O-Phosphonatomethylcholine, Its Analogues, Alkyl Esters, and Their Biological Activity[1]

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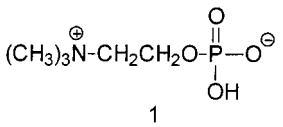
O-Phosphonatomethylcholine, an isopolar phosphocholine analogue with a phosphonomethyl ether group replacing a phosphomonoester residue, was prepared by reaction of diisopropyl 2-chloroethoxymethylphosphonate with dimethylamine followed by quaternization of the thus-obtained diisopropyl 2-dimethylaminoethoxymethylphosphonate with iodomethane; the ester groups in the quaternary intermediate were cleaved with bromotrimethylsilane. Replacement of dimethylamine in the reaction sequence by morpholine and/or pyrrolidine gave the *N*-methylmorpholinium or *N*-methylpyrrolidinium analogues of *O*-phosphonatomethylcholine. Reaction of *O*-phosphonomethylcholine monotetrabutylammonium salt with 1-bromoalkanes in acetonitrile afforded a series of the corresponding monoalkyl (C_{10}−C_{16}) esters. None of these compounds except for the hexadecyl ester exhibited any appreciable cytostatic activity against DU-145, H460, HT-29, or MES-SA cell lines in vitro (evaluated by ^{3}H−Thd incorporation assay). The hexadecyl ester exhibited modest in vitro cytotoxic activity comparable to that of the anticancer drug miltefosine (hexadecyl *O*-phosphocholine). In vivo evaluation of hexadecyl *O*-phosphonomethylcholine [transplanted SD lymphoma in inbred SD/cub rats, 10 mg kg^{-1} day^{-1} intratumoral injection for 10 days] resulted in a 40% decrease in lymphoma mass.

Introduction

Phosphocholine (**1**) is one of the fundamental metabo-



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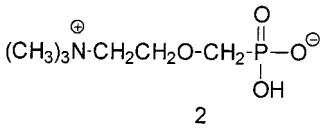

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lites. In biosynthetic pathways relevant to a complex lipid metabolism, phosphocholine constitutes a part of the structure of phosphatidylcholine wherefrom it is formed by hydrolysis catalyzed with phospholipase C. CTP/phosphocholine cytidylyltransferase catalyzes the formation of CDP−choline from CTP and phosphocholine. CDP−choline in turn transfers the phosphocholine residue to diacylglycerols, leading to phosphatidylcholine; this reaction is catalyzed by CDP−choline/1,2-diacylglycerol cholinephosphotransferase. The CDP−choline analogue is also formed from the antiviral acyclic nucleotide analogue, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (cidofovir, Vistide),[2] which was discovered and studied in detail in this laboratory,[3] as well as in others.[4] This drug is approved for treatment of CMV retinitis;[5] however, it is active against most other DNA viruses as well and its application presently involves other indications (e.g., papillomatoses, etc.[6]). It is generally accepted that this metabolite (HPMPCpcholine) generates the HPMPC diphosphate (dCTP analogue) by phosphorolysis catalyzed with CTP/phosphocholine cytidylyltransferase. This slow process might explain the long duration of the antiviral effect and the resulting infrequent regimen of drug application.

In this context we considered it of interest to synthesize an analogue of phosphocholine **2** bearing an enzymatically nondegradable phosphonomethyl ether linkage instead of the phosphoric acid ester group and to investigate its biological activity. Compound **2** is isopolar with phosphocholine; its structure contains a methylene group inserted between the phosphorus atom and choline hydroxyl group, which warrants easy adaptability of the conformation and resistance against enzymatic degradation.

The phosphocholine structure is also present in anticancer compounds derived from membrane phospholipids. Lysophospholipids or etherphospholipids are also clinically used in the therapy of selected tumors. Among these compounds there are, for example, eldefosine (phosphocholine 3-*O*-octadecyl-2-*O*-methylglyceryl ester), its 3-octadecylsulfanyl analogue ilmofosine, and, in particular, miltefosine (phosphocholine hexadecyl ester),[7] which is used in topical application (Miltex) in the therapy of skin neoplasias, malignant melanoma, skin forms of B- and T-lymphomas, and skin metastases of solid tumors.[8] Supposedly these drugs are incorporated into the lipid bilayer of the cellular membrane, thereby affecting the transfer of cellular signals.[9] Erucylphosphocholine is another prospective alkylphosphocholine derivative that shows cytostatic activity against leukemic cell lines. It is highly active against human brain tumor cell lines[10] and human urinary bladder carcinoma cell lines[11] in vitro. It also demonstrates a distinctly improved therapeutic efficacy on iv administration.[12] Therefore, we also targeted our study

