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Synthesis and antiproliferative activity of cytidine-5′-alkylphosphonophosphates and structurally related compounds

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Abstract

The chemical synthesis of cytidine-5′-alkyl- and cytidine-5′-alkyl(acyl)deoxyglycerophosphonophosphates is reported. The compounds obtained represent a novel class of cytostatically active agents based on phospholipids, which inhibit the growth of various tumor cell lines in vitro. They are phosphono analogs of the cytidine-5′-diphosphate-diacylglycerol (CDP-DAG) possessing a structurally modified lipid moiety and a phospholipase C-resistant P-C bond. The antiproliferative efficacy of the cytidine-5′-alkylphosphonophosphates strongly depends on the alkyl chain length. The cytidine-5′-hexadecylphosphonophosphate was found to be the most effective compound tested in this study. Its cytostatic effect was distinctly higher than that of the alkyl(acyl)deoxyglycero derivatives and of the corresponding diphosphates. The structures of the new compounds were confirmed by fast atom bombardment mass spectrometry (FAB). The FAB fragmentation pattern is discussed.

Keywords: Cytidine-5′-alkylphosphonophosphate; Cytidine-5′-alkyl(acyl)deoxyglycerophosphonophosphates; Phospholipid nucleoside conjugates; Antiproliferative activity; Fast atom bombardment mass spectrometry

1. Introduction

Recent work has indicated a much improved antitumor efficacy, relative to the parent drug, of some nucleoside-phospholipid conjugates such as ara-cytidinediphosphate-alkyl(acyl)glycerols and

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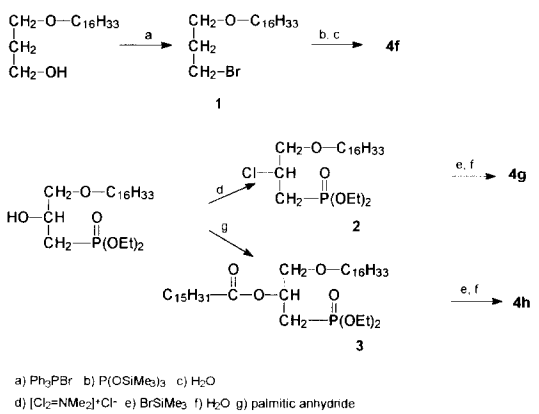


Fig. 1. Synthetic routes of novel 3-hexadecyloxypropyl-1-phosphonate **4f**, 2-chloro-3-hexadecyloxypropyl-1-phosphonate **4g** and 3-hexadecyloxy-2-palmitoyloxypropyl-1-phosphonate **4h**.

-thioglycerols [1–3]. These conjugates function as prodrugs of the nucleoside, which are able to overcome practical limitations of the parent nucleoside, such as rapid cleavage to biologically ineffective metabolites. In addition, they are more efficiently incorporated into cells and are converted enzymatically into the corresponding nucleoside monophosphate, the precursor of the effective nucleoside triphosphate. This concept of intracellular conversion of nucleoside-phospholipid conjugates into the nucleoside monophosphates and triphosphates has also been used in the case of virostatically active 3′-deoxythymidine and 2′,3′-dideoxycytidine derivatives [4–6].

Recently we have found that some ether lipid analogs of the naturally occurring cytidinediphosphate-diacylglycerol (CDP-DAG) exhibit antitumor activity though lacking a cytostatically active nucleoside component [7–9]. Apparently, the effects are mainly or exclusively attributed to the lipid component or their metabolites rather than to the nucleoside component.

