−1.5 (m, 2H), 1.4−1.2 (m, 22 H), 0.90 (t, 3H, J = 6.9 Hz) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ 173.2, 172.3, 171.9, 171.3, 130.9, 130.8, 102.1, 74.2, 72.8, 70.9, 70.2, 63.3, 55.5, 33.6, 33.1, 30.9, 30.8, 30.8, 30.8, 30.7, 30.6, 30.5, 30.3, 30.2, 28.2, 28.1, 27.1, 23.7, 22.8, 20.7, 20.6, 20.6, 14.5 ppm. HRMS (ESI) m/z calcd for $C_{32}H_{55}NO_9$, 597.3897; found, 598.3970 $(M + H)^+$.

*4.1.2. Oleyl 2-Acetamido-2-deoxy-β-D-glucopyraroside (**2**).* Compound **6** (800 mg, 1.30 mmol) was dissolved in MeOH (2 mL) and treated with a 0.1 M solution of NaOMe (10 mL). The reaction was stirred at room temperature for 2 h. After this time, the mixture was neutralized with Amberlite IR-120 (H^+ form), filtered off, and concentrated. The residue was purified by silica gel column chromatography (EtOAc−MeOH, 10:0 → 10:1) to give **2** (840 mg, quantitative) as a white solid; mp 155−160 °C; $[\alpha]_D$ −9.0° (*c* 0.5, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 5.4−5.3 (m, 2H), 4.38 (d, 1H, J = 8.4 Hz), 3.9−3.8 (m, 2H), 3.69 (dd, 1H, J = 11.9, 5.5 Hz), 3.62 (dd, 1H, J = 10.3, 8.4), 3.5−3.4 (m, 1H), 3.34 (d, 1H, J = 9.6 Hz), 3.25 (ddd, 1H, J = 9.6, 5.5, 2.4 Hz), 2.1−1.9 (m, 7H), 1.5−1.4 (m, 2H), 1.3−1.2 (m, 26H), 0.88 (t, 3H, J = 7.0 Hz) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ 173.4, 130.6, 130.5, 102.3, 79.1, 78.8, 78.5, 77.4, 75.7, 71.8, 70.5, 62.5, 57.1, 33,4, 32.8, 30.6, 30.6, 30.5, 30.5, 30.4, 30.40, 30.3, 30.3, 30.2, 30.9, 30.1, 30.1, 29.9, 28.0, 26.9, 23.5, 23.0, 14.4 ppm. HRMS (ESI) m/z calcd for $C_{26}H_{49}NO_6$, 471.3570; found, 472.3643 $(M + H)^+$, 494.3461 $(M + Na)^+$.

*4.1.3. Oleyl Mesylate (**8**).* Oleic alcohol (4 mL, 10.8 mmol) was dissolved in anhydrous CH_2Cl_2 (108 mL), and Et_3N (4.5 mL, 32.4 mmol) was added. Mesyl chloride (2.3 mL, 32.4 mmol) was added dropwise, and the mixture was stirred at room temperature for 23 h. After the completion of the mesylation (TLC: hexane−EtOAc, 2:1), the reaction mixture was concentrated in vacuo and purified by silica gel column chromatography (hexane−EtOAc, 3:1) to afford **8** (3.13 g, 84%) as a yellow liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.3−5.2 (m, 2H), 4.19 (t, 2H, J = 6.6 Hz), 3.0 (s, 3H), 2.0−1.9 (m, 4H), 1.9−1.6 (m, 2H), 1.3−1.1 (m, 22H), 0.85 (t, 3H, J = 6.6 Hz) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 130.2, 129.9, 70.5, 37.5, 32.8, 32.1, 30.0, 29.9, 29.7, 29.5, 29.3, 29.3, 29.3, 29.2, 27.4, 27.4, 25.6, 22.9, 21.2, 14.4 ppm.

*4.1.4. Oleyl 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-α- and β-D-Glucopyranoside (**9** and **10**).* To a mixture of **7**[23] (1.9 g, 4.30 mmol) and Et_3N (1.8 mL, 12.9 mmol) in anhydrous DMF (6 mL), **8** (2.2 g, 6.5 mmol) was added, under stirring at 60 °C and under Ar atmosphere for 8 h. The reaction mixture was concentrated, and the residue was purified by silica gel column chromatography (hexane−EtOAc 5:1 → 2:1) to give **9** (0.72 g 27%) as a yellow oil and **10** (0.89 g, 33%) as a white solid.

Compound **9**: $[\alpha]_D$ +112.4° (*c* 0.5, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.71 (d, 1H, J = 8.7 Hz), 5.39 (d, 1H, J = 5.4 Hz), 5.4−5.3 (m, 1H), 5.1−5.0 (m, 2H), 4.5−4.4 (m, 1H), 4.4−4.3 (m, 2H), 4.2−4.0 (m, 2H), 2.6−2.5 (m, 2H), 2.1−1.9 (m, 16H), 1.7−1.4 (m, 2H), 1.4−1.2 (m, 22H), 0.88 (t, 3H, J = 5.7 Hz) ppm. ^{13}C NMR (75 MHz, CD_3OD): δ 171.6, 170.7, 169.8, 169.3, 130.0, 129.7, 84.6, 71.4, 68.3, 68.1, 62.8, 52.3, 36.5, 32.5, 31.9, 31.5, 29.7, 28.8, 27.2, 23.2, 22.7, 20.7, 14.6, 14.1 ppm. HRMS (ESI) m/z calcd for $C_{32}H_{55}NO_8S$, 613.85; found, 614.5 $(M + H)^+$.

Compound **10**: mp 132−143 °C; $[\alpha]_D$: −13.1° (*c* 1.5, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.46 (d, 1H, J = 9.3 Hz), 5.4−5.3 (m, 2H), 5.2 5.0 (m, 2H), 4.57 (d, 1H, J = 10.5 Hz), 4.24 (dd, 1H, J = 4.9, 12.3 Hz), 4.2−4.0 (m, 2H), 4.1−4.0 (m, 1H), 3.7−3.6 (m, 1H), 2.7−2.6 (m, 2H), 2.0−1.9 (m, 16H), 1.7−1.5 (m, 2H), 1.4−1.2 (m, 22 H), 0.88 (t, 3H, J = 6.3 Hz) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ 171.1, 170.7, 170.0, 169.3, 130.0, 129.8, 84.6, 76.7, 75.9, 73.8, 68.3, 62.30, 53.3, 32.6, 31.9, 30.1, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 28.9, 27.2, 27.1, 23.3,

22.7, 20.8, 20.7, 20.6, 14.1 ppm. HRMS (ESI) m/z calcd for $C_{32}H_{55}NO_8S$, 613.85; found, 614.5 $(M + H)^+$.

*4.1.5. Oleyl 2-Acetamido-2-deoxy-1-thio-α-D-glucopyranoside (**3**).* Compound **9** (810 mg, 1.30 mmol) was dissolved in MeOH (2 mL) and treated with a 0.1 M solution of NaOMe (10 mL), and the reaction mixture was stirred at room temperature for 1 h. After this time, the mixture was neutralized with Amberlite IR-120 (H^+ form) and concentrated. The crude was purified by silica gel column chromatography (EtOAc−MeOH 10:1) to give **3** (0.47 g, 73%) as a white solid; mp 185−190 °C; $[\alpha]_D$ +132.0° (c 1.0, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 5.45 (d, 1H, J = 5.4 Hz), 5.4−5.3 (m, 2H), 4.00 (dd, 1H, J = 5.4, 11.1 Hz), 4.0−3.9 (m, 1H), 3.81 (dd, 1H, J = 2.0, 12.0 Hz), 3.70 (dd, 1H, J = 5.4, 12.0 Hz), 3.60 (t, 1H, J = 9.0 Hz), 3.3 (t, 1H, J = 9.3 Hz), 2.6−2.5 (m, 2H), 2.1−2.0 (m, 4H), 2.0 (s, 3H), 1.6−1.5 (m, 2H), 1.3−1.2 (m, 22H), 0.90 (t, 3H, J = 6.6 Hz) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ 173.6, 130.9, 130.8, 85.1, 74.2, 72.7, 72.6, 62.6, 55.9, 33.6, 33.1, 31.6, 30.9, 30.8, 30.6, 30.6, 30.5, 30.3, 29.9, 28.2, 28.1, 23.8, 22.6, 14.9 ppm. MS (ESI) m/z calcd for $C_{26}H_{49}NO_5S$, 487.5; found, 488.5 $(M + H)^+$.

*4.1.6. Oleyl 2-Acetamido-2-deoxy-1-thio-β-D-glucopyranoside (**4**).* Using the same procedure for the preparation of **3**, starting from **10** (1.4 g, 2.23 mmol), **4** (0.84 g, 77%) was obtained as a white solid; mp 170−175 °C; $[\alpha]_D$ −18.3° (c 1.1, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 5.4−5.3 (m, 2H), 4.47 (d, 1H, J = 10.3 Hz), 3.86 (dd, 1H, J = 2.2, 12.0 Hz), 3.73 (t, 1H, J = 10.1 Hz), 3.67 (dd, 1H, J = 5.7, 12.0 Hz), 3.43 (t, 1H, J = 9.7 Hz), 3.3−3.2 (m, 2H), 2.7−2.6 (m, 2H), 2.1−2.0 (m, 4H), 2.00 (s, 3H), 1.6−1.5 (m, 2H), 1.4−1.2 (m, 22H), 0.90 (t, 3H, J = 6.6 Hz) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ 173.5, 130.9, 130.8, 85.7, 82.1, 77.4, 71.9, 62.9, 56.3, 33.6, 33.1, 30.9, 30.8, 30.8, 30.6, 30.5, 30.3, 30.2, 30.0, 28.2, 28.1, 23.8, 23.0, 14.5 ppm. HRMS (ESI) m/z calcd for $C_{26}H_{49}NO_5S$, 487.3341; found, 488.3415 $(M + H)^+$.

4.2. In Vitro Activity of the Compounds: Inhibition of A549 and C6 Tumor Cell Proliferation. Human A549 line was cultured as previously described.[25] Rat glioma (C6 line) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) complete medium (Sigma-Aldrich, St. Louis, MO), supplemented with fetal bovine serum (FBS, 10%; GLinus, Madrid, Spain), glutamine (2 mM), penicillin (50 IU/mL), and streptomycin (50 mg/mL), at 37 °C in a 5% CO_2 humidified atmosphere. Exponentially growing C6 cells were seeded on 96-well plates (Beckton Dickinson, Le Pont de Claix, France), in complete DMEM medium, at a density of 5×10^4 cells/well. The cells were allowed to attach with 5% of CO_2 at 37 °C for overnight, and then, the medium was replaced by 100 μL of fresh medium. Stock solutions in EtOH of the compounds **1**−**4** (100 mM) were finally dissolved in DMEM medium containing EGF or basic FGF (10 ng/mL) for the treatments. The cells were treated with serial dilutions of compounds (**1**−**4**) for 48 h. Cell proliferation was evaluated with an MTT assay (Sigma-Aldrich), based on the conversion of the water-soluble MTT into an insoluble formazan. Briefly, after 48 h of compound treatment, the medium was replaced by 100 μL of fresh DMEM without phenol red containing MTT (5 mg/mL) solution, and cells were incubated for 3 h at 37 °C with 5% CO_2. After this time, the medium was removed, the precipitated formazan was dissolved in 100 μL of DMSO, and the solution optical density was measured at 595 nm in a Spectramax Plus (Molecular Devices Corporation) for 96-well plates equipment. Absorption values were referred to positive proliferation controls, cultured in DMEM medium supplemented with EGF or basic FGF. Inhibition was expressed as an ID_{50} value, the compound concentration that reduced maximal proliferation by 50%.

4.3. Liquid Chromatography−Mass Spectrometry Analysis of Glycosphingolipids (A549). The glycosphingolipids used as internal standards were from Avanti Polar Lipids. A549 cells (2.5×10^5 cells/mL) were seeded in 1 mL of medium with 10% FBS in 6-well plates. Twenty-four hours later, media were replaced with fresh medium containing test compound or vehicle solvent and cultured for 48 h at 37 °C/5% CO_2. Then, cells were washed in PBS, collected by brief trypsinization, and transferred to glass vials. An aliquot of the cells was taken for cell counting, using a Countess automatic cell counter. Sphingolipid extracts, spiked with internal standards (0.2 nmol), were prepared as described[24] and analyzed by liquid chromatography−mass spectrometry using a Waters Aquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time-of-flight mass spectrometer (Waters, Milford, MA), operated in positive electrospray ionization mode in the conditions previously reported.[25]

4.4. *N*-Acetyl-D-hexosaminidase Assay. *4.4.1. Chemicals.* *N*-Acetyl-β-D-hexosaminidase from Jack bean (1.2 mg protein/mL), 2-acetamido-2-deoxy-β-D-glucopiranose (GlcNAc), and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopiranoside (pNP-GlcNAc) were purchased from Sigma-Aldrich. Phosphate buffers were prepared by mixing appropriately concentrated aqueous sodium dihydrogenphosphate and disodium hydrogen phosphate solutions to reach the required pH.

4.4.2. HPLC Analysis. All analyses were performed using a Jasco Pu-2089 Plus, Dual Gradient Pump chromatograph, equipped with an ultraviolet Jasco UV-2075 Plus detector and a 20 μL Rheodyne injector. Data acquisition and processing were accomplished with the Jasco ChromPass Chromatography Data System 1.8.6.1 software. A reverse phase Lichrosorb C18 (5 μM, 4.6 mm × 250 mm) column was used as the stationary phase, using a C18 guard cartridge. GlcNAc separation was carried out with a mobile phase of water−methanol mixture, following a gradient elution program: 95−5 (from 0 to 4 min) to 0−100 (from 8 to 20 min) to 95−5 (from 25 to 30 min). The flow rate was 1.0 mL min^{-1}, and the elution profile was monitored by recording the UV absorbance at 205 nm. The amount of liberated GlcNAc was calculated from a standard curve obtained using unlinked GlcNAc, with a retention time of 2.8 min in 95% water−5% methanol.

4.4.3. Enzyme Assay. *N*-Acetyl-β-D-hexosaminidase activity was determined using pNP-GlcNAc as substrate, by measuring the release of *p*-nitrophenol (pNP) from pNP-GlcNAc. UV/Visible spectra were recorded at 405 nm on a SpectraMax Plus 384 spectrophotometer at 37 °C. One unit was defined as that amount of enzyme that released 1 μmol *p*-nitrophenol min^{-1}. Reaction mixtures consisted of 500 μL of 40 mM sodium phosphate buffer (pH 5) and 20% isopropanol, containing 1 mM of substrate (**2** or **4**) and 1 unit/ml of enzyme and were incubated at 37 °C (170 rpm in an orbital shaker). Fifty microliter aliquots were collected at different time periods, varying between 0.25 and 48 h. The reaction was stopped by freezing at −80 °C and then analyzed by HPLC under the conditions described above. All experiments were performed in triplicate. Product formation was calculated as % percentage and expressed as the mean ± SEM (standard error of the mean).

4.5. Evaluation of the in Vivo Effect. The activity of compounds **1** and **3** on tumor growth was evaluated using an orthotopic model in female nude mice (Foxn1$^{nu/nu}$, 6−8 weeks old, Harlan Iberica, Barcelona, Spain). Mice were maintained in the animalhouse of the Cajal Institute (Madrid, Spain) with ad libitum food and water in a 12 h light/dark cycle. Animals were handled complying with the European Union guidelines for care and handling of experimental animals (86/609/EEC), and the protocols were approved by the Cajal Institute animal welfare committee. Mice were injected into a flank, sc, a suspension of 2×10^6 cells of rat glioma C6. The tumor volume was calculated according to the following formula: $V\,(cm^3) = (L \times W^2 \times \pi)/6$, where L is the length and W is the width of the tumor, respectively. When tumors reached 250 mm^3, the animals were treated with a daily intratumoral injection of the compounds for 14 days. Stock solutions in DMSO of the compounds (100 mM) were finally dissolved in PBS buffer containing fatty acid-free BSA (5 mg/mL; Sigma-Aldrich, Steinheim, Germany) for the treatments. Control animals received injections with the same solution of PBS buffer with BSA and DMSO as other groups of animals treated with test compounds. The animals were sacrificed by cervical dislocation after the treatments, and the tumors were resected on ice until use. Animal weight

was controlled to evaluate the general health state during treatments (see the Supporting Information).

4.6. ^{1}H MAS NMR Spectroscopy. High-resolution proton MAS NMR measurements were performed on a Bruker Avance 400 wide bore (89 mm Ø) spectrometer (Bruker Instruments, Karlsruhe, Germany) operating at 9.4 T (proton Larmor frecuency at 400.14 MHz). The spectra were acquired at 20 °C (293 K) using a Bruker double tuned broad-band solid state CP/MAS probe head. After the in vivo experiment, the animals were sacrificed, and the tumors were resected on ice. The tumor samples were washed with deuterated phosphate-buffered solution and placed into a 4 mm Ø zirconia rotor. The spectra were acquired using a 90° pulse of 3.35 μs followed by a T_2 filter of 60 ms. The spinning rate was 3 kHz. The spectral width was 8.3 kHz, 16 k data points, and the number of scans was 256 with a repetition rate of 5 s. The residual water signal was suppressed by presaturation on the water resonance. All FIDs were processed with a 2 Hz line-broadening, and the chemical shifts were referenced to internal creatine (3.026 ppm).

■ ASSOCIATED CONTENT

Supporting Information. Glucosylceramide, lactosylceramide, and ganglioside content in A549 cells as a function of the acyl chain length and the number of insaturations; weight gain curves of control and treated animals; and NMR spectra and elemental analysis data of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

UPLC-MS, ultra performance liquid chromatography−mass spectrometry; ^{1}H MAS NMR, proton magic angle spining nuclear magnetic resonance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ID_{50}, 50% inhibitory dose

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