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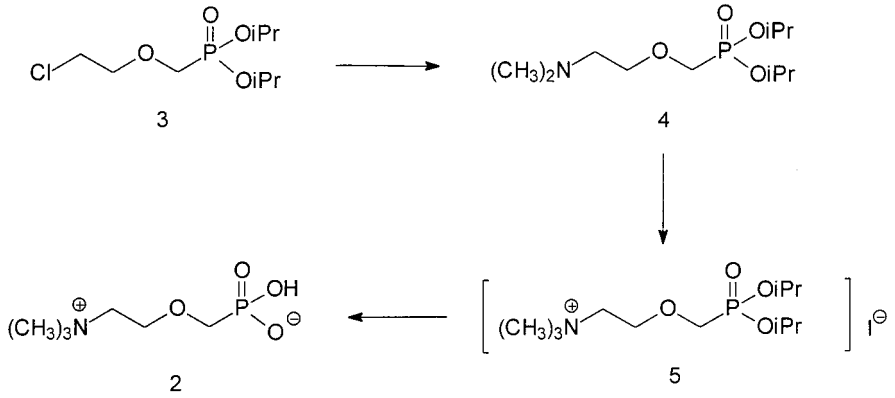
Scheme 1



toward the synthesis of long-chain alkyl esters **8** derived from the phosphocholine analogue **2** and the verification of their cytostatic activity.

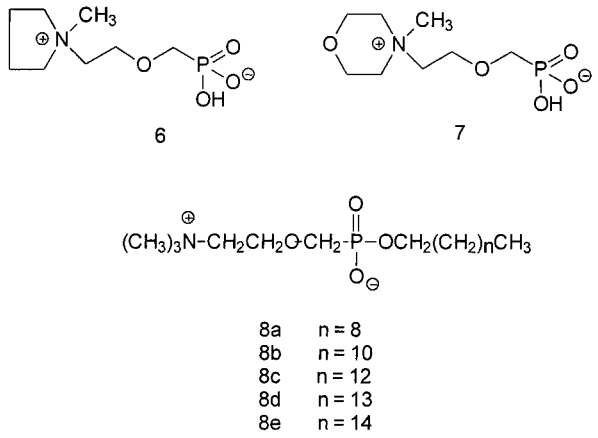
Chemistry

Synthesis of Phosphocholine Analogues. Instead of introducing the phosphonomethyl ether group by condensation of the hydroxyl group of the polar choline with dialkyl *p*-toluenesulfonyloxymethylphosphonate,[13] we made use of the conversion of the C−Cl linkage to the $(CH_3)_3N^+$−C grouping characteristic of the choline molecule. The synthesis was performed in three steps starting with diisopropyl 2-chloroethoxymethylphosphonate ester **3**,[14] whose ester linkage is stable during the treatment with amines (Scheme 1). It was heated with excess ethanolic[15] dimethylamine in an autoclave and was evaporated. After removal of the inorganic salt, the crude intermediate **4** was treated with neat iodomethane. The quaternary salt **5**, which was formed in an exothermic reaction, was readily crystallized by addition of ether. It was then treated with bromotrimethylsilane and subsequently hydrolyzed to afford crude compound **2**. It was desalted by chromatography on a Dowex 1 in OH^- form; compound **2** was eluted with water or with diluted acetic acid solutions. It crystallized as a zwitterionic monohydrate.

Analogously, the 2-chloroethoxymethylphosphonate **3** gave, by treatment with pyrrolidine and subsequent methylation followed by ester cleavage with bromotrimethylsilane, the *N*-methylpyrrolidinium analogue **6** (Chart 1), while analogous replacement of dimethylamine in the first reaction step by morpholine afforded, after quaternization with iodomethane, the *N*-methylmorpholinium derivative **7** (Chart 1).

Synthesis of Alkyl *O*-Phosphonomethylcholines (8). Direct esterification based on the phosphomonoester-activating agents, such as *N*,*N*′-dicyclohexylcarbodiimide, was not applicable to the zwitterionic compounds. Nor did the reaction of the disodium salt of the compound **2** with 1-haloalkanes offer satisfactory ester yields. A procedure analogous to the method described for the synthesis of alkyl phosphocholines[16] was therefore used to prepare the monoalkyl *O*-phosphonomethylcholines (**8**; Chart 1). Compound **2** was first transformed to its monotetrabutylammonium salt by neutralization of its aqueous solution with 1 equiv of aqueous tetrabutylammonium hydroxide. The dried salt was treated with excess 1-bromoalkane in acetonitrile at reflux temperature. The purification of the product

Chart 1



from tetrabutylammonium bromide formed in the reaction was achieved by chromatography of the crude reaction product on silica gel. This method was applied to the preparation of a series of monoalkyl esters of *O*-phosphonomethylcholine **8** with primary alcohols.

It is also possible to prepare the esters **8** by activation of the parent zwitterionic compound **2** with Vilsmeier−Haack−Arnold reagent (dimethylchloromethyleneammonium chloride), prepared in situ by the reaction of triphosgene (trichloromethyl chlorocarbonate) with dimethylformamide and subsequent treatment with excess aliphatic alcohol.[17] However, it is difficult to control the exothermic activation reaction in larger scale.

Structures of all prepared compounds **8** were confirmed by ^1H and ^{13}C NMR spectra. NMR data are given in Experimental Section. The presence of the phosphorus atom is manifested by characteristic *J*(P,H) and *J*(P,C) couplings over one to three bonds (Figure 1), which were also helpful for the structural assignment of the involved proton and carbon signals together with ^1H,^{13}C-heterocorrelated HMQC spectra. Carbon and mainly proton signals of aliphatic chains in **8** are heavily overlapping and therefore could not be completely structurally assigned.