In order to elucidate the additional structural requirements of the antitumoral efficiency in more detail and in search of more effective and selective antitumor compounds, we have prepared a number of cytidine-5′-phosphonophosphate-lipid conjugates of type **5** (Fig. 2) possessing a non-hydrolysable bond between the alkyl(acyl)-deoxyglyceryl or alkyl and the phosphono moiety, resistant to phospholipase C. Some of the newly synthesized compounds exhibit a strong cytostatic activity on various tumor cell lines in vitro. The antiproliferative efficacy of the cytidine-5′-alkylphosphonophosphates (**5a–e**) strongly depends on the length of the aliphatic alkyl chain and was in certain cases distinctly higher than that of the structurally related alkyldeoxyglycero analogs (**5f–i**) as well as of the corresponding diphosphates (**6**). The cytidine-5′-hexadecyl- and cytidine-5′-octadecylphosphonophosphate (**5d, e**) were found to be the most effective compounds tested in this study.

2. Experimental procedures

2.1. Material and methods

2.1.1. Compounds

Tris(trimethylsilyl)phosphite, trimethylsilylbro-

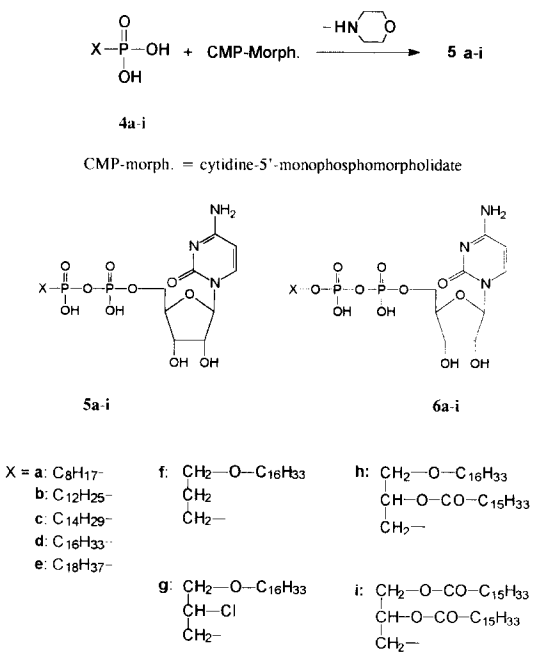


Fig. 2. The chemical synthesis of cytidine-5′-alkyl- (and alkyldeoxyglycero-) phosphono-phosphates (**5**), the chemical structures of **5** and their diphosphate analogs **6**.

mide, N-methyl-N-trismethylsilyl-acetamide, dichloromethylene-dimethylimmonium chloride were purchased from Fluka. Cytidine-5′-monophosphomorpholidate (4-morpholine-N,N'-dicyclohexylcarboxamidinium salt) was obtained from Sigma. Triphenylphosphinedibromide was prepared according to Schmitt et al. [10].

The alkylphosphonates (**4a–e**) were prepared by Michaelis-Arbuzov reaction of alkylbromides with triethylphosphite via the diethyl alkylphosphonates [11] and bis(trimethylsilyl) alkylphosphonates [12].

2,3-Dipalmitoyloxypropyl-1-phosphonate (**4i**) was prepared by a similar reaction starting from 2,3-dipalmitoyloxy-1-iodopropane and tris(trimethylsilyl)phosphite according to the procedure of P. W. Deroo et al. [13].

The diethyl 3-hexadecyloxy-2-hydroxypropyl-1-phosphonate was obtained from 2,3-epoxypropylphosphonate [14] by a BF_3 etherate-catalyzed ring-opening reaction in the presence of hexadecanol [15].

The cytidine-5′-(2-chloro-1-hexadecyloxypropyl-3)-diphosphate (**6g**), the cytidine-5′-alkyl (octyl, dodecyl, tetradecyl, hexadecyl, octadecyl) diphosphates (**6a–e**) were synthesized as previously described [9].

3-O-Hexadecylpropanediol-1,3 was prepared by alkylation of propanediol-1,3 with 1-bromohexadecane in the presence of sodium hydride in dimethyl formamide followed by column chromatographical resolution of the reaction mixture (results not published).

