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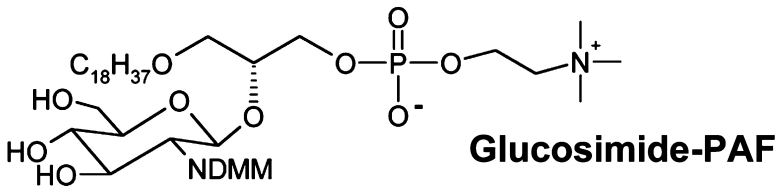
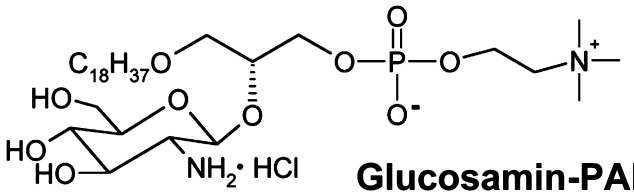


Glucosimide-PAF



Glucosamin-PAF

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Glucosamine-glycerophospholipids That Activate Cell-Matrix Adhesion and Migration

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Two new analogues derived from the platelet activating factor (PAF), containing glucosamine instead of the acetyl group, were synthesized, and their effect on the human keratinocyte cell line HaCaT was evaluated with respect to cytotoxicity, proliferation, adhesion, and migration. Starting with (*R*)-1,2-isopropylideneglycerol (**3**), the glycosylation acceptor 1-*O*-octadecyl-3-*O*-*tert*-butyldimethylsilyl-*sn*-glycerol (**6**) was synthesized in three steps. Glycosylation of **6** with the already known *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-dimethylmaleimido-β-D-glycopyranosyl)-trichloracetimidate gave 1-*O*-octadecyl-2-*O*-(3′,4′,6′-tri-*O*-acetyl-2′-deoxy-2′-dimethylmaleimido-β-D-glucopyranosyl)-3-*O*-*tert*-butyldimethylsilyl-*sn*-glycerol (**7**). After removing the (*tert*-butyldimethyl)silyl (TBDMS) group with $FeCl_3 \cdot 6H_2O$, phosphoryl choline was introduced, yielding [1-*O*-octadecyl-2-*O*-(2′-deoxy-2′-dimethylmaleimido-β-D-glucopyranosyl)-*sn*-glycero(3)]phosphorylcholine (**2**) (glucosimide-PAF). pH controlled cleavage of the amino protection group gave [1-*O*-octadecyl-2-*O*-(2′-deoxy-2′-amino-β-D-glucopyranosyl)-*sn*-glycero(3)]phosphorylcholine hydrochloride (**1**) (glucosamine-PAF). **2** inhibited proliferation of HaCaT cells by 26% at nontoxic concentrations, while **1** increased the proliferation rate by 30% at low concentrations. At higher concentrations, both compounds showed cytotoxic properties with $LD_{50} = 30$ μmol/L (**1**) and $LD_{50} = 5-6$ μmol/L (**2**). Both **1** and **2** were potent promoters of cell adhesion and migration of HaCaT cells.

Introduction

Phospholipids are basic membrane components. Furthermore, some of their metabolites, such as lysolecithin, inositol trisphosphate, and platelet activating factor (PAF), are important mediators in the regulation of physiological and pathological processes.[1,2] Very recently the role of lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) in promoting endothelial cell migration and angiogenesis was demonstrated.[3,4] For many years, PAF [1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero(3)]phosphorylcholine has been a chemical lead for the development of synthetic ether lipids. Racemic [1-*O*-octadecyl-2-*O*-methoxy-glycero(3)]phosphorylcholine (Edelfosine, ET-18-OCH_3) and hexadecylphosphocholine (Miltefosine, HePC) have been widely investigated for their tumor suppressing properties. Their primary target is the cell membrane, which clearly distinguishes these synthetic phospholipids from classical tumor therapeutic agents. Many mechanisms have been proposed,[5,6] ranging from a selective cytotoxicity against tumor cells, due to their higher endocytosis rate, to more sophisticated interference with signaling pathways, resulting in suppression of proliferation, immune stimulation, and induction of apoptosis or cell differentiation. Despite promising in vitro results, the cytotoxicity of these amphiphilic agents to normal cells has hitherto restricted (with one exception[7]) their use to topical or ex vivo treatment, e.g., therapy of skin metastases or purgitation of bone marrow as a leukemia treatment.[8] With this in mind, we investigated the effects of two newly synthesized phospholipids on a human keratinocyte cell line. Apart from cytotoxicity and proliferation, we further analyzed cell-matrix adhesion and migration, which are important events in wound healing, angiogenesis, metastasis, and embryogenesis.

Recently, we described the novel glycosidated phospholipids that inhibit cell proliferation. These compounds contain glucose in the *sn*-2 position of the glycerol backbone of lysophosphatidylcholine and lysoplatelet activating factor, giving rise to glycerogluco-phosphocholine (Glc-PC) and 1-*O*-octadecyl-2-*O*-α-D-glucopyranosyl-*sn*-2-glycero-3-phosphocholine (Glc-PAF), respectively. Glc-PC and Glc-PAF inhibit growth of HaCaT cells at nontoxic concentrations.[9,10] Here, we present two new compounds of this group with glucosamine instead of glucose at the *sn*-2-position: glucosamine-PAF {[1-*O*-octadecyl-2-*O*-(2′-deoxy-2′-amino-β-D-glucopyranosyl)-*sn*-glycero(3)]phosphorylcholine hydrochloride (**1**)} and glucosimide-PAF {[1-*O*-octadecyl-2-*O*-(2′-deoxy-2′-dimethylmaleimido-β-D-glucopyranosyl)-*sn*-glycero(3)]phosphorylcholine (**2**)}, which differ in the protecting group on the characteristic amino function. The effect of both compounds on proliferation as well as on adhesion and migration was studied using HaCaT cells.

Results and Discussion

Chemistry. glucosamine-PAF [1-*O*-octadecyl-2-*O*-(2′-deoxy-2′-amino-β-D-glucopyranosyl)-*sn*-glycero(3)]-phosphorylcholine (**1**) and glucosimide-PAF [1-*O*-octadecyl-2-*O*-(2′-deoxy-2′-dimethylmaleimido-β-D-glucopyranosyl)-*sn*-glycero(3)]phosphorylcholine (**2**) were

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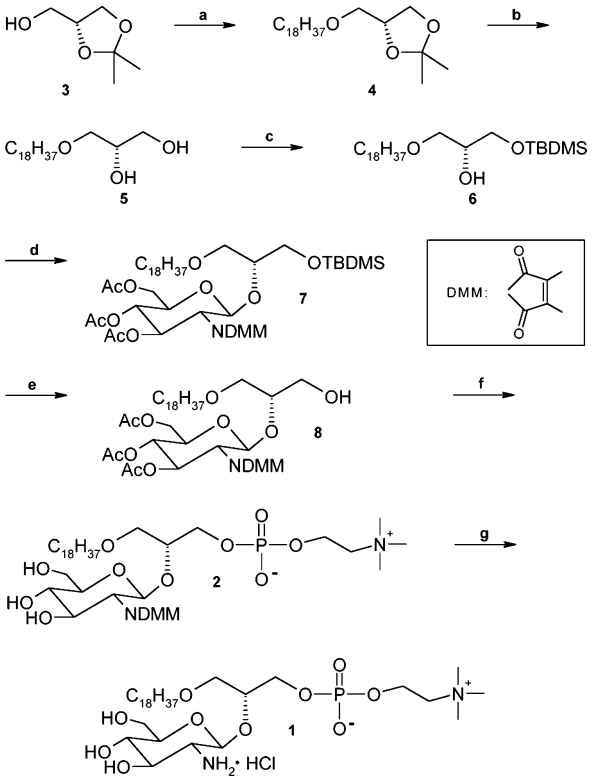
Scheme 1. Synthesis of glucosamine-PAF (**1**) and glucosimide-PAF (**2**)[a]



[a] (a) NaH, $C_{18}H_{37}Br$, THF, 80 °C (75%). (b) AcOH/H_2O (60:40) (98%). (c) TBDMS-chloride, imidazole, CH_2Cl_2, 0 °C (97%). (d) *O*-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-dimethylmaleimido-β-D-glucopyranosyl)trichloracetimidate, TMS-triflate, CH_2Cl_2 (97%). (e) $FeCl_3$·$6H_2O$, CH_2Cl_2 (81%). (f) (1) $POCl_3$, Et_3N, $CHCl_3$, 0 °C; (2) choline tosylate, pyridine, 0 °C; (3) $NaHCO_3$, 35 °C (78%). (g) (1) NaOH, dioxane/H_2O (4:1); (2) ethylamine, HCl (pH 5); (3) K_2CO_3 (63%).

prepared in a sequence that utilized (*R*)-1,2-isopropylideneglycerol (**3**) as a starting material, thus affording the same chirality as the natural phosphoglycerides (Scheme 1). Formation of the octadecyl ether derivative required temperatures above 80 °C and ensured the differentiation of the primary hydroxyl groups before acid hydrolysis of acetonide **4**. The primary hydroxyl group of diol **5** was converted selectively into the (*tert*-butyldimethyl)silyl (TBDMS) ether. Coupling of glycosyl acceptor **6** with *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-dimethylmaleimido-β-D-glycopyranosyl)trichloracetimidate[11] in the presence of trimethylsilyl-triflate (TMS-triflate) in catalytic amounts gave glycoside **7**. As expected, only the β-anomer was formed. Surprisingly, the removal of the TBDMS ether caused some problems; poor yields were obtained with tetra-*n*-butylammonium fluoride or HF_x·pyridine. A method with 2 equiv of $FeCl_3$·$6H_2O$ described originally for the cleavage of the trityl protection group[12] proved very useful for our purposes. Alcohol **8** was then esterified with phosphoryl chloride by carefully controlling the temperature to avoid formation of disubstituted byproducts.[13] The resulting dichloro ester was directly reacted with choline tosylate. Basic hydrolysis in an aqueous sodium bicarbonate solution gave glucosimide-PAF (**2**). Final cleavage of the amino protection group was carried out by sequential treatment with sodium hydroxide and hydrochloric acid.[11] glucosamine-PAF (**1**) was obtained in an overall yield of 27%.

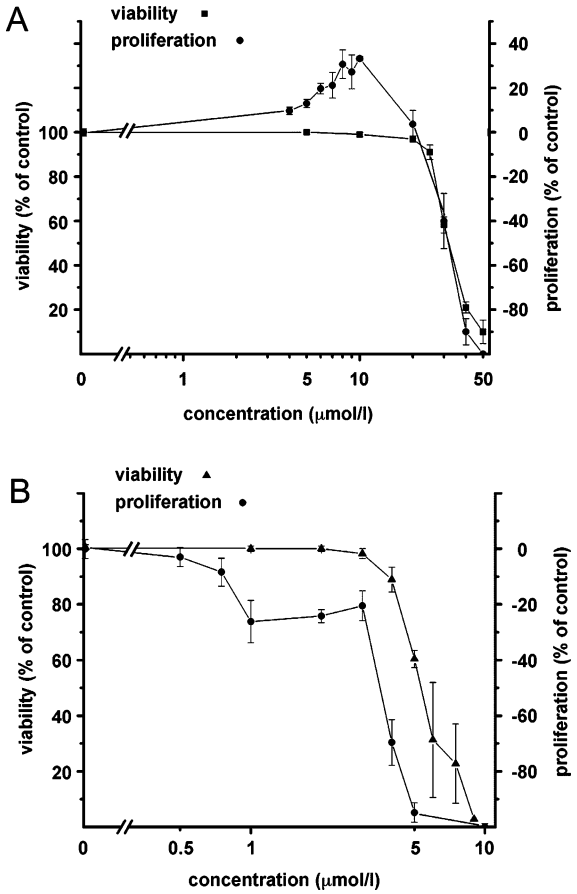


Figure 1. Viability and proliferation upon treatment with glucosamine-PAF (A) or glucosimide-PAF (B). For viability, subconfluent HaCaT cells were incubated for 24 h with the substances dissolved in serum-free keratinocyte growth medium. Cells were stained with trypan blue (0.1%), and the ratio of unstained to total cell count was determined on four fields. Three independent experiments were performed. The proliferation was determined by incorporation of 5-bromo-2′-deoxyuridine (BRDU). Subconfluent cells were incubated for 24 h with the respective compounds. Data are mean values of at least three independent experiments done in quadruplicate.

Cytotoxic Properties and Effects on Proliferation. The biological activities of **1** and **2** were determined by use of a human keratinocyte-derived cell line (HaCaT[14]). glucosamine-PAF (**1**) showed no cytotoxic effects after 24 h at concentrations $c \leq 10$ μmol/L (viability > 98%). Higher concentrations, however, resulted in rising cytotoxicity with a mean lethal dose $LD_{50} = 30$ μmol/L (Figure 1B). The corresponding value for glucosimide-PAF (**2**) was $LD_{50} = 5.5$ μmol/L, and it was nontoxic at or below 2 μmol/L (Figure 1A). Taking into consideration the different incubation times (t_{inc}), these LD_{50} values are of the same order of magnitude as those previously published for similar compounds (Table 1). The higher cytotoxicity of **2** is probably due to its more lipophilic structure, which could result in a stronger enrichment in the cell membrane. However, Erukulla et al.[15] investigated two glucosamine-containing glycero-etherlipids (without phosphate), which differed only by the presence or absence of an acetyl group at the amino function of the sugar moiety. The less lipophilic compound without the *N*-acetyl group was found to have the higher cytotoxicity.

Proliferation of HaCaT cells after 24 h of treatment with glucosimide-PAF (**2**) was maximally inhibited by

Table 1. Published LD_{50} Values of Known Phospholipids[a]

substance	LD_{50} (μmol/L)	t_{inc} (h)	cells	ref
glucosamine-PAF	30	24	HaCaT	
glucosimide-PAF	5.5	24	HaCaT	
Glc-PAF	9	6	HaCaT	10
Glc-PC	17	6	HaCaT	9
HePC	20 (LD_{10})	24	HaCaT	19
	200	6	MDCK	20
ET-18-OCH_3	90	6	MDCK	21

[a] Abbreviations: LD, lethal dose; t_{inc}, incubation time; MDCK, Madin−Darby canine kidney cells; HePC, hexadecylphosphocholine.

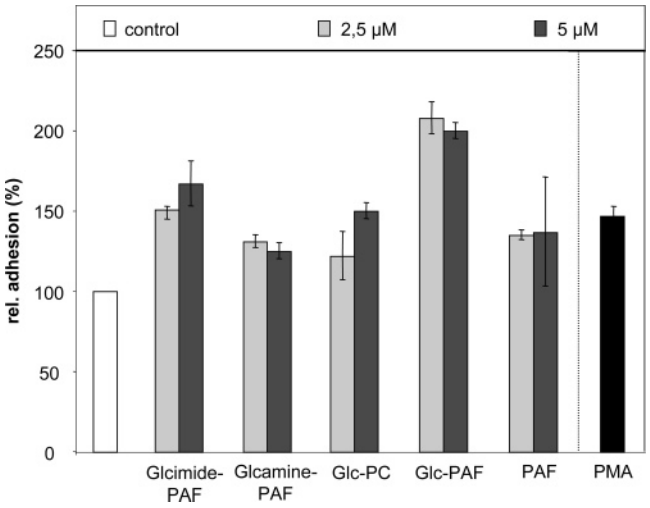


Figure 2. Adhesion upon treatment with glucosimide-PAF, glucosamine-PAF, Glc-PC, Glc-PAF, and PAF to collagen after treatment with two different concentrations of the respective substance for 3 h. As a positive control, cells were treated with 10 nM PMA, a known activator of cell-matrix adhesion. Cells were fixed and stained with crystal violet. Values were determined photometrically from at least three different experiments done in quadruplicate. Adhesion of untreated cells was taken as 100%.

26% at nontoxic concentrations, whereas glucosamine-PAF (**1**) increased the growth rate in the same time period by as much as 30% (Figure 1). Treatment for 48 h showed almost the same results for **2**, whereas compound **1** inhibited by 16% (data not shown). The increase of proliferation at low concentrations that is caused by **1** had already been described for Glc-PC,[9] but not for Glc-PAF. The mechanism which underlies this stimulating effect remains to be clarified. These data were surprising, since they show that the presence of the free amino group of glucosamine diminishes the antiproliferative effect of Glc-PAF [10] dramatically.

Adhesion- and Migration-Stimulating Properties. Cell adhesion on a surface coated with collagen, an important component of the basal membrane, was significantly increased after treatment with the PAF derivatives including the naturally occurring PAF. Here, the strongest effect can be obtained by treatment with Glc-PAF and glucosimide-PAF. This increase is stronger than the adhesion-stimulating effect observed in the presence of 10 nM phorbolmyristilacetate (PMA), an activator of protein kinase C and a known stimulator of adhesion processes (Figure 2). The results obtained with the glycosidic PAF analogues differ from the finding that HePC inhibits the adhesion of HaCaT cells on all matrix proteins by more than 50%.[16] One reason

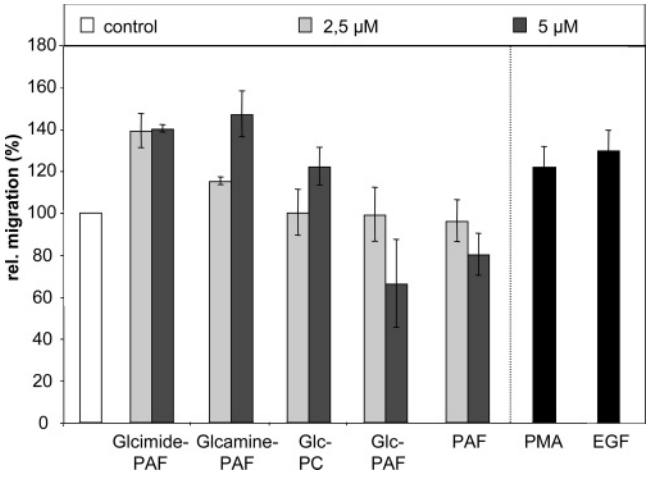


Figure 3. Migration toward collagen monitored after treatment with two different concentrations of the respective substance for 3 h. Transwell chambers were coated with collagen for 30 min, and 10^6 cells/mL were allowed to migrate for 6 h. For comparison, HaCaT cells were treated with EGF (1 nM) or PMA (10 nM), which are known activators of cell migration. Data represent mean values of at least three experiments in which four fields were counted per filter.

for these different results could be different experimental conditions, but the effect might also be due to structural differences between HePC and the PAF-derived phospholipids.

Migration of HaCaT cells toward a collagen gradient was elevated after treatment with either glucosamine-PAF (**1**) or glucosimide-PAF (**2**) (Figure 3). This effect on migration is even stronger than the stimulation of migration obtained by 1 nM epidermal growth factor (EGF) or 10 nM PMA. Stimulation of migration by micromolar concentrations of the natural phospholipids lysophosphatidic acid (LPA)[3] and sphingosine-1-phosphate[4] was described recently. Since Glc-PC has no effect on cell migration and Glc-PAF even slightly attenuates cell motility, it is unlikely that all compounds act in the same way. By which mechanisms glucosamine- and glucosimide-PAF affect cell migration remains to be elucidated. Very recently, it has been shown that LPA induces focal adhesion kinase (FAK) phosphorylation, a prerequisite of adhesion and migration.[3]

The coincident occurrence of a higher adhesion and migration after treatment with the analogues is not necessarily a contradiction, since a stronger adhesion increases the tractive power as part of the mechanism of migration.[17] The influence on these processes may be explained by intercalation of the compounds into the plasma membranes with subsequent modulation of signaling systems.[18] Therefore, target molecules modulated by these substances need to be identified.

Conclusion

These data give important information about the structure−function relationship of the glycosidic phospholipids. Introduction of an amino group into the glucose, in the form of glucosamine, diminishes the antiproliferative effect of Glc-PAF significantly. Additionally, our studies have identified the presented phospholipids as effective inducers of adhesion and migration. Further investigation should reveal whether glucosamine has a unique effect in promoting these cell

functions and whether this has any significance for physiological processes such as wound healing and angiogenesis. Since glucosamine-PAF (**1**) and glucosimide-PAF (**2**) have been found to have remarkably different cytotoxic and proliferative properties, further chemical modifications of the sugar moiety of these phospholipids can be expected to produce more interesting and useful compounds.

Experimental Section

All solutions were purified and dried in the usual way. Thin-layer chromatography (TLC) was performed on aluminum plates coated with silica gel 60 F_{254} (E. Merck), with visualization by treatment with a solution of 1% anisaldehyde/acetic acid and 10% sulfuric acid and heating at 150 °C. Flash chromatography was carried out with silica gel 60 (E. Merck, 40−63 μm). Optical rotations were determined at room temperature with a Perkin-Elmer P 241 polarimeter. NMR spectra were recorded with Bruker AC 250 or AM 270 instruments using TMS as an internal standard. Mass spectra (MS-FAB) were recorded with a Finnigan MAT 112 S. Elemental analyses were performed with a Perkin-Elmer 2400 CHN elemental analyzer. All chemicals were purchased from Aldrich.

1-*O*-Octadecyl-2,3-*O*-isopropylidene-*sn*-glycerol (4). 1 mL (8.06 mmol) of (*R*)-isopropylidene glycerol dissolved in 50 mL of dry tetrahydrofuran (THF) was added to a stirred suspension of 2 equiv of NaH (80% in paraffin, 0.5 g) in 50 mL of dry THF. After hydrogen generation had finished, 3.2 g (1.2 equiv) of octadecylbromide in 20 mL of dry THF was added and the reaction mixture was stirred overnight. The reaction mixture was heated to 80 °C, and further NaH (about 0.5 g) was added under TLC control. A complete conversion could not be achieved. The mixture was cooled in ice, and then, H_2O was added dropwise. The mixture was extracted with diethyl ether. After it was dried over sodium sulfate, the diethyl ether was evaporated and the residue was purified by flash chromatography (*n*-hexane/ethyl acetate, 10:1) to yield **4** (1.96 g, 74.5% yield) as a white wax. $C_{24}H_{48}O_3$ (384.641). TLC (*n*-hexane/ethyl acetate, 10:1): R_f 0.51. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.8 (t, J = 6.5 Hz, 3 H), 1.2−1.3 (m, 30 H), 1.35 (s, 3 H), 1.4 (s, 3 H), 1.5 (multiplet centerosymmetric (mc), 2 H), 3.3−3.5 (m, 4 H), 3.7 (dd, J_1 = 10 Hz, J_2 = 1 Hz, 1 H), 4.0 (dd, J_1 = 10 Hz, J_2 = 1 Hz, 1 H), 4.2 (mc, 1 H).

1-*O*-Octadecyl-*sn*-glycerol (5). 1.96 g (5.1 mmol) of acetonide **4** was dissolved in a solution of 100 mL of 60% AcOH/H_2O and 50 mL of THF. After being stirred overnight at 50 °C, the solvent was coevaporated three times with toluene to yield **5** (1.73 g, 98.5% yield) as a white powder. $C_{21}H_{44}O_3$ (344.577). TLC (*n*-hexane/ethyl acetate, 1:1): R_f 0.21. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.85 (t, J = 6.5 Hz, 3 H), 1.2−1.35 (m, 30 H), 1.5 (mc, 2 H), 2.3 (br, 1 H), 2.75 (br, 1 H), 3.4−3.55 (m, 4 H), 3.6−3.8 (m, 2 H), 3.85 (mc, 1 H).

1-*O*-Octadecyl-3-*O*-*tert*-butyldimethylsilyl-*sn*-glycerol (6). To a solution of 1.70 g (4.92 mmol) of diol **5** and 0.67 g (2 equiv) of imidazole in 30 mL of CH_2Cl_2 was added *tert*-butylchlorodimethylsilane (1.2 g, 8 mmol in 10 mL of CH_2Cl_2) at 0 °C. After being stirred at room temperature for 5 h, the reaction mixture was diluted with water (10 mL), then extracted with CH_2Cl_2, and dried over $CaCO_3$. The solvent was evaporated, and the residue was purified by flash chromatography (*n*-hexane/ethyl acetate, 6:1), giving 2.19 g (97% yield) of **6** as a white solid. $C_{27}H_{58}O_3Si$ (458.839). TLC (*n*-hexane/ethyl acetate, 6:1): R_f 0.31. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.05 (s, 6 H), 0.85 (m, 12 H), 1.2−1.3 (m, 30 H), 1.55 (mc, 2 H), 2.65 (br, 1 H), 3.35−3.45 (m, 4 H), 3.6 (m, 2 H), 3.8 (mc, 1 H).

1-*O*-Octadecyl-2-*O*-(3′,4′,6′-tri-*O*-acetyl-2′-deoxy-2′-dimethylmaleimido-β-D-glucopyranosyl)-3-*O*-*tert*-butyldimethylsilyl-*sn*-glycerol (7). 2.19 g (4.77 mmol) of **6** and 2.9 g (5.2 mmol) of *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-dimethylmaleimido-β-D-glucopyranosyl)trichloroacetimidate (prepared according to a published procedure[11]) were dissolved in 40 mL of dry CH_2Cl_2 and stirred for 10 min in an argon atmosphere. A 5 mL portion of TMSOTf (0.01 M in CH_2Cl_2) was added dropwise. After 3 h, the reaction mixture was neutralized by triethylamine and dried in vacuo. Flash chromatography (*n*-hexane/ethyl acetate, 3:1) yielded **7** (3.94 g, 97% yield) as a colorless solid. $C_{45}H_{79}O_{12}NSi$ (854.204). TLC (*n*-hexane/ethyl acetate, 2:1): R_f 0.33. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.00 (s, 6 H), 0.85 (m, 12 H), 1.15−1.30 (m, 30 H), 1.40 (m, 2 H), 1.85−2.10 (4 s, 15 H), 3.20 (m, 3 H), 3.30−3.50 (m, 2 H), 3.60−3.80 (m, 3 H), 3.90−4.10 (m, 2 H), 4.20 (dd, J_{gem} = 12.5 Hz, $J_{5,6}$ = 2.5 Hz, 1 H, 6-H), 5.05 (t, J = 10 Hz, 1 H, 4-H), 5.30 (d, J = 10 Hz, 1 H, 1-H), 5.60 (t, J = 10 Hz, 1 H, 3-H).

1-*O*-Octadecyl-2-*O*-(3′,4′,6′-tri-*O*-acetyl-2′-deoxy-2′-dimethylmaleimido-β-D-glucopyranosyl)-*sn*-glycerol (8). To a solution of 3.9 g (4.57 mmol) of **7** in 100 mL of CH_2Cl_2 was added $FeCl_3 \cdot 6H_2O$ (2.7 g, 2 equiv). After being stirred for 3.5 h at room temperature, the reaction mixture was diluted with 20 mL of H_2O and extracted with CH_2Cl_2. The organic layer was dried over sodium sulfate and the solvent evaporated in vacuo. The residue was purified by flash chromatography (*n*-hexane/ethyl acetate, 3:1), giving 2.75 g (81% yield) of **8**. $C_{39}H_{65}O_{12}N$ (739.942). TLC (*n*-hexane/ethyl acetate, 2:1): R_f 0.17. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.85 (t, J = 6.25, 3 H), 1.15−1.30 (m, 30 H), 1.40 (m, 2 H), 1.90−2.10 (4 s, 15 H), 2.80 (br, 1 H), 3.2 (m, 4 H), 3.50−3.60 (m, 2 H), 3.70 (m, 1 H), 3.80 (m, 1 H), 4.00−4.20 (m, 3 H), 5.05 (t, J = 10 Hz, 1 H, 4-H), 5.30 (d, J = 10 Hz, 1 H, 1-H), 5.65 (t, J = 10 Hz, 1 H, 3-H).

[1-*O*-Octadecyl-2-*O*-(2′-deoxy-2′-dimethylmaleimido-β-D-glucopyranosyl)-*sn*-glycero(3)]phosphorylcholine (2). Phosphoryl chloride (0.45 mL, 4.9 mmol) was distilled and diluted in 40 mL of dry chloroform. 2.5 mL (18 mmol) of dry triethylamine was added dropwise. The reaction mixture was cooled to 0 °C and treated with a solution of **8** (2.75 g, 3.72 mmol in 50 mL of dry $CHCl_3$). After being stirred for 3 h at 0 °C, a solution of choline tosylate (2.5 g, 9 mmol in 170 mL of dry pyridine) was added. The reaction mixture was stirred overnight at room temperature and diluted with a saturated solution of 3.5 g of $NaHCO_3$. The solvent was evaporated at 35 °C in vacuo and the residue dissolved in 100 mL of CH_2Cl_2/toluene (1:1). The insoluble ammonium salt was removed by filtration, and the solution was concentrated under reduced pressure. The residue was dissolved in 100 mL of THF and filtered to remove more ammonium salt. After evaporation in vacuo, the residue was purified by flash chromatography ((1) $CHCl_3/CH_3OH$, 5:1; (2) CH_3OH), giving 2.25 g (78% yield) of **2** as a white salt, which was recrystallized from methanol/acetone. $C_{38}H_{71}O_{12}N_2P$ (778.959). TLC (CH_3OH/H_2O, 5:1): R_f 0.27. $[\alpha]^{20}_D$: −12.38°. ^{1}H NMR (CD_3OD, 270 MHz): δ 0.90 (t, J = 6.25 Hz, 3 H), 1.20−1.35 (m, 30 H), 1.45 (mc, 2 H), 1.95 (s, 6 H), 3.25 (s, 9 H), 3.25−3.50 (m, 6 H), 3.60−3.75 (m, 4 H), 3.80−4.05 (m, 4 H), 4.20 (m, 1 H), 4.40 (m, 2 H), 5.20 (d, J = 7.5 Hz, 1 H, 1-H). ^{13}C NMR (CD_3OD, 63 MHz): δ 8.7, 14.5, 23.8, 27.2, 30.5, 30.8, 33.0, 54.8, 58.5, 60.4, 60.5, 62.4, 67.0, 67.1, 67.8, 71.4, 72.4, 72.5, 72.7, 78.2, 79.0, 79.1, 99.8, 138.2, 173.3. ^{31}P NMR (CD_3OD/D_2O, 1:1, 202 MHz): δ −0.623. IR (KBr): ν 3406 (s), 2923 (vs), 2853 (vs), 2361 (w), 1771 (w), 1706 (vs), 1640 (w), 1468 (m), 1407 (s), 1235 (s), 1088 (vs), 969 (m), 925 (w), 874 (w), 828 (w), 768 (w), 735 (m), 712 (s), 522 (m). MS (FAB pos., xenon, 3 kV, H_2O/DMSO/*m*-NO_2-benzyl alcohol), *m/z* (%): 801 (3.8), 779 (19.4), 742 (7.4), 510 (20.5), 224 (24.4), 184 (84.7). Anal. Calcd for $C_{38}H_{71}O_{12}N_2P$: C, 58.59; H, 9.19; N, 3.60. Found: C, 58.70; H, 9.21; N, 3.52.

[1-*O*-Octadecyl-2-*O*-(2′-deoxy-2′-amino-β-D-glucopyranosyl)-*sn*-glycero(3)]phosphorylcholine Hydrochloride (1). 250 mg (0.321 mmol) of imide **2** was dissolved in 6 mL of dioxane/H_2O (4:1). Sodium hydroxide (0.12 g, 3 mmol) was added, and the mixture was stirred for 24 h at room temperature. Ethylamine (70%, 0.06 mL, 0.75 mmol) was added and the pH adjusted to 5 with 1 N hydrochloric acid. After being stirred an additional 24 h, the solution was neutralized with potassium carbonate and dried in vacuo. The residue was purified by flash chromatography (CH_3OH/H_2O, 5:1) and recrystallized in methanol/acetone, yielding 135 mg (0.201, 63% yield) of **1** as a colorless solid. $[\alpha]^{20}_D$: −7.63°. TLC (CH_3OH/H_2O, 5:1): R_f 0.05. ^{1}H NMR (CD_3OD, 270 MHz): δ

0.80 (t, J = 6.25, 3 H), 1.10−1.25 (m, 30 H), 1.45 (mc, 2 H), 2.40 (m, 1 H), 3.10 (s, 9 H), 3.10−3.20 (m, 4 H), 3.35 (m, 2 H), 3.45−3.60 (m, 5 H), 3.70 (d, J = 12.5 Hz, 1 H), 3.90 (m, 3 H), 4.20 (m, 2 H), 4.30 (d, J = 7.5 Hz, 1 H, 1-H). ^{13}C NMR (CD_3OD, 63 MHz): δ 14.8, 24.0, 27.6, 30.8, 31.0, 31.1, 33.4, 55.0, 59.0, 60.8, 62.8, 67.0, 67.8, 71.5, 71.9, 72.9, 78.0, 78.6, 79.4, 79.5, 105.5. ^{31} P NMR (CD_3OD/D_2O, 1:1, 202 MHz): δ −0.547. IR (KBr): ν 3378 (s), 2923 (vs), 2853 (vs), 1708 (w), 1572 (m), 1468 (s), 1413 (m), 1384 (m), 1245 (s), 1087 (vs), 971 (s), 924 (w), 874 (w), 826 (w), 767 (w), 720 (w), 582 (w), 507 (w). MS (FAB pos., xenon, 3 kV, H_2O/DMSO/*m*-NO_2-benzyl alcohol), *m/z* (%): 692 (7.1), 671 (8.7), 670 (13.5), 633 (8.3), 510 (32.6), 224 (26.0), 184 (100). Anal. Calcd for $C_{32}H_{67}O_{10}N_2P\cdot HCl\cdot 1.5H_2O$: C, 52.34; H, 9.74; N, 3.81. Found: C, 52.22; H, 9.77; N, 3.64.

Cell Culture. HaCaT cells were grown in RPMI-medium, supplemented with heat inactivated fetal bovine serum (10%), penicillin/streptomycin (100 U/mL and 0.1 mg/mL, respectively), and l-glutamine (440 mg/L). Two days before an experiment, cells were adapted to defined keratinocyte-serum free medium + growth supplement (including insulin, EGF, and fibroblast growth factor) (GibcoBRL).

Cytotoxicity Test. As a marker for cytotoxicity, the loss of membrane integrity in dead cells was determined by staining with trypan blue. Subconfluent HaCaT cells were incubated for 24 h with glucosamine-PAF (**1**) and glucosimide-PAF (**2**), dissolved in serum-free keratinocyte growth medium in different concentrations. All but 500 μL of the medium was removed, and 500 μL of trypan blue (0.1%) was added. After 60 s, the dye was removed and the stained and unstained cells were determined.

Proliferation Test. The proliferation was determined by a colorimetric immunoassay, which is based on the incorporation of 5-bromo-2′-deoxyuridine (BRDU from Roche) into newly synthesized DNA. Subconfluent cells were incubated for 24 h in a 96-well microtiter plate with different concentrations of **1** or **2**. BRDU was added to a final concentration of 10 μmol/L. All the following steps were performed as described in the instruction manual. The cells were fixed, and the DNA was denaturated. Incorporated BRDU was detected by a peroxidase-conjugated BRDU antibody. Tetramethylbenzidine was added as a substrate, and the peroxidase-catalyzed color reaction was quantified at 405 nm in the enzyme-linked immunosorbent assay (ELISA) reader and compared to the untreated control.

Cell Adhesion Assay. Adherent HaCaT cells were incubated for 3 h with different concentrations of the analogues. For attachment assays, 96-well dishes were coated with collagen (20 μg/mL phosphate-buffered saline (PBS); Sigma, Munich, Germany) for 16 h at 4 °C. Unspecific binding was blocked with 1% bovine serum albumin (BSA) for 4 h at 4 °C. A concentration of 2.5×10^5 cells/mL was preincubated with a serum-free medium for 30 min and subsequently plated on the indicated matrix proteins. After 1 h of incubation at 37 °C, nonattached cells were removed by washing with PBS. Attached cells were fixed with 1% glutardialdehyde in PBS, stained with 0.1% crystal violet, and photometrically measured at 570 nm after Triton X-100 dye solubilization.[19] Untreated cells were taken as 100%.

Migration Assay. The tests were carried out with Transwell porous bottom dishes (Corning Costar Corp., diameter 6.5 mm, pore size 8.0 μm). To generate a positive matrix protein gradient, the following liquids were applied from the outside of the porous bottom dishes. First, they were coated for 30 min at room temperature with 50 μL of collagen (20 μg/mL of PBS; Sigma). The liquid was removed, and unspecific binding was blocked by 50 μL of 1% BSA. After 30 min, it was washed once with PBS. The lower chamber was filled with 500 μL/well keratinocyte growth medium, and the Transwell dishes were added. HaCaT cells had been incubated with the analogues for 3 h before the assay started. The cells were then trypsinized, and 100 μL of cell suspension (10^6 cells/mL keratinocyte growth medium) was plated inside each prepared porous bottom dish. After 6 h of incubation at 37 °C, the cell suspension was removed and the dishes were cleaned on the inside. The remaining cells outside the porous bottom dish were fixed in 500 μL of 3.7% paraformaldehyde, 0.025% saponin in PBS. After 30 min, the cells were washed with distilled water and stained with 500 μL of crystal violet (0.1%) for 30 min. The colored cells were counted under the microscope.

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References

(1) Demopoulos, C.; Pinckard, R.; Hanahan, D. Platelet activating factor: evidence for 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J. Biol. Chem.* **1979**, *254*, 9355−9358.

(2) Prescott, S. M.; Zimmerman, G.; Stafforini, D.; McIntyre T. Platelet-Activating Factor and Related Lipid Mediators. *Annu. Rev. Biochem.* **2000**, *69*, 419−445.

(3) Jung, I. D.; Lee, J.; Kyong, B. L.; Park, C. G.; Kim, Y. K.; Seo, D. W.; Park, D.; Lee, H. W.; Han, J.-W.; Lee, H. Y. Activation of p21-activated kinase 1 is required for lysophosphatidic acid-induced focal adhesion kinase phosphorylation and cell motility in human melanoma A2058 cells. *Eur. J. Biochem.* **2004**, *271*, 1557−1565.

(4) Bernatchez, P. N.; Tremblay, F.; Rollin, S.; Neagoe, P.-E.; Sirois, M. G. Sphingosine 1-Phosphate Effect on Endothelial Cell PAF Synthesis: Role in Cellular Migration. *J. Cell. Biochem.* **2003**, *90*, 719−731.

(5) Brachwitz, H.; Vollgraf, C. Analogs of Alkyllysophospholipids: Chemistry, Effects on the Molecular Level and Their Consequences for Normal and Malignant Cells. *Pharmacol. Ther.* **1995**, *66*, 39−82.

(6) Arthur, G.; Bittman, R. The inhibition of cell signaling pathways by antitumor ether lipids. *Biochim. Biophys. Acta* **1998**, *1390*, 85−102.

(7) Jha, T.; Sundar, S.; Thakur, C.; Bachmann, P.; Karbwang, J.; Fischer, C.; Voss, A.; Berman, J. Miltefosine, an Oral Agent, for the Treatment of Indian Visceral Leishmaniasis. *N. Engl. J. Med.* **1999**, *341*, 1795−1797.

(8) Wieder, T.; Reutter, W.; Orfanos, C. E.; Geilen, C. C. Mechanisms of action of phospholipid analogs as anticancer compounds. *Prog. Lipid Res.* **1999**, *38*, 249−259.

(9) Mickeleit, M.; Wieder, T.; Buchner, K.; Geilen, C.; Mulzer, J.; Reutter, W. Glc-PC, a New Type of Glucosidic Phospholipid. *Angew. Chem.* **1996**, *34*, 2667−2669.

(10) Mickeleit, M.; Wieder, T.; Arnold, M.; Geilen, C.; Mulzer, J.; Reutter, W. A Glucose-Containing Ether Lipid (Glc-PAF) as an Antiproliferative Analogue of the Platelet-Activating Factor. *Angew. Chem., Int. Ed.* **1998**, *37*, 351−353.

(11) Aly, M.; Castro-Palomino, J.; Ibrahim, E.; El-Ashry, E.; Schmidt; R. R. The Dimethylmaleoyl Group as Amino Protective Group − Application to the Synthesis of Glucosamine-Containing Oligosaccharide. *Eur. J. Org. Chem.* **1998**, 2305−2316.

(12) Ding, X.; Wang, W.; Kong, F. Detritylation of mono- and di-saccharide derivatives using ferric chloride hydrate. *Carbohydr. Res.* **1997**, *303*, 445−448.

(13) Hirth, G.; Saroka, H.; Bannwarth, W.; Barner, R. Synthese von Glycerylätherphosphatiden. Herstellung von 2-*O*-Acetyl-1-*O*-[(Z)-9-octadecenyl]-*sn*-glyceryl-3-phosphorylcholin (Oleyl-PAF), des Enantiomeren sowie einiger analoger, ungesättigter Verbindungen. *Helv. Chim. Acta* **1983**, *66*, 1210−1240.

(14) Boukamp, P.; Petrussevska, R.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig; N. E. Normal Keratinization in a Spontaneously Immortalized Aneuploid Human Keratinocyte Cell Line. *J. Cell Biol.* **1988**, *106*, 761−771.

(15) Erukulla, R.; Zhou, X.; Samadder, P.; Arthur, G.; Bittman, R. 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. *J. Med. Chem.* **1996**, *39*, 1545−1548.

(16) Schön, M.; Schön, M. P.; Geilen, C. C.; Hakyl, N.; Orfanos, C. E.; Reutter, W. Cell−matrix interactions of normal and transformed human keratinocytes in vitro are modulated by the synthetic phospholipid analogue hexadecylphosphocholine. *Br. J. Dermatol.* **1996**, *135*, 696−703.

(17) Libotte, T.; Kaiser, H.-W.; Alt, W.; Bretschneider, T. Polarity, Protrusion-Retraction Dynamics and Their Interplay during Keratinocyte Cell Migration. *Exp. Cell Res.* **2001**, *270*, 129−137.
(18) Wiese, A., Wieder, T.; Mickeleit, M.; Reinöhl, S.; Geilen, C. C.; Seydel, U.; Reutter, W. Structure-Dependent Effects of Glucose-Containing Analogs of Platelet Activating Factor (PAF) on Membrane Integrity. *Biol. Chem*. **2000**, *381*, 135−144.
(19) Danker, K.; Gabriel, B.; Heidrich, C.; Reutter, W. Focal Adhesion Kinase pp125^{FAK} and the α1 Integrin Subunit Are Constitutively Complexed in HaCaT Cells. *Exp. Cell Res.* **1998**, *239*, 326−331.
(20) Geilen, C. C.; Haase, A.; Wieder, T.; Arndt, D.; Zeisig, R.; Reutter, W. Phospholipid analogues: side chain- and polar head group-dependent effects on phosphatidylcholine biosynthesis. *J. Lipid Res.* **1994**, *35*, 625−632.
(21) Wieder, T.; Haase, A.; Geilen, C. C.; Orfanos, C. E. The Effect of Two Synthetic Phospholipids on Cell Proliferation and Phosphatidylcholine Biosynthesis in Madin-Darby canine Kidney Cells. *Lipids* **1995**, *30*, 389−393.

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