Biology

In vitro evaluation of the thus-prepared *O*-phosphonomethylcholines **8** was made in DU-145 (prostate carcinoma), HT-29 (colon adenocarcinoma), NCI-H460 (nonsmall cell lung carcinoma), and MES-SA (uterine sarcoma) cells by determination of ^3H-labeled thymidine

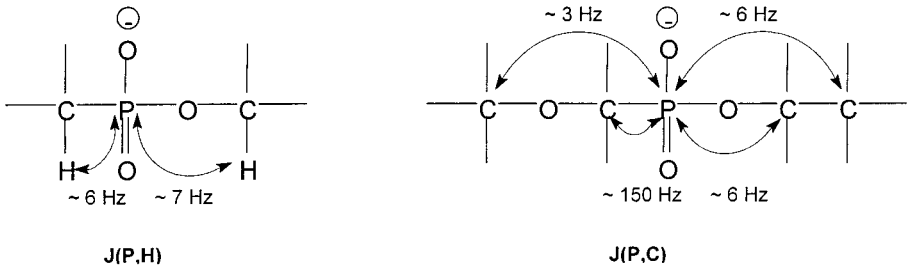


Figure 1. Typical *J*(P,H) and *J*(P,C) couplings in the structural fragment of compounds **8**.

Table 1. Cytotoxic Activity in Vitro (Average IC_{50} [μM]) of Series **8** Compounds[a]

agent	cell line			
	DU-145	H460	HT-29	MES−SA
8a	*b*	*b*	*b*	*b*
8b	132.98	137.59	*b*	*b*
8c	*b*	40.31	12.17	*b*
8d	19.855	17.997	NC	9.594
8e	17.163	15.709	4.755	2.109
gemcitabine	0.0356	0.0078	0.0030	0.0092
miltefosine	24.92	6.62	6.15	5.35
paclitaxel	0.00489	0.00014	0.00016	0.00004
vincristine	0.00112	0.00013	0.00001	0.00002

[a] Average of three to five determinations. Assayed by inhibition of ^{3}H−Thd incorporation. [b] Not calculable generally because of a lack of sufficient compound activity.

incorporation into the DNA of the cells grown in the presence of the tested compounds. The series of test compounds **8** was supplemented with standard cytotoxic agents (paclitaxel, vincristine, and gemcitabine) and with the structurally related miltefosine (hdPC) as positive controls. The IC_{50} (50% inhibitory concentration) data summarized in Table 1 demonstrate that the shorter-chain (C_{10}−C_{14}) alkyl esters (**8a**−**c**) are essentially inactive in vitro. A marginal cytotoxic effect was indicated for the pentadecyl ester **8d**, and more significant activity was observed with the hexadecyl ester **8e** only. Under the assay conditions used, the IC_{50} value for hexadecyl ester **8e** was considerably greater than for vincristine, gemcitabine, or paclitaxel; however, IC_{50} values obtained for hexadecyl ester **8e** were comparable to IC_{50} values obtained with miltefosine.

Therefore, the anticancer activity of alkyl *O*-phosphonomethylcholines was also examined in an animal model in vivo, evaluating the tumor progression of transplanted SD lymphoma in inbred rats.[18] When the tumor mass data for nontreated animals were compared with data for animals treated for 10 days once daily at 10 mg/kg dosage with hexadecyl (**8e**) and tetradecyl ester (**8c**) directly to the site of the growing lymphoma, an approximately 40% tumor mass decrease was encountered with hexadecyl *O*-phosphonomethylcholine (**8e**) (Table 2).

In conclusion, this paper describes a synthesis of *O*-phosphonatomethylcholine (**2**), a novel isopolar analogue of phosphocholine with catabolically stable P−C linkage replacing phosphate ester bond. It also opens a synthetic approach to its long-chain alkyl esters **8** as potential membrane-modifying cytostatics. The cytostatic activity was experimentally proven for the hexadecyl ester **8e** in the animal model of transplantable SD leukemia.

Analogues of alkyl phosphocholines have another use in chemotherapy. Hexadecyl phosphocholine (miltefosine) exhibits significant therapeutic effect on the intestinal form of leishmaniasis,[19] the disease caused by the *Leishmania donovani* parasite, which is transmitted by *Phlebotomus* fly (kala-azar fever afflicts 3 million patients a year in India, with mortality reaching 98% in untreated cases). The antiparasitic activity of the products described in this paper remains to be examined.

Table 2. Lymphoma Mass of Animals Treated with Compounds **8**

		O-phosphonomethylcholine alkyl ester (**8**)	
	control	tetradecyl (**8c**)	hexadecyl (**8e**)
lymphoma mass (g)	13.45	9.38	7.95
standard deviation	2.62	3.57	1.29
t-test		0.083	0.014
lymphoma mass decrease (%)	0	30	41

Experimental Section

Unless otherwise stated, solvents were evaporated at 40 °C at 2 kPa and compounds were dried at 2 kPa over P_2O_5. Melting points were determined on a Büchi melting point apparatus. TLC was performed on Silufol UV254 plates (Kavalier Votice, Czech Republic) in chloroform/ethanol (4:1). NMR spectra were measured on an FT NMR spectrometer, Varian UNITY 500 (^{1}H at 500 MHz and ^{13}C at 125.7 MHz frequency), in $CDCl_3$ or D_2O. Mass spectra were measured on a ZAB-EQ (VG Analytical) spectrometer using FAB (ionization by Xe, accelerating voltage 8 kV, glycerol matrix).

Materials. Bromotrimethylsilane, trichloromethyl chlorocarbonate, 1-hexadecanol, iodomethane, and dimethylamine (30% solution in ethanol or in water) were purchased from Fluka (Switzerland). Pyrrolidine, morpholine, and all 1-bromoalkanes were obtained from Sigma-Aldrich (Praha, Czech Republic). Dimethylformamide and acetonitrile were distilled from P_2O_5 and stored over molecular sieves (4 Å). Gemcitabine (Eli Lilly Co., Indianapolis, Indiana), paclitaxel and vincristine (both from Mead-Johnson, Evansville, Illinois), and hexadecylphosphocholine (hdPC, Anatrace, Maumee, Ohio) were obtained as commercial pharmaceutical preparations.

***O*-Phosphonatomethylcholine (2). Method A.** Diisopropyl 2-chloroethoxymethylphosphonate (**3**) (50 g, 0.193 mol) and 30% dimethylamine solution in ethanol (140 mL) were heated in an autoclave under stirring for 24 h at 120 °C, and the mixture was evaporated in vacuo. The residue was dissolved in benzene (250 mL) and washed with saturated potassium carbonate solution (2 × 50 mL). The organic layer was dried with potassium carbonate, filtered, and evaporated in vacuo. The oily residue of compound **4** was placed in a 500 mL flask with a magnetic stirrer and reflux condenser. Iodomethane (30 mL) was rapidly added through the condenser under vigorous stirring, and after the exothermic reaction subsided, the mixture was left to stand for 16 h at ambient temperature. Dry ether (200 mL) was added, and the mixture was stirred for 20 min. The crystalline product was filtered by suction, washed with dry ether, and dried in vacuo.

Yield, 46 g (58.2%) of quaternary salt **5**, mp 72 °C. Anal. ($C_{12}H_{29}INO_4P$) C, H, I, N, P. ^{1}H NMR spectrum ($CDCl_3$): δ 4.72 dh, 2 H, J(H,P) = 7.7, J(H,H) = 6.2 (2 × C*H*(CH_3)$_2$); 4.15 m, 2H (N^+−CH_2); 4.05 m, 2H (N−CH_2-*CH_2*−O); 3.82 d, 2H, J(H,P) = 7.6 (P−CH_2−O); 3.53 s, 9H [N^+(CH_3)$_3$]; 1.34 d, 12 H, J = 6.2 [(*CH_3*)$_2$CH]. ^{13}C NMR ($CDCl_3$): δ 71.47 d, ^2J(C,P) = 16.8 (P(O−*C*H(CH_3)$_2$)$_2$); 66.83 d, ^3J(C,P) = 8.8 (−CH_2−O−); 65.94 d, ^1J(C,P) = 165.0 (O−CH_2−P); 65.80 (N−CH_2−); 55.03 (N(CH_3)$_3$); 24.16 d, ^3J(C,P) = 4.4 and 24.08 d, ^3J(C,P) = 3.9 (P(O−CH(*C*H_3)$_2$)$_2$).

Bromotrimethylsilane (40 mL) was added to a solution of this compound in acetonitrile (250 mL), and the mixture was left to stand overnight at ambient temperature. The volatiles were evaporated in vacuo, and the residue was codistilled with toluene (3 × 100 mL). Water (200 mL) was added, and the solution was evaporated in vacuo. The residue in water (200 mL) was neutralized by addition of Dowex 1 × 2 (OH^- form), and the resulting suspension was poured onto the column (200 mL) of the same resin. The column was washed with water (500 mL), the combined eluates were evaporated to dryness, the residue was coevaporated with ethanol (2 × 100 mL) and finally crystallized from ethanol. Yield, 23 g (95%) of *O*-phosphonatomethylcholine monohydrate (**2**), mp 265 °C. Anal. ($C_6H_{16}NO_4P·H_2O$) C, H, N, P. ^{1}H NMR (D_2O): δ 3.99 m, 2H (N^+−CH_2); 3.64 d, 2H, J(H,P) = 8.9 (P−CH_2−O); 3.58 m, 2H (N−CH_2-*CH_2*−O); 3.17 s, 9H [N^+(CH_3)$_3$]. ^{13}C NMR (D_2O): δ 71.65 d, ^1J(C,P) = 151.3 (O−CH_2−P); 68.88 d, ^3J(C,P) = 11.7 (−CH_2−O-); 68.35 (N−CH_2−); 56.85 (N(CH_3)$_3$). MS, *m*/*z*: 198 (MH^+).

Method B. A mixture of diisopropyl 2-chloroethoxymethylphosphonate (**3**) (50 g, 0.193 mol) and 30% aqueous dimethylamine solution (100 mL) was heated in an autoclave for 8 h at 120 °C under stirring and thereafter was evaporated in vacuo. The residue was codistilled with water (100 mL) and 20% aqueous KOH solution (100 mL) was added, and the mixture was extracted with benzene (2 × 100 mL). The combined extracts were dried with potassium carbonate, filtered, and evaporated in vacuo. The oily crude compound **4** was placed in a 500 mL flask equipped with a magnetic stirrer and efficient reflux condenser. Iodomethane (30 mL) was rapidly added through the condenser under stirring, and after the exothermic reaction had ceased, the mixture was left to stand at ambient temperature overnight. Dry ether (200 mL) was added, and the mixture was stirred for 20 min. The crystalline product **5** was filtered by suction, washed with dry ether, and dried in vacuo. Further workup was performed as described in method A. Yield, 18 g (43.3%) of *O*-phosphonatomethylcholine monohydrate (**2**), mp 265 °C.

Decyl *O*-Phosphonomethylcholine (8a). General Method for Preparation of Alkyl *O*-Phosphonomethylcholines (8). A solution of compound **2** (4.3 g, 20 mmol) in water (50 mL) was neutralized by 20% aqueous tetrabutylammonium hydroxide solution to pH 7.5, the resulting solution was evaporated in vacuo, and the residue was codistilled successively with ethanol (3 × 50 mL) and toluene (2 × 50 mL) and was dried overnight at 13 Pa over phosphorus pentoxide. Acetonitrile (80 mL) and 1-bromodecane (4.2 mL, 20 mmol) were added to the residue, and the mixture was stirred for 2 h in a closed flask and then heated at reflux temperature for an additional 3 h. The mixture was evaporated in vacuo, and the residue was decanted with ether (3 × 100 mL) and adsorbed from methanolic solution on silica gel (50 g). This material was applied on a column (300 mL) of silica gel in chloroform, and the column was eluted with a chloroform/methanol mixture (4:1) to remove tetrabutylammonium bromide (TLC silica gel, chloroform/methanol 4:1, R_f = 0.40). The column was then eluted with methanol to afford the product fraction. It was evaporated in vacuo, and the residue was stirred with hexane to afford crystalline compound **8a**, which was filtered by suction and dried in vacuo. Yield, 5.2 g (77%), mp 131 °C. Anal. ($C_{16}H_{36}NO_4P$) C, H, N, P. ^{1}H NMR ($CDCl_3$): δ 4.19 m, 2H (N^+−CH_2); 3.80 q, 2H, J = 6.7 (P−O−CH_2); 3.74 m, 2H (N−CH_2-*CH_2*−O); 3.62 d, 2H, J = 6.1 (P−CH_2−O); 3.48 s, 9H [N^+(CH_3)$_3$]; 1.55 m, 2H, and 1.24−1.31 m, 14 H [−(CH_2)$_8$−]; 0.88 t, 3H, J = 7.0 [−(CH_2)$_8$−*CH_3*]. ^{13}C NMR ($CDCl_3$): δ 66.73 d, ^1J(C,P) = 149.4 (O−CH_2−P); 65.90 (N−CH_2−); 65.46 d, ^3J(C,P) = 3.4 (−CH_2−O−); 64.43 d, ^2J(C,P) = 5.9 (P−O−CH_2); 54.26 (N(CH_3)$_3$); 31.35 d, ^3J(C,P) = 5.8 (−P−O−C−CH_2); 31.81; 29.59; 29.52; 29.38; 29.23; 25.83 and 22.58 (−(CH_2)$_7$−); 14.02 (CH_3). MS, *m*/*z*: 338 (MH^+).

Dodecyl *O*-Phosphonomethylcholine (8b). Yield, 5.2 g (71%), mp 243 °C. Anal. ($C_{18}H_{40}NO_4P$) C, H, N, P. ^{1}H NMR ($CDCl_3$): δ 4.18 m, 2H (N^+−CH_2); 3.80 q, 2H, J = 7.1 (P−O−CH_2); 3.74 m, 2H (N−CH_2-*CH_2*−O); 3.62 d, 2H, J = 6.1 (O−CH_2−P); 3.49 s, 9H [N^+(CH_3)$_3$]; 1.55 m, 2H, and 1.24−1.31 m, 18 H [−(CH_2)$_{10}$−]; 0.88 t, 3H, J = 7.0 [C−*CH_3*]. ^{13}C NMR ($CDCl_3$): δ 66.74 d, ^1J(C,P) = 149.9 (O−CH_2−P); 65.92 (N−CH_2−); 65.47 d, ^3J(C,P) = 2.9 (−CH_2−O−); 64.44 d, ^2J(C,P) = 5.4 (P−O−CH_2); 54.26 (N(CH_3)$_3$); 31.36 d, ^3J(C,P) = 5.8 (−P−O−C−CH_2); 31.82; 29.61; 29.59; 29.57(2); 29.39; 29.26; 25.84 and 22.59 (−(CH_2)$_9$−); 14.02 (CH_3). MS, *m*/*z*: 336 (MH^+).

Tetradecyl *O*-Phosphonomethylcholine (8c). Yield, 5.6 g (71%), mp 244 °C. Anal. ($C_{20}H_{44}NO_4P$) C, H, N, P. ^{1}H NMR ($CDCl_3$): δ 4.18 m, 2H (N^+−CH_2); 3.80 q, 2H, J = 6.7 (P−O−CH_2); 3.75 m, 2H (N−CH_2-*CH_2*−O); 3.62 d, 2H, J = 6.0 (P−CH_2−O); 3.49 s, 9H [N^+(CH_3)$_3$]; 1.55 m, 2H, 1.25−1.30 m, 20 H [−(CH_2)$_{11}$−]; 0.88 t, 3H, J = 7.0 [−(CH_2)$_{12}$−*CH_3*]. ^{13}C NMR ($CDCl_3$): δ 66.71 d, ^1J(C,P) = 150.4 (O−CH_2−P); 65.95 (N−CH_2−); 65.43 d, ^3J(C,P) = 3.0 (−CH_2−O−); 64.45 d, ^2J(C,P) = 5.9 (P−O−CH_2); 54.27 (N(CH_3)$_3$); 31.36 d, ^3J(C,P) = 5.9 (−P−O−C−CH_2); 31.84; 29.62 (3); 29.60; 29.59(2); 29.40; 29.28; 25.84 and 22.60 (−(CH_2)$_{11}$−); 14.04 (CH_3). MS, *m*/*z*: 394 (MH^+).

Pentadecyl *O*-Phosphonomethylcholine (8d). Yield, 5.8 g (71%), mp 143 °C. Anal. ($C_{21}H_{46}NO_4P$) C, H, N, P. ^{1}H NMR ($CDCl_3$): δ 4.19 m, 2H (N^+−CH_2); 3.80 q, 2H, J = 7.1 (P−O−CH_2); 3.75 m, 2H (N−CH_2-*CH_2*−O); 3.63 d, 2H, J = 6.2 (P−CH_2−O); 3.49 s, 9H [N^+(CH_3)$_3$]; 1.56 m, 2H, 1.25−1.30 m, 24 H [−(CH_2)$_{13}$−]; 0.88 t, 3H, J = 7.0 [−(CH_2)$_{13}$−*CH_3*]. ^{13}C NMR ($CDCl_3$): δ 66.73 d, ^1J(C,P) = 149.9 (O−CH_2−P); 65.89 (N−CH_2-); 65.47 d, ^3J(C,P) = 3.4 (−CH_2−O−); 64.43 d, ^2J(C,P) = 5.9 (P−O−CH_2); 54.24 (N(CH_3)$_3$); 31.36 d, ^3J(C,P) = 5.8 (−P−O−C−CH_2); 31.83; 29.61 (4); 29.58 (2); 29.56; 29.40; 29.26; 25.84 and 22.58 (−(CH_2)$_{12}$−); 14.01 (CH_3). MS, *m*/*z*: 408 (MH^+).

Hexadecyl *O*-Phosphonomethylcholine (8e). Yield, 5.9 g (70%), mp 144 °C. Anal. ($C_{22}H_{48}NO_4P$) C, H, N, P. ^{1}H NMR ($CDCl_3$): δ 4.20 m, 2H (N^+−CH_2); 3.81 q, 2H, J = 6.7 (P−O−CH_2); 3.72 m, 2H (N−CH_2-*CH_2*−O); 3.63 d, 2H, J = 6.0 (P−CH_2−O); 3.49 s, 9H [N^+(CH_3)$_3$]; 1.56 m, 2H, 1.25−1.30 m, 26 H [−(CH_2)$_{14}$−]; 0.88 t, 3H, J = 6.8 [−(CH_2)$_{14}$−*CH_3*]. ^{13}C NMR ($CDCl_3$): δ 66.68 d, ^1J(C,P) = 150.4 (O−CH_2−P); 66.00 (N−CH_2−); 65.46 d, ^3J(C,P) = 3.0 (−CH_2−O−); 64.47 d, ^2J(C,P) = 5.9 (P−O−CH_2); 54.30 (N(CH_3)$_3$); 31.37 d, ^3J(C,P) = 5.9 (−P−O−C−CH_2); 31.86; 29.64 (7); 29.61; 29.60; 29.43 and 29.29 (−(CH_2)$_{12}$−); 14.06 (CH_3). MS, *m*/*z*: 422 (MH^+).

Tetradecyl *O*-Phosphonomethylcholine (8c). Finely powdered compound **2** (2.1 g, 0.01 mol) was codistilled with toluene (3 × 100 mL) and suspended in dimethylformamide (100 mL) in a 500 mL flask equipped with a magnetic stirrer, closed funnel for addition of solids, and calcium chloride protecting tube. Trichloromethyl chlorocarbonate (11.9 g, 0.04 mol) was gradually added to the suspension under stirring and exclusion of moisture at 0 °C. After the vigorous reaction accompanied by gas evolution subsided, the mixture was stirred without cooling to complete dissolution (approximately 30 min). 1-Tetradecanol (21.5 g, 0.1 mol) was added in one portion, and the mixture was stirred until dissolution and was left to stand for 2 days at ambient temperature. Water (20 mL) was added, the volatiles were evaporated at 50 °C in vacuo, and the residue was treated with water (200 mL). The mixture was alkalized by addition of concentrated aqueous ammonia solution to pH 9 and was extracted with ether (3 × 100 mL), and the aqueous phase was concentrated in vacuo to approximately 70 mL. This solution was applied onto a column (150 mL) of Dowex 1 × 2 (OH^- form), and the column was washed with water (300 mL). The eluate was evaporated in vacuo, the residue was codistilled with ethanol (3 × 100 mL) and dried over phosphorus pentoxide in vacuo. The residue was stirred with hexane (100 mL) to crystallization,

and the product was filtered, washed with hexane (100 mL), and dried in vacuo. Yield, 1,4 g (17.8%) tetradecyl *O*-phosphonomethylcholine (**8c**), mp 244 °C. Anal. ($C_{20}H_{44}NO_4P$) C, H, N, P. ^{1}H NMR and ^{13}C NMR spectra were identical with those described for compound **8c** above.

***O*-Phosphonatomethyl-2-(*N*-methylpyrrolidinium)ethanol (6).** Compound **3** (25 mL, 0.1 mol) and pyrrolidine (20 mL, 0.24 mol) in ethanol (100 mL) were heated in an autoclave under stirring for 16 h at 110 °C. The mixture was evaporated to dryness. The residue in toluene (200 mL) was washed with saturated aqueous K_2CO_3 solution (3 × 50 mL), dried with K_2CO_3, and evaporated (at last at 50 °C at 13 Pa). Iodomethane (25 mL) was added through the reflux condenser under stirring, and the mixture was left to stand overnight at ambient temperature. The volatiles were evaporated in vacuo, and acetonitrile (150 mL) and bromotrimethylsilane (40 mL) were added to the residue. After standing overnight at ambient temperature, the mixture was evaporated in vacuo, and the residue codistilled with toluene (3 × 50 mL) and with water (3 × 50 mL). The residue in water was neutralized by addition of Dowex 1 × 2 (OH^- form), and the suspension was applied onto a column (150 mL) of the same resin. The column was eluted with water (total, 1 L), and the eluate was evaporated in vacuo. The residue was dried by repeated codistillation with an ethanol/toluene mixture (1:3) (3 × 50 mL) and was left to stand in vacuo over phosphorus pentoxide to crystallization. Acetonitrile (100 mL) was then added, and the mixture was stirred until the crystallization was complete. The product was filtered, washed with acetonitrile and ether, and dried in vacuo. Yield, 11.6 g (52%), mp 223 °C. Anal. ($C_8H_{18}NO_4P$) C, H, N, P. ^{1}H NMR (D_2O): δ 4.00 m, 2H (O−CH_2−C); 3.64 d, 2H, J(H,P) = 8.8 (P−CH_2−O); 3.59 m, 6H (N−CH_2 and 2 × N−CH_2 [pyrrol]); 3.11 s, 3H [N−CH_3]; 2.20 m, 4H (2 × C−CH_2 [pyrrol]). ^{13}C NMR (D_2O): δ 69.91 d, ^1J(C,P) = 156.7 (O−CH_2−P); 69.51 d, ^3J(C,P) = 12.7 (−CH_2−O); 68.14 (2 × N−CH_2− [pyrrol]); 65.84 (N−CH_2−); 51.39 (N−CH_3); 23.94 (2 × C−CH_2 [pyrrol]). MS, *m*/*z*: 224 (MH^+).

***O*-Phosphonatomethyl-2-(*N*-methylmorpholinium)ethanol (7).** Compound **3** (25 mL, 0.1 mol) and morpholine (22 mL, ∼0.25 mol) in ethanol (100 mL) were heated in an autoclave under stirring 16 h at 120 °C. The crystalline morpholine hydrochloride was filtered off and washed with ethanol, and the combined filtrates were evaporated to dryness. The residue in toluene (200 mL) was washed with a saturated aqueous K_2CO_3 solution (3 × 50 mL), dried with K_2CO_3, and evaporated (at last at 60 °C at 13 Pa). Iodomethane (30 mL) was added through the reflux condenser under stirring, and the mixture was left to stand overnight at ambient temperature. The volatiles were evaporated in vacuo, and acetonitrile (150 mL) and bromotrimethylsilane (40 mL) were added to the residue. After standing overnight at ambient temperature, the mixture was evaporated in vacuo, and the residue was codistilled with toluene (3 × 50 mL) and with water (3 × 50 mL). The residue in water was neutralized by addition of Dowex 1 × 2 (OH^- form), and the suspension was applied onto a column (150 mL) of the same resin. The column was eluted with water (total, 1 L), and the eluate was evaporated in vacuo. The residue was dried by repeated codistillation with an ethanol/toluene mixture (1:3) (3 × 50 mL) and left to stand in vacuo over phosphorus pentoxide to crystallization. Acetonitrile (100 mL) was then added, and the mixture was stirred till the crystallization was complete. The product was filtered, washed with acetonitrile, and ether and was dried in vacuo. Yield, 4.8 g (20%), mp 228 °C. Anal. ($C_8H_{18}NO_5P$) C, H, N, P. ^{1}H NMR (D_2O): δ 4.06 m, 6H (−CH_2O + 2 × O−CH_2 [morph]); 3.76 m, 2H (N−CH_2−); 3.66 d, 2H, J = 8.9 (P−CH_2−O); 3.65 dt, 2H, 3.54 dtd, 2H (2 × N−CH_2- [morph]); 3.30 s, 3H [N−CH_3]. ^{13}C NMR (D_2O): δ 69.97 d, ^1J(C,P) = 157.2 (O−CH_2−P); 68.43 d, ^3J(C,P) = 12.7 (−CH_2−O−); 66.59 (N−CH_2−); 63.45 (2 × N−CH_2−[morph]); 63.32 (2 × O−CH_2 [morph]); 51.01 (N−CH_3). MS, *m*/*z*: 240 (MH^+).

Cytotoxic Activity in Vitro. 1. Tumor Cell Culture. Tumor cell lines were obtained from commercial sources. All tumor cells used were of human origin. The following cell lines were used (origin and medium is given in parentheses): DU-145 (prostate, carcinoma, MEM with Earle's salts); HT-29 (colon adenocarcinoma; McCoy's medium 5a); NCI−H460 (nonsmall cell lung carcinoma (RPMI 1640); MES-SA (uterine sarcoma; McCoy's medium 5a). All medium types contained 10% fetal bovine serum. Tumor cell lines were maintained at 37 °C with 5% CO_2 in standard humidified tissue culture incubators unless otherwise indicated. The culture medium for each cell type was changed every 48−72h.

2. Cell Plating. Adherent tumor cells were removed from the culture substrate following exposure to trypsin (25−30 U/mL) with EDTA (0.2 mg/mL) in Dulbecco's phosphate buffered saline (Ca^{2+}/Mg^{2+}-free), pH 7.4, 22 °C. Harvested cells were then suspended in a 2× volume of supplemented media (see above) to inactivate trypsin, pelleted by centrifugation (∼200*g*, 5 min), suspended in supplemented media, and dispersed into 96-well tissue culture plates (1×10^4 cells/well, 100 μL aliquots). Consistent cell densities were maintained by determining viable cell counts for each cell type immediately prior to cell plating [by hemocytometer, using Trypan Blue exclusion (50 μL of 0.4% Trypan Blue in 0.85% saline, 0.45 μm filtered, was added to 50 μL of the cell suspension)]. All tumor cell cultures were maintained for ∼4 h following cell plating, under tissue culture conditions, to ensure cell attachment to substrate prior to experimentation. Cell attachment was confirmed microscopically.

3. Treatments. Stock solutions of all agents were diluted with an appropriate supplemented medium (see above) to produce working stock solutions from which serial dilutions (n = 8 wells per dilution concentration) were prepared in 96-well plates immediately before addition of treatments to cell-containing 96-well plates. Aliquots (100 μL) of each serially diluted concentration of agent were placed into wells (8 wells/concentration) of a 96-well plate containing tumor cell cultures in 100 μL of medium). A positive (supplemented medium) growth control (8 wells/group) was included on each assay plate during experimentation.

After 24 h of treatment additions, 0.25 μCi of [methyl-^{3}H]-thymidine (20 μL of 12.5 μCi/mL stock [methyl-^{3}H]thymidine diluted in appropriate medium) was added to each well. Thymidine-labeled cells were then incubated for an additional 48 h under tissue culture conditions (37 °C, 5% CO_2, 100% relative humidity) for a total assay duration of 72 h. Following incubations, cells were lysed by addition of 0.1% Triton X-100 (15 min of incubation under room-temperature conditions) and harvested onto glass-fiber filters (96-well GF/B unifilter glass-fiber plates, Packard Instrument Company, Meriden, CT) using a 96-well cell harvester (Packard Instrument Company). Unincorporated [methyl-^{3}H]thymidine was removed from cellular lysates by quadruplicate washes of each plate with deionized water. Filter plates were air-dried, 50 μL of scintillation cocktail (MicroScint-20, Packard Instrument Company) was added to each filter well, and counts per minute (CPM) of incorporated [methyl-^{3}H]thymidine were determined (TopCount, Packard Instrument Company; ^{3}H channel, CPM B, 1 min counts). The data are summarized in Table 1.

In Vivo Cytostatic Activity of Alkyl *O*-Phosphonomethylcholines (8). The study was performed with three groups of 2-month-old male SD cub rats (four animals each) originating from the breeding in the animal house of Institute of Biology, First Faculty of Medicine, Prague. The average animal body weight was 300 g. The animals were subcutaneously injected with 10^6 cells of SD lymphoma to the right flank. The therapy was started 10 days after lymphoma cell injection. Sterile drug solution in PBS buffered to pH 7.2 (3 mL, 1 mg/mL) was applied 1× daily to the vicinity of growing lymphoma. One group of animals received the same volume of PBS only and served as a control. The therapy was interrupted at day 10. After 24 h, the animals were sacrificed and lymphomas were isolated and weighed. The evaluation of biological activity consisted of the estimation of average lymphoma mass decrease in the group of drug-treated animals compared to average lymphoma mass in the control group of animals. The average lymphoma masses, standard deviations, *t*-test values,

and lymphoma mass decrease expressed as a percentage of the control are in the Table 2.

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