Pyridine was distilled and stored over calcium hydride. Toluene was distilled over calcium hydride and was stored over sodium. Dry ethanol-free chloroform was obtained by chromatography on Al_2O_3. Dry acetone was prepared by treatment with Na_2SO_4. All other solvents were distilled prior to use.

2.1.2. Cell cultures

Ehrlich ascites tumor cells (5×10^4) were suspended in 0.5 ml MEM supplemented with 5×10^{-5} M mercaptoethanol and 4% calf serum added to a microplate and grown for 2 days at 37°C in the presence of varying concentrations of the compounds to be tested. After incubation, the increase in cell number was evaluated with a cell counter (PS-4, Medical Budapest). Increase in cell number of the untreated controls was about threefold at this time.

H184 A1N4, a benzpyrene induced, immortalized, but nontumorigenic human breast epithelial cell line, was obtained from Dr. M. Stampfer, Lawrence Berkeley Laboratory, University of California, Berkeley, USA. MATU-cells and H184 A1N4-cells were grown as described in [8]. After addition of the compounds, the cell-cultures were continued for another day, the monolayers trypsinized and the cells were counted in a cell counter (PS-4, Medical Budapest). Increase in cell number of the untreated controls was about threefold at this time.

2.2. Analytic methods

Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates using solvent system A: hexane/ethyl acetate (9:1, v/v); solvent system B: hexane/ethyl acetate (1:1, v/v); solvent system C: heptane/ethyl acetate (2:3, v/v); solvent system D: chloroform/methanol/water (65:25:4, v/v/v); solvent system E: chloroform/methanol/ acetic acid/acetone/water (50:10:20:10:5, v/v/v/v/v); solvent system F: chloroform/methanol/water/acetic acid (50:30:4:8, v/v/v/v).

Phosphorus containing compounds were visualized with Zinzade's reagent modified according to Beiss [16].

Flash chromatography was performed with Merck silica gel 60 (0.015–0.040 mm, ASTM).

The purification of cytidine-5′-diphosphate and -phosphonophosphate-lipid derivatives of type **5** and **6** was performed by carboxymethylcellulose column chromatography (Servacel CM 52, sodium form) using chloroform/methanol mixtures with increasing polarity as eluent.

The melting points of all solids were determined on a Boetius melting point apparatus and are uncorrected.

The fast atom bombardment mass spectrometry was performed on a Finnigan MAT 212 reversed

geometry mass spectrometer coupled to a SS 188 data system. The instrument was operated in the negative or positive ion mode. Argon was fed into an ION TECH gun to produce neutrals of 6 keV energy. The monitor current and the discharge current were 0.04 and 2 mA, respectively. The substances dissolved in glycerol were loaded onto a copper target. The FAB mass spectra of the substances were obtained by subtracting the solvent spectrum from that of the solution using the appropriate computer program of the data system.

2.3. Chemical synthesis

2.3.1. 3-Bromo-1-hexadecyloxypropane (1) ([17] modified procedure)

1-O-Hexadecylpropanediol-1,3 (3.0 g, 10 mmol) in acetonitrile/dimethyl formamide (40 ml, 1:1, v/v) was dropped into a solution of triphenylphosphine dibromide [10] (13.7 g, 32.5 mmol) in dry acetone (90 ml). After 1 h the acetonitrile was rotary evaporated followed by the addition of water (100 ml). The slurry was extracted twice with heptane/diethyl ether (3:1, v/v). The combined upper layers were dried over Na_2SO_4 and were removed in vacuo. The residue was treated with heptane (6 ml) and the remaining solid was filtered off. Heptane evaporation yielded 3.4 g of a crude product which was purified by silicagel flash chromatography using heptane/ethyl acetate (98:2, v/v) as eluent.

Yield: 3.1 g (85%), m. p. 13–14°C, TLC: R_f 0.51 (solvent system A), high resolution mass spectra: m/z 364.3832 (364.3705 calc. for $C_{19}H_{39}^{81}BrO$) (M^+.).

2.3.2. Diethyl 2-chloro-3-hexadecyloxypropyl-1-phosphonate (2)

The solution of diethyl 2-hydroxy-3-O-hexadecyloxypropyl-1-phosphonate (2.183 g, 5 mmol) in 200 ml of dry pyridine was heated at 110°C followed by the addition of dichloromethylenedimethylimmonium chloride (2.233 g, 13.75 mmol). The mixture was stirred for 30 min at 110°C, 2 h at 80°C and 14 h at room temperature. Then pyridine was removed in vacuo and the residue was treated with ether/water (300 ml, 1:1 v/v). The upper phase was dried over Na_2SO_4, decoloured with charcoal and evaporated under reduced pressure. Purification was accomplished using silica gel column chromatography with solvent system C as eluent.

Yield: 0.746 g (32.8%), TLC: R_f 0.45 (solvent system C), elemental analysis: calc. for $C_{23}H_{48}ClO_4P$ (455.06) C 60.71 H 10.68; found C 60.49 H 10.68, pos. ion FAB-MS: m/z 455 $(M+H)^+$, neg. ion FAB-MS: m/z 425 $(M\text{-}C_2H_5)^-$.

2.3.3. Diethyl 2-hexadecanoyloxy-3-hexadecyloxypropyl-1-phosphonate (3)

A mixture of diethyl 2-hydroxy-3-hexadecyloxypropyl-1-phosphonate (4.7 g, 10.8 mmol), palmitic anhydride (8.27 g, 16.7 mmol) and pyrrolidinopyridine (1.63 g, 11 mmol) were combined in dry chloroform (180 ml) and stirred for 3 h at 35°C and then for 14 h at room temperature under a nitrogen atmosphere. Then chloroform (220 ml), methanol (120 ml) and 0.1 N H_2SO_4 (80 ml) were added. The mixture was shaken in a separation funnel and the organic phase was separated, dried over Na_2SO_4 and evaporated to dryness. Purification of the resulting product was carried out by column chromatography on 180 g silicagel (for 7 g of the crude product) using ethyl acetate/heptane (1:4 to 1:1, v/v) as eluent.

Yield: 6.41 g (88%), white solid, m. p. 33–38°C, TLC: R_f 0.46 (solvent system B), elemental analysis: calc. for $C_{39}H_{79}O_6P$ (675.03) C 69.39 H 11.80 P 4.59; found C 69.14 H 11.68 P 4.40, pos. ion FAB-MS: m/z 697 $(M+Na)^+$, m/z 675 $(M+H)^+$.

2.3.4. 3-Hexadecyloxypropyl-1-phosphonate (4f)

A mixture of 3-bromo-1-O-hexadecyloxypropane (1, 2 g, 15.5 mmol) and tris(trimethylsilyl)phosphite (16.4 g, 18.4 ml) was heated at 150°C with stirring under nitrogen for 18 h. Unreacted tris(trimethylsilyl)phosphite was removed in vacuo. The bis(trimethylsilyl) 3-hexa-decyloxypropyl-1-phosphonate was hydrolyzed by stirring with tetrahydrofurane/ water (35 ml, 4:1, v/v) for 12 h. The solvents were removed by evaporation in vacuo. The resulting residue was recrystallized from heptane and ethanol.

Yield 1.58 g (79%), white solid, m. p.: 88–91°C, TLC: R_f 0.68 (solvent system F), elemental analysis: calc. for C_{19} H_{41} O_4P (364.51) C 62.61 H 11.34, found C 62.35 H 11.50.

