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Cationic Amphiphiles of Methylphosphonates and Cholesterol Phosphocholines and Their Homo Derivatives

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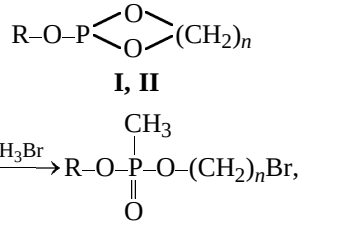
Abstract — A method of synthesis of (P → O) and (P → N)-choline cholesteryl methylphosphonates and their homo derivatives of the cationic type is developed. These compounds were obtained by the Arbuzov reaction between bromomethane and cyclic cholesteryl phosphorous esters and amidoesters and subsequent aminolysis of the intermediate bromoalkyl phosphonates. Alkylation of trimethylamine with cholesteryl ethanediyl and propanediyl phosphates gives cholesterol phosphocholines and their homo derivatives.

In the last ten years there has been considerable synthetic work on cholesterol-containing phospohorus-free cationic amphiphiles [1, 2]. Such lipids are being actively studied as components of cationic liposoms for transport of genetic material into the cell, as well as medicinals [3, 4]. At the same time, we have been the only who synthesized phosphorus lipids with cholesterol as structural domain. Hence, the synthesis of cholesterol-containing thiophospholipids, such as phosphocholines, phosphomethylcolamines, and phosphodiols [5], as well as of amidodiester (P → N)-cholesterol phospholipids of the cationic type [6] has been accompished. Cholesteryl phospholipids of the cationic type, owing to specific properties of cholesterol, may present considerable interest in terms of transfection effectiveness [7].

Proceeding with our research, we set ourselves the task to develop synthetic approaches to formerly unknown cationic methylphosphonate analogs of cholesterol, as well as to cholesterol phosphocholines and their homo derivatives.

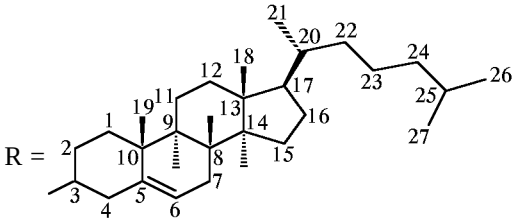
The first line of out work was based on the use of alkanediyl cholesteryl phosphites in the synthesis of cationic cholesteryl methylphosphonates. To this end, we performed Arbuzov alkylation of the available ethanediyl and propanediyl phosphites (**I, II**) with bromomethane.

The reaction was carried out at 110–120°C. The yield of bromoalkyl phosphonates **III, IV** reached 40–65%. The products are colorless oils. Importantly, these compounds are stable and can be handled in dark closed vessels at 5°C for several months. Reaction progress was controlled by ^{31}P NMR spectroscopy. The ^{31}P–{^{1}H} NMR spectra of cholesteryl methylphosphonates **III, IV** contain singlets at δ_P 30.34 and 30.02 ppm, respectively. The ^{1}H NMR spectra of these compounds show expected signals of all proton groups.[1] Hence, the PCH_3 protons, due to coupling with phosphorus, appear as a doublet at δ 1.47 ppm ($^{2}J_{PH}$ 17.04–17.50 Hz). The CH_2Br methylene protons give characteristic triplet signals at δ 3.48–3.49 ppm, the cholesteryl C^3HOP proton, a multiplet at δ 4.38 ppm, and the cholesteryl C^6H= proton, due to coupling with the C^7H_2 methylene protons, a doublet at δ 5.38 ppm. The rest signals in the spectra of the acyclic cholesteryl phosphonates were roughly the same as in the spectra of the starting phosphites **I**, **II** (see Experimental).



I, II

III, IV



n = 2 (**I, III**), 2 (**II, IV**).

[1] Analysis of the ^{1}H NMR spectra was carried out with account for data of Facke and Berger [8] who performed detailed assignment of the ^{1}H NMR spectra of cholesterol.

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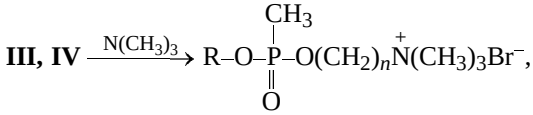
Bromoalkyl phosphonates **III, IV** were used for preparing cationic amphiphiles **V, VI**. To this end, phosphonates **III, IV** were reacted with *i*th trimethylamine.

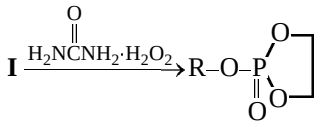


V, VI

Quarternization of trimethylamine occurred at room temperature (about 22°C) within 12 h. Earlier quarternization of glycerol bromoalkyl phosphonates in the synthesis of cationic amphiphiles of phosphoglycerols was also performed at room temperature, but for a longer time (70–72 h) [9]. Reaction progress was controlled by TLC. The yields of chromatographically pure ammonium compounds **V, VI** were 52–65%. They are solid amorphous substances.

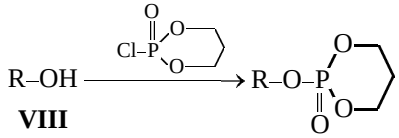
The ^{1}H NMR spectra of phosphonates **V, VI** contain singlets of the $N^+(CH_3)_3$ group at δ 3.32–3.40 ppm. The triplets of the CH_2Br methylene protons have disappeared, and, instead of them, multiplet signals of the $CH_2N^+(CH_3)_3$ methylene protons have appeared. The other signals in the spectra of compounds **V, VI** practically coincide with those of the starting bromoalkanols (see Experimental).

Cholesteryl alkanediyl phosphates were also used for preparing cholesterol phosphocholine and its homo derivative. As the starting material for preparing cholesterol phosphocholine we used cholesteryl ethanediyl phosphite (**I**) which was oxidized to cholesteryl ethanediyl phosphate (**VII**) with urea hydrogen peroxide adduct (20°C, 5 h). The product was isolated by column chromatograpghy in 40% yield.



VII

To extend the scope of the method of synthesis of cholesterol phosphocholines, we made an attempt to oxidize cholesteryl propanediyl phosphite (**II**). Unfortunately, this oxidation failed. Therefore, the target product was prepared by reacting cholesterol (**VIII**) with propanediyl phosphorochloridate in the presence of triethylamine.



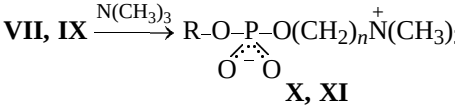
IX

Propanediyl phosphorochloridate proved to be much less reactive compared to propanediyl phosphorochloridite toward cholesterol (**VIII**) and could only be reacted at 80°C within 8 h. The yield of the target product **IX** isolated on a silica gel column was as low as 35%.

Cholesteryl alkanediyl phosphates **VII, IX**, unlike the corresponding thiophosphates [5], are unstable hygroscopic crystalline substances. They can be stored without decomposition in closed vials only for several days at –5°C.

The purity and structure of phosphates **VII, IX** was proved by TLC and ^{31}P and ^{1}H NMR spectroscopy. The ^{31}P NMR spectra of compounds **VII, IX** contained singlets at δ_P 15.98 and –8.76 ppm, respectively. The ^{1}H NMR spectra of phosphates **VII, IX** showed signals characteristic of all proton groups. Thus, the spectrum of ethanediyl phosphate **VII** contained a multiplet of methylene protons of the phospholane ring at δ 4.40 ppm, whereas the spectrum of propanediyl phosphate **IX**, characteristic multiplets of protons of the phosphorinane ring at δ 2.20 and 4.46 ppm. The other signals in the spectra of phosphates **VII, IX** were consistent with the proposed structures (see Experimental).

To pass to cholesterol phosphatide structures of the betain type, we performed alkylation of trimethylamine with phosphates **VII, IX**.



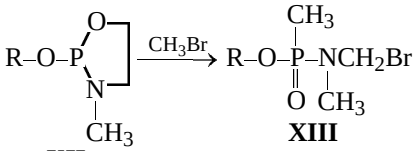
X, XI

n = 2 (**III, V**), 3 (**IV, VI**).

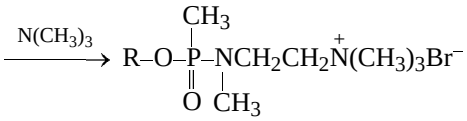
The reactions with trimethylamine were carried out in benzene at 90–100°C for 30–35 h. Then benzene was removed in a vacuum, and the remaining target products were washed with hexane. Cholesterol phosphocholine **X** and phosphohomocholine **XI** were obtained as colorless crystals in 30 and 42% yields, respectively. Note that in the case of cholesterol thiophosphocholines prepared by the analogous procedure from cholesteryl thiophosphates the yields of the target products were higher, 40 and 80%, respectively [5]. The ^{31}P NMR spectra of phosphocholines **X, XI** contained singlet signals at δ_P –0.82 and 0.27 ppm, respectively. The ^{1}H NMR spectra of these compounds displayed singlets of ammonium methyl protons at δ 3.32–3.40 ppm. In addition, the spectrum of betain **X** contained multiplets of β-methylene protons of the $CH_2N^+(CH_3)_3$ group at δ 3.65 ppm, that of betaine **XI**, multiplets of β- and γ-methylene protons of the $CH_2CH_2N^+(CH_3)_3$ group at δ 2.38 ppm

and 2.85 ppm. The other signals in the ^{1}H NMR spectra had only slightly different chemical shifts compared to the respective signals of the starting cyclic phosphates **VII, IX** (see Experimental).

In the final stage of this work we applied the proposed synthetic scheme for preparing cholesteryl ethylamidophosphonate of the cationic type. Such (P→N) phospholipids, unlike (P→O) phospholipids, have a more polar phosphoryl group due to the presence of a phosphamide fragment in its structure, and this may influence the properties of membrane constructions on their basis.



XII **XIII**



XIV

The synthesis of lipid aminophosphonate amphiphiles was begun from alkylation of the previously prepared by us [5] cholesteryl phosphoramidite **XII** with bromomethane (90°C, 8 h). Then cholesteryl phosphonamidate **XIII** was purified by column chromatography on silica gel and reacted with trimethylamine to obtain cationic ammonium phospholipid **XIV** (22°C, 12 h). This compound was precipitated from benzene as a white powder. The yield of chromatographically pure product **XIV** was 42%.

The ^{31}P NMR spectra of compounds **XIII, XIV** contained two singlet signals at δ_P 33.12 and 33.42 (**XIII**) and 34.39 and 34.69 ppm (**XIV**). Such a complification of the spectra is connected with the fact that these compounds are present as diastereomeric pairs due to the cholesteryl radicals and racemic phosphorus atom. The ^{1}H NMR spectra of phosphonates **XIII, XIV** contained signals characteristic of all proton groups, sometimes complicated by stereomeric anisochronicity. Hence, the PCH_3 protons appear as doublets at δ 1.45 ppm, and the NCH_3 protons, as a doublet at δ 2.75–3.40 ppm. In addition, the ^{1}H NMR spectrum of compound **XIII** showed a triplet at δ 3.02 ppm, characteristic of CH_2Br groups. The spectrum of choline phosphonate **XIV** differed from the spectrum of the acyclic compound **XIII** by the presence of a singlet at δ 3.50 ppm from the $N^+(CH_3)_3$ methyl protons, a multiplet of the $CH_2N^+ \cdot (CH_3)_3$ methylene protons at δ 3.56 ppm, as well as by the absence of the CH_3Br triplet. All the other proton signals of compounds **XIII, XIV** almost did not differ from each other (see Experimental).

The present work resulted in the synthesis of a group of original phosphocholesterol systems belonging to cationic phospholipids. The synthesized compounds offer interest as objects for research into lipid exchange. Moreover, they can be used for studying the behavior of a polar head incorporated into the rigid lipid matrix.

EXPERIMENTAL

The ^{1}H NMR spectra were obtained on a Bruker WM-250 (250 MHz) spectrometer against internal HMDS. Signal assignment was made on the basis of double-resonance data. The ^{31}P–{^{1}H} spectra were measured on a Bruker WP-80 SY spectrometer (32.4 MHz) against external 85% phosphoric acid. Column chromatography was performed on a column 15 mm in diameter, filled on Silica gel L (100–160 μm). Thin-layer chromatography was performed on Silufol UV-254 plates, eluents 3:1 benzene–dioxane (A), