Synthesis and Evaluation of the Antiproliferative Effects of 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl)-*sn*-glycerol and 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-amino-2′-deoxy-β-D-glucopyranosyl)-*sn*-glycerol on Epithelial Cancer Cell Growth

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Two ether glucosyl diglyceride analogs were synthesized, and their antiproliferative activity against four epithelial cancer cell lines was evaluated. 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl)-*sn*-glycerol (**4**) was synthesized by reaction of 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride with 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol followed by deacetylation by methanolic hydrolysis. The *N*-acetyl group of **4** was removed by hydrolysis with ethanolic potassium hydroxide to form 1-*O*-hexadecyl-2-*O*-methyl-3-*O*-(2′-amino-2′-deoxy-β-D-glucopyranosyl)-*sn*-glycerol (**5**). Compounds **4** and **5** inhibited the proliferation of MCF-7, A549, A427, and T84 cancer cell lines. The IC_{50} values for **5** ranged from 6.5 to 12.2 μM, whereas **4** was more effective against A549 cells (IC_{50} 9 μM) than against MCF-7 (IC_{50} 17 μM) and A427 (IC_{50} 25 μM) cells and was inactive against T84 cells. Under identical incubation conditions, compounds **4** and **5** were potent inhibitors of the proliferation of OVCAR-3 cells with IC_{50} values of 12 and 4 μM, respectively, whereas ET-18-OCH_3, hexadecylphosphocholine, and erucylphosphocholine had IC_{50} values of 24, >30, and >30 μM, respectively. The cell-inhibitory profile of these ether-linked glucosyl diglycerides strengthens the hypothesis that such glycolipids represent a distinct group of antitumor ether lipids, having antineoplastic activities that differ from the well-known alkylphosphocholines and alkyllysophospholipids.

Introduction

Alkyllysophospholipids and alkylphosphocholines possess anticancer properties and have the potential of providing a new therapeutic approach to cancer treatment, since their growth-inhibitory effects are apparently achieved via interaction with cellular membranes rather than with DNA (for recent reviews, see refs 1 and 2). Synthetic diglycerides with a long chain alkyl group at the *sn*-1 position, a methoxy at the *sn*-2 position, and a glucosyl moiety linked via an *O* or *S* bond to the *sn*-3 position may represent a subclass of antitumor ether lipids. These compounds displayed a selective cytotoxicity against leukemic cells that was dependent on the configuration at the anomeric position.[3] The α anomer of 1-*O*-hexadecyl-2-*O*-methylthioglucosyl-*sn*-glycerol inhibited the growth of various cancer cells, but the β analog had no effect on the growth of the same cells.[3] One potential advantage of the glucosyl diglycerides may be their lower potential for inducing thrombogenesis compared with other antineoplastic ether lipids.[3]

We have recently examined the effect of the α and β anomers of 1-*O*-hexadecyl-2-*O*-methyl-3-*S*-thioglucosyl-*sn*-glycerol on a number of epithelial cells.[4] Both compounds inhibited the proliferation of the epithelial cancer cell lines examined but were not cytotoxic. Furthermore, relative to the other tested cells, A549 cells were most sensitive to the effects of the compounds even though they were the least sensitive to the inhibitory effects of ET-18-OCH_3. These observations suggest that ether glucosyl diglycerides may be effective antineoplastic agents with a cytotoxicity profile different from that of ET-18-OCH_3, the prototypic alkyllysophospholipid.[1]

In view of the modest inhibition of epithelial cell proliferation obtained with the ether thioglycolipids and their potential usefulness, studies were initiated to synthesize new analogs of ether-linked glucosyl diglycerides that possessed antiproliferative activity toward epithelial cancer cell lines. We report here the synthesis of a glucosamine diglyceride (**5**), which was found to show activity against a variety of epithelial cell lines. Compound **5** also effectively inhibited the proliferation of an ovarian cancer cell line, OVCAR-3, at concentrations at which two alkylphosphocholines, hexadecyl- and erucylphosphocholine, and the alkylphospholipid ET-18-OCH_3 had limited or no effects.

Results and Discussion

Chemistry. Alkoxy Glycolipid 5. 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-amino-2′-deoxy-β-D-glucopyranosyl)-*sn*-glycerol (**5**) was prepared in a sequence that utilized 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride (**1**) and 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol (**2**) as starting materials (Scheme 1). Coupling of the glycosyl donor, 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride (**1**), with di-*O*-alkylglycerol **2** in the presence of 1 equiv each of zinc chloride

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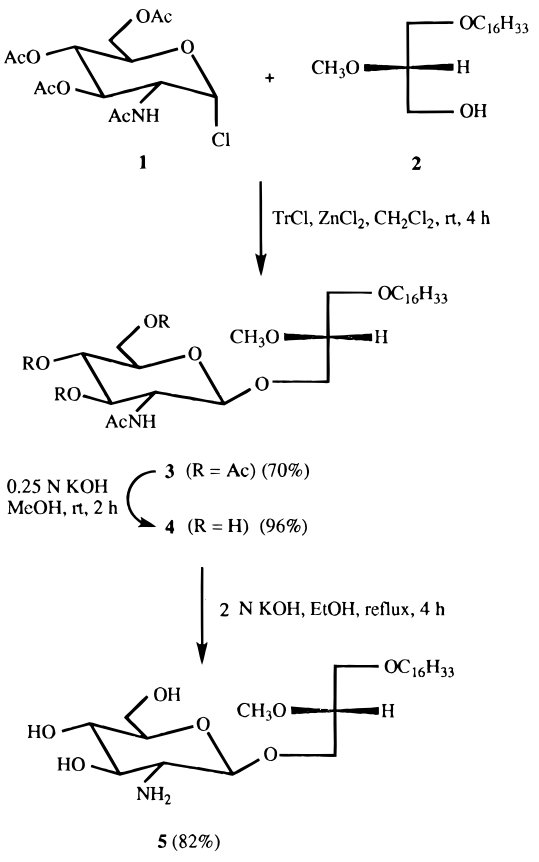
Scheme 1. Synthesis of **4** and **5**



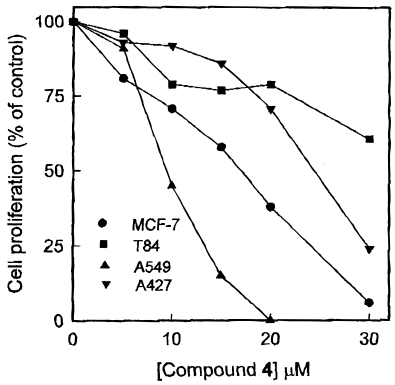


Figure 1. Effect of *N*-acetylglucosamine **4** on the proliferation of MCF-7 (●), T84 (■), A549 (▲), and A427 (▼) cells. Proliferating cells growing in 24-well plates were incubated with **4** (0−30 μM). The increase in cell number after 48 h was determined and expressed as a percent of controls. The results are the means of eight different determinations. Standard deviations from the mean were <12%.

and trityl chloride in methylene chloride, as described previously,[5] gave β-glycolipid conjugate **3** in 70% yield. Hydrolysis of the acetyl groups of glycolipid **3** in methanolic potassium hydroxide at room temperature gave 2-acetamido glycolipid **4** in 96% yield, which on further hydrolysis with 2 N ethanolic potassium hydroxide under reflux gave the target glycolipid **5** in 82% yield.

Antiproliferative and Cytotoxic Properties of 4 and 5. The effects of 1-*O*-hexadecyl-2-*O*-methyl-3-*O*-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl)-*sn*-glycerol (**4**) on the proliferation of MCF-7, A549, T84, and A427 cell lines after 48-h treatment is displayed in Figure 1. The concentrations of **4** that inhibited cell proliferation by

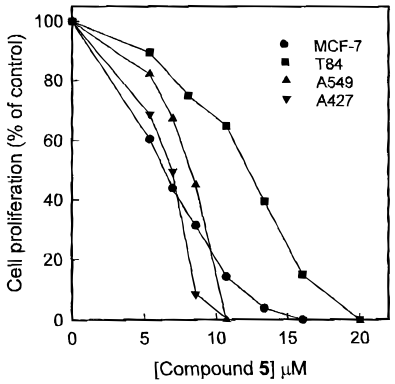


Figure 2. Effect of glucosamine **5** on the proliferation of MCF-7 (●), T84 (■), A549 (▲), and A427 (▼) cells. Cells were treated with **5** for 48 h. The results are the means of eight different determinations with standard deviations of <9%.

Table 1. IC_{50} Values (μM) of **4** and **5** against Epithelial Cancer Cell Lines[a]

cells	4	5	cells	4	5
A549	9.0	8.3	T84	>30	12.2
MCF-7	17.0	6.5	OVCAR-3	12.0	4.0
A427	24.5	7.0			

[a]Cells were treated with varying concentrations of **4** or **5** (0−30 μM) for 48 h.

50% (IC_{50}) were 9, 17, 25, and >30 μM for A549, MCF-7, A427, and T84 cell lines, respectively (Table 1). A549 cells were therefore the most sensitive to the inhibitory effects of **4**. Indeed, A549 cells were the only cells tested here to which the drug reduced cell numbers to levels below those present at the time of drug addition, which is an indication that **4** was cytotoxic to these cells, albeit at concentrations of 20 μM and higher. Compound **4** was more effective against A549 cells than were the α and β anomers of 1-*O*-hexadecyl-2-*O*-methyl-3-*S*-D-glucopyranosyl-*sn*-thioglycerol (ET-16-OCH_3-thio-Glc),[4] whereas its effects on MCF-7 and T84 cells were similar to those observed with ET-16-OCH_3-thio-Glc.[4]

Figure 2 shows the effect of compound **5** on the proliferation of the cell lines proliferating in 10% FBS-supplemented medium. The IC_{50} values of **5** against MCF-7, A427, and A549 cells were in the range 6.5−8.3 μM (Table 1). In contrast to **4**, which was most effective against A549 cells and inactive against T84 cells, **5** was active against all four cell lines shown in Table 1. It is worth noting that T84 cells were the least sensitive cells we tested to the effects of both **4** and **5**, even though this cell line was one of the most sensitive to ET-18-OCH_3.[6,7]

Concentrations of **5** that reduced the cell numbers below those present at the time of drug addition were 10.5 μM for both A549 and A427, 15 μM for MCF-7, and 20 μM for T84 (Figure 2). Concentrations of 10 μM or higher of **5** also produced similar results with the prostate cancer cell line DU 145, neuroblastoma SK-N-MC, and melanoma SK-MEL-28 (results not shown). Thus **5** was effective against epithelial cancer cells derived from various tissue sources.

Inhibition of OVCAR-3 Cell Growth by 4, 5, ET-18-OCH_3, HPC, and EPC. The effects of **4** and **5** on the growth of the ovarian cancer cell line OVCAR-3 were compared with those of ET-18-OCH_3, hexadecylphosphocholine, and erucylphosphocholine (Figure 3). The

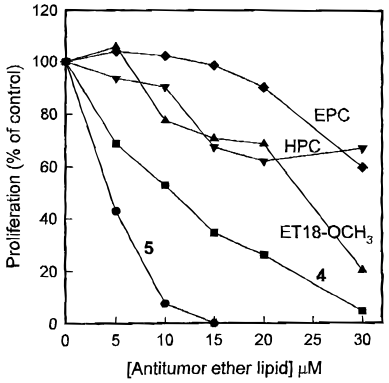


Figure 3. Effects of antitumor ether lipids on the proliferation of OVCAR-3 cells. The cells were treated with varying concentrations of **4** (■), **5** (●), ET-18-OCH_3 (▲), hexadecylphosphocholine (HPC; ▼), and erucylphosphocholine (EPC; ◆) for 48 h. The results represent the means of eight different determinations with standard deviations of <15%.

antiproliferative effects of **4** and **5** were remarkably superior to the two established classes of antitumor ether lipids. The IC_{50} values for **4** and **5** were 12 and 4 μM, respectively, whereas the corresponding IC_{50} value for ET-18-OCH_3 was 24 μM and >30 μM for both hexadecylphosphocholine and erucylphosphocholine. The effectiveness of the glucosyl diglycerides **4** and **5** is even more significant in light of the fact that the incubation medium consisted of 20% FBS-supplemented medium; the high protein content of the medium would decrease the effective free concentration of the compounds.[8]

Structurally, the only difference between **4** and **5** is the absence of an *N*-acetyl group on the amine of the sugar moiety of **5**, yet there is a marked difference in the activities of **4** and **5**. The relative lipophilicity of these compounds could obviously determine the quantities that accumulate in the membrane; however, since the exposed amine group would render **5** more polar (less lipophilic) than **4**, the higher cytotoxicity of **5** is probably not related to the extent of incorporation into the membranes of sensitive cells. Elucidation of the mechanism of action of these novel compounds would help explain the differences in activity between the two analogs.

Conclusion

Our studies have identified β-glucosamine diglyceride **5** as an effective antiproliferative and cytotoxic agent against epithelial cancer cells from different tissues, whereas its *N*-acetyl analog **4** was generally much less growth-inhibitory and less cytotoxic (Table 1). However, the latter compound could be useful if its selectivity against A549, a non-small-cell lung adenocarcinoma, is confirmed in future studies employing a wider panel of cells. Compound **5** was highly active in inhibition of OVCAR-3 cell growth (IC_{50} 4 μM; Figure 3), whereas compound **4** was moderately active (IC_{50} 12 μM); in contrast, ET-18-OCH_3 (IC_{50} 24 μM) and HPC and EPC (IC_{50} >30 μM) were much less active.

Experimental Section

Silica gel GF TLC plates of 0.25-mm thickness (Analtech, Newark, DE) were used to monitor reactions, with visualization by charring using 10% sulfuric acid in ethanol. Flash chromatography was carried out with silica gel 60 (230−400 ASTM mesh) of E. Merck, purchased from Aldrich. ^{1}H-NMR spectra were recorded on a Bruker 200-MHz spectrometer, and chemical shifts are in parts per million from tetramethylsilane as internal standard. Optical rotations were measured on a Jasco DIP-140 digital polarimeter. The melting points are uncorrected.

Trityl chloride was obtained from Aldrich. Zinc chloride was obtained from Fluka. Dichloromethane was dried over P_2O_5 and distilled just before use. 2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride (**1**) was prepared according to a published procedure.[9] 1-*O*-Hexadecyl-2-*O*-methyl-*sn*-glycerol (**2**) was prepared as described previously[10] or obtained from Sigma Chemical Co. HPC and EPC were prepared as described previously.[11] ET-18-OCH_3 was obtained from Medmark (Grünwald, Germany).

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-*β*-D-glucopyranosyl)-*sn*-glycerol (3). To a solution of 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride (**1**) (219.4 mg, 0.6 mmol), 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol (**2**) (100 mg, 0.3 mmol), and trityl chloride (83.6 mg, 0.3 mmol) was added a suspension of 41.2 mg (0.3 mmol) of zinc chloride in dry dichloromethane (5 mL). The reaction mixture was stirred for 4 h at room temperature. The progress of the reaction was monitored by TLC in ethyl acetate. The reaction mixture was diluted with ethyl acetate (50 mL), washed with 5% aqueous sodium bicarbonate solution and water, dried over sodium sulfate, and concentrated under reduced pressure. The residue was purified by flash chromatography (elution with hexane/ethyl acetate, 1:1), giving 140 mg (70%) of **3** as a white solid: for R_f, $[\alpha]^{25}_D$, and ^{1}H-NMR, see ref 5.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-acetamido-2′-deoxy-*β*-D-glucopyranosyl)-*sn*-glycerol (4). 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-*β*-D-glucopyranosyl)-*sn*-glycerol (**3**) (140 mg, 0.21 mmol) was dissolved in 3 mL of 0.25 N methanolic KOH, and the mixture was stirred for 2 h at room temperature. The reaction mixture was neutralized with saturated aqueous ammonium chloride solution and extracted with chloroform (10 mL). The chloroform layer was dried over $MgSO_4$ and concentrated under reduced pressure, and the residue was purified by flash chromatography (elution with 10% methanol in chloroform), giving 109 mg (96%) of **4** as a white solid: mp 150−153 °C; R_f 0.56 ($CHCl_3$−CH_3OH, 4:1); $[\alpha]^{25}_D$ −2.26° (*c* 5.25, $CHCl_3$−CH_3OH, 1:1, v/v); ^{1}H-NMR ($CDCl_3$ and a few drops of CD_3OD) δ 0.80 (t, 3H, J = 6.33 Hz, C*H*$_3$), 1.25 (br m, 26H, (C*H*$_2$)$_{13}$CH$_3$), 1.56 (2H, OCH$_2$C*H*$_2$), 2.01 (s, 3H, NHCOC*H*$_3$), 3.23−3.83 (m, 19H, with a singlet at δ 3.45, C*H*$_2$OC*H*$_2$C$_{15}$H$_{31}$, C*H*$_3$OC*H*, OC*H*$_2$, -C*H*O-'s of sugar moiety), 4.43 (d, 1H, J = 6.84 Hz, H-1), 7.51 (d, 1H, J = 8.51 Hz, N*H*).

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-amino-2′-deoxy-*β*-D-glucopyranosyl)-*sn*-glycerol (5). 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2′-acetamido-2′-deoxy-*β*-D-glucopyranosyl)-*sn*-glycerol (**4**) (24 mg, 45.3 μmol) was dissolved in 2 mL of 2 N ethanolic KOH, and the mixture was heated for 4 h at 120 °C (bath temperature). The mixture was cooled and then neutralized with saturated aqueous ammonium chloride solution, and the product was extracted with chloroform. The chloroform layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (elution with 20% methanol in chloroform), giving 18 mg (82%) of **5** as a white solid: R_f 0.28 ($CHCl_3$−CH_3OH, 4:1); $[\alpha]^{25}_D$ −14.04° (*c* 7.5, $CHCl_3$−CH_3OH, 1:1, v/v); ^{1}H NMR ($CDCl_3$ and a few drops of CD_3OD) δ 0.85 (t, 3H, J = 6.34 Hz, C*H*$_3$), 1.23 (br, 26H, (C*H*$_2$)$_{13}$CH$_3$), 1.53 (2H, OCH$_2$C*H*$_2$), 3.40−3.91 (m, 20H, with a singlet at δ 3.45, C*H*$_2$OC*H*$_2$C$_{15}$H$_{31}$, C*H*$_3$OC*H*, OC*H*$_2$, -C*H*O-'s of sugar moiety), 4.82 (br s, 2H, N*H*$_2$); HRMS (FAB, MH$^+$) calcd for $C_{26}H_{54}NO_7$ 492.3900, found 492.3899.

Antiproliferative Studies. The human epithelial cancer cell lines were grown from frozen stocks originally obtained from ATCC. A549 cells (non-small-cell lung adenocarcinoma) were cultured in Ham's F-12 medium, T84 cells (colon carcinoma) were cultured in a 1:1 mixture of F-12 and Dulbecco's modified Eagle medium (DMEM), and MCF-7 (breast adenocarcinoma) and A427 (large cell lung carcinoma) cells were cultured in DMEM. DU 145 (prostate carcinoma) was grown in Eagle minimum essential medium (MEM), SK-MEL-28 (melanoma) was grown in MEM with nonessential amino acids

and Hanks balanced salt solution (BSS), and SK-N-MC (neuroblastoma) was grown in Eagle MEM with nonessential amino acids and Earles BSS. The media were supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/mL), streptomycin (50 μg/mL), and fungizone (0.5 μg/mL). OVCAR-3 (ovarian adenocarcinoma) cells were cultured in RPMI 1640 medium supplemented with 20% FBS and 10 μg/mL insulin. Cells were subcultured into 24-well plates, and the cell number was monitored daily with a Coulter ZM counter. When the cells were in log phase, the medium was replaced with one containing the required drug concentration, and the cells were incubated for 48 h. The increase in cell numbers relative to control wells without any drug was determined after the incubation.[4]

Stock solutions of the drugs (30 mM) were prepared in ethanol and stored at −20 °C. A 30 μM solution of the drug in the appropriate media was prepared fresh on the day of the experiment and serially diluted to give the required concentrations. The final concentration of ethanol in all wells was 0.1% (v/v).

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