*2.3.5. 2-Chloro-3-hexadecyloxypropyl-1-phosphonate (**4g**)*

To a solution of diethyl 2-chloro-3-hexadecyloxypropyl-1-phosphonate (**2**) (0.746 g, 1.64 mmol) in alcohol-free, dry chloroform (25 ml), freshly distilled trimethylsilyl bromide (1.4 g, 9 mmol) in chloroform (5 ml) was added dropwise at 40°C under stirring in a nitrogen atmosphere over a period of 1 h. Then the solvents were removed in vacuo, and the resulting oil was taken up in tetrahydrofurane/water (20 ml, 4:1, v/v). The mixture was stirred at room temperature for 14 h. The solvents were removed in vacuo and the dark residue was recrystallized twice from heptane (15 ml) to give the pure product.

Yield: 0.4033 g (62%), white solid, m. p.: 65–68°C, TLC: R_f 0.36 (solvent system D), R_f 0.11 (solvent system C), elemental analysis: calc. for $C_{19}H_{40}ClO_4P$ (427.00) C 57.20 H 10.63, found C 57.46 H 10.68, neg. ion FAB–MS: 426 $(M - H)^-$.

*2.3.6. 2-Hexadecanoyl-3-hexadecyloxypropyl-1-phosphonate (**4h**)*

This compound was prepared as described for the synthesis of **4g** starting from diethyl 2-hexadecanoyl-3-hexadecyloxypropyl-1-phosphonate (**3**) (2.7 g, 4 mmol) and trimethylsilylbromide (3.4 g, 22 mmol). The crude product was recrystallized from heptane and twice from ethanol.

Yield: 1.8 g (74%), white crystals, m. p.: 75–77°C, TLC: R_f 0.38 (solvent system D), elemental analysis: calc. for $C_{35}H_{71}O_6P$ (618.92) C 67.92 H 11.566 P 5.01, found C 68.02 H 11.65 P 5.16, neg. ion FAB-MS: 617 $(M-H)^-$.

*2.3.7. 5′-Cytidine-alkyl (and -O-alkyldeoxyglycero) phosphonophosphates (**5a-i**)*

2.3.7.1. General procedure. A mixture of cytidine-5′-monophosphomorpholidate (4-morpholine-N,N′-dicyclohexylcarboxamidine salt containing 0.5 mmol H_2O, 1.15 mmol) and phosphonate (**4a-i**, 1 mmol) was dried by repeated evaporation from dry pyridine/toluene (4:1, 3x40 ml) under reduced pressure. The residue obtained was suspended in pyridine (40 ml) and the reaction mixture was stirred at 25–35°C for about 60–80 h under an anhydrous atmosphere. The progress of the reaction was monitored by TLC (silicagel, solvent system F). Then pyridine was evaporated to dryness. The residue was dissolved in chloroform/methanol/water (40 ml, 2:3:1, v/v/v) and the pH was adjusted to 2–3 by addition of 0.1 n HCl. The aqueous layer was extracted with chloroform (3x8 ml). The combined organic layers containing the crude product were washed with water (2x6 ml), dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was treated with dry acetone. The solid was filtered off and washed with 4-ml portions of dry acetone. The crude product was purified by column chromatography on Servacel CM 52 using chloroform/methanol mixtures with increasing polarity of the solvent similar to the procedure described by Comfurius et al. [18]. Fractions containing the pure product were evaporated to dryness to give the di-sodium salts of **5** as colourless solids.

The substances were obtained with yields of 20–40%.

UV-spectrum λ_{max}: 272 (methanol).

TLC: **5a–5d** R_f 0.28 (solvent system E), R_f 0.51 (solvent system F); **5e, 5g** R_f 0.33 (solvent system E), R_f 0.53 (solvent system F); **5f, 5h** R_f 0.41 (solvent system E), **5f** R_f 0.20 (solvent system F), **5h** R_f 0.24; substance **5i**, R_f 0.44 (solvent system E), R_f 0.27 (solvent system F).

2.3.7.2. Elemental analysis.

(1) **5a**: calc. for $C_{17}H_{29}Na_2O_{10}P_2 \times 2H_2O$ (579.39) C 35.24 H 5.74 N 7.25, found C 34.78 H 55.37 N 7.37.

(2) **5b**: calc. for $C_{21}H_{37}Na_2N_3O_{10}P_2 \times 2H_2O$ (635.50) C 39.69 H 6.50 N 6.61, found C 40.28 H 6.66 N 7.08.

(3) **5c**: calc. for $C_{23}H_{41}Na_2O_{10}P_2 \times 3H_2O$ (681.57) C 40.43 H 6.95 N 6.17, found C 39.92 H 6.64 N 5.82.

(4) **5d**: calc. for $C_{25}H_{45}Na_2O_{10}P_2 \times 2H_2O$ (691.60) C 43.42 H 7.14 N 6.08, found C 43.56 H 7.13 N 6.06.

Table 1
Diagnostic peaks and fragment ions in the negative FAB-MS-pectra of cytidine-5′-alkylphosphonophosphates (**5a–e**) with relative intensities (%) in parentheses

Compound	$[M-2H+Na]^-$	$[M-H]^-$	$[A+2H]^-$	$[A+H+Na]^-$	$[A+2H-3H_2O]^-$
5a	520 (82,93)	498 (100)	391 (36,58)	413 (19,51)	337 (39,02)
5b	576 (21,09)	554 (100)	447 (114,06)	469 (28,13)	393 (24,22)
5c	604 (25,93)	582 (100)	475 (20,99)	497 (12,3)	421 (27,16)
5d	632 (21,54)	610 (100)	503 (54,61)	525 (18,46)	449 (31,54)
5e	660 (37,04)	638 (100)	531 (37,04)	553 (33,33)	477 (44,44)
5f	690 (13,00)	668 (100)	561 (2,00)	—	507 (6,90)
5g	724 (400,0)	702 (100)	—	—	—
5h	944 (87,79)	922 (100)	—	—	—
5i	959 (41,18)	937 (100)	—	—	—
Compound	$[B]^-$	$[B-H_2O]^-$	$[B-H+Na]^-$	$[C]^-$	$[D]^-$
5a	273 (80,49)	255 (85,37)	295 (151,22)	322 (41,46)	193 (80,49)
5b	329 (24,22)	311 (87,11)	351 (70,70)	322 (50,00)	249 (56,64)
5c	357 (31,48)	339 (54,94)	379 (61,73)	322 (49,01)	277 (56,79)
5d	385 (40,00)	367 (105,38)	407 (53,85)	322 (112,31)	305 (84,61)
5e	413 (51,85)	395 (96,30)	435 (111,11)	322 (122,22)	333 (122,22)
5f	443 (6,20)	425 (18,10)	465 (12,80)	322 (55,50)	363 (16,70)
5g	—	—	—	322 (1,00)	—
5h	697 (1,20)	679 (0,75)	719 (1,48)	322 (2,50)	617 (1,75)
5i	712 (20,59)	—	—	322 (54,90)	632 (22,55)

(5) **5e**: calc. for $C_{27}H_{49}Na_2O_{10}P_2 \times 3H_2O$ (737.67) C 43.96 H 7.52 N 5.70, found C 43.95 H 7.19 N 5.71.

Scheme 1 shows the characteristic fragment ions in the negative FAB-MS of CDP-lipid compounds **5a–i** and their fragmentation pattern.

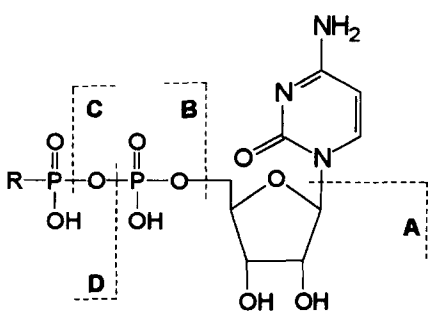
3. Results and discussion

3.1. Chemistry

The chemical synthesis and the structure of the newly synthesized phosphono analogs (**5a–i**) of the cytidine-5′-alkyl (and O-alkyldeoxyglycero) diphosphates (**6**) [9] used in this study are outlined in Fig. 2. The conjugates include cytidine-5′-alkylphosphonophosphates (**5a–e**) and the cytidine-5′-O-alkyldeoxyglycerophosphonophosphates (**5f–i**).

The new compounds represent phosphono analogs of the CDP-DAG possessing one long-chain O-alkyl group in the deoxyglycerol moiety and hydrogen (**5f**) or chlorine (**5g**) as additional substituents. Furthermore, the 2,3-di-O-acyl derivative (**5i**) as well as the alkyl-acyl derivative (**5h**) were synthesized for comparison. In addition it was of great interest to prepare compounds in which the modified glycerol part is substituted by only one long-chain alkyl, because in other cases the substitution of the glycerol moiety by a long alkyl group has resulted in compounds with an enlarged effective range at the molecular and cellular level [8,24].

The new compounds were obtained by condensation of the corresponding alkylphosphonates (**4**)



Scheme 1.

with CMP-morpholidate according to the procedure described for the synthesis of the CDP-DAG analogs [9,19]. The compounds were prepared in this fashion in yields of 25–35%. They were purified by chromatography on CM-52-cellulose using chloroform/methanol as eluent.

The synthetic routes to the new phosphonates **4f, g** and **h** are outlined in Fig. 1.

The starting compound for the preparation of 3-hexadecyloxypropyl-1-phosphonate (**4f**) was the commercially available 3-hexadecyloxypropane-1-ol, which was converted into the 1-bromo-3-hexadecyloxy-propane (**1**) by treatment with triphenylphosphine dibromide according to a modified procedure of S. L. Morris-Natschke et al. [17] with 85% yield. We found this method to be superior to that using PBr_3, since this reagent gave only poor yields and many side products. The Michael-Arbusov reaction of the 1-bromo-3-hexadecyloxypropane with tris(trimethylsilyl)-phosphite led to the formation of the corresponding bis-trimethylsilyl 3-hexadecyloxypropyl phosphonate which was easily hydrolyzed by stirring in THF/water at room temperature to give the 3-hexadecyloxypropyl-1- phosphonate (**4f**) with a yield of 79%.

The preparation of the 2-chloro- and 2-palmitoyloxy-substituted derivatives **4g** and **4h** started from the known diethyl 3-hexadecyloxy-2-hydroxypropyl-1-phosphonate [15]. Two types of reactions were attempted to obtain the diethyl 3-hexadecyloxy-2-chloropropyl-1-phosphonate (**2**). The diethyl 3-hexadecyloxy-2-hydroxypropyl-1-phosphonate was tosylated to give the 2-tosyloxy derivative. Nucleophilic substitution of tosylate by use of tetrabutylammonium chloride similar to the synthesis of alkylchlorodeoxyglycerols [20] resulted in a mixture containing considerable amounts of impurities which could not be easily separated from the desired product. A convenient method for the preparation of **2** was the one-step reaction of the OH-derivative with phosgeniminium chloride (dichloromethylenedimethylimmonium chloride). This procedure has already been successfully used for the preparation of chlorodeoxy carbohydrates [21].

The acylation of the hydroxy derivative to give **3** was performed using palmitic anhydride in the presence of pyrrolidinopyridine similar to the procedure described by J. T. Mason et al. [22]. In order to obtain the phosphonates **4**, the diethyl esters were converted into the bis-trimethylsilyl esters since the latter are easily hydrolyzable to the free acids under mild conditions [23]. The main difficulty in the preparation of the phosphonates **4g** and **4h** has been the incomplete transesterification of the diethyl ester into the bis-trimethylsilylester. Thus, considerable amounts of a chromatographically faster running side products have been isolated from the hydrolysis mixture, which could be identified as monoethyl esters of **4g** and **4h** by FAB-MS.

3.2. Mass Spectrometry (see Table 1)

The structure of the cytidine-5′-alkylphosphonophosphates **5a–i** was confirmed by negative ion fast atom bombardment (FAB) mass spectrometry. The major diagnostic peaks, their relative intensities and the fragmentation pattern of the compounds are summarized in Table 1. The spectra are characterized by the presence of intensive peaks of the $(M\text{-}H)^-$ ion and of the sodium adduct $(M\text{-}2H + Na)^-$. A further characteristic peak found at m/z 322 is derived from the cytidinemonophosphate (CMP) backbone and represents the $(CMP\text{-}H)^-$ ion (fragment C) produced by the loss of the R-P(O)OH moiety from the molecular ion.

In the case of the alkyl derivatives **5a–f**, elimination of the cytidyl residue from the molecular ion led to some fragment ions derived from A, e.g. $(A + 2H)^-$, $(A + H + Na\text{-}H_2O)^-$ and $(A + 2H\text{-}3H_2O)^-$. The fragment ions of type B are generated by expulsion of the nucleoside moiety and reflect the presence of the alkylphosphonophosphate group. In addition, fragment ions corresponding to $(B\text{-}H_2O)^-$ and $(B\text{-}H + Na)^-$ were observed in all spectra. Intensive peaks attributable to the $(alkylphosphonate\text{-}H)^-$ ion (fragment D) were also found in all spectra.

The spectra of the glycero derivatives **5g–i** do not show any fragment ions derived from A. Further fragments containing the glycero moiety (fragment B and D) are either completely lacking or are only present with low intensity, apparently due to their low stability.

Table 2
Cytostatic activity (halfmaximal inhibitory concentration, ID_{50}) of cytidinephosphonophosphate lipid conjugates **5a–i** and of cytidinediphosphate lipid conjugates **6a–i** against Ehrlich ascites tumor cells (EATC), the human immortalized mammary epithelian cell H184 A1N4 (H184) and the human mammary tumor cell MATU

Compound	ID_{50} [μM]		
	EATC	H184	MATU
5a	>100	>100	>100
5b	95	>100	>100
5c	65	35	48
5d	23	17	16
5e	37	12	16
5f	—	12	15
5g	—	46	30
5h	>100	>100	>100
5i	>100	>100	>100
6a	>100	>100	>100
6b	76	>100	>100
6c	—	>100	>100
6d	—	100	85
6e	>100	38	47
6f	4	—	—
6g	—	52	57
6i	>100	—	—

3.3. Cytostatic activity

All newly prepared compounds including the cytidine-5′-alkylphosphonophosphates **5a–e** and the O-alkyldeoxyglycero derivatives **5f–i** were tested for their antiproliferative activity against Ehrlich ascites tumor cells, the human immortalized mammary epithelian cell H184 A1N4 and the human mammary tumor cell MATU. In Table 2 the concentration of the compounds resulting in 50% inhibition of cell growth (IC_{50}) are listed. The data were compared with those of the corresponding diphosphates **6** in order to determine the significance of the phosphonophosphate group for activity. A significant increase of the cytostatic activity with the elongation of the alkyl group was observed beginning at C_{16}-alkyl and C_{14}-alkyl in the case of the diphosphates and the phosphonophosphates, respectively. In comparison to the diphosphates, the corresponding phosphonophosphates show a distinctly higher antiproliferative efficacy presumably due to the increased biostability of the P-C bond against cleavage by phospholipase C. The introduction of a halogen-containing glycerol or deoxyglycerol moiety into both the phosphonates and the diphosphates to give **5g** and **6g** causes a decrease of the cytostatic activity compared with the C_{16}-alkyl derivative **5d** and the C_{18}-alkyl derivative **6e**, respectively. In contrast, the 3-O-hexadecylpropyl derivative, structurally stronger related to the alkyl derivatives, is of similar efficacy as the C_{18}-alkyl derivative.

By comparing the antiproliferative activity of the compounds on the different cell lines studied, we found no obvious differences with the exception of the compound **6e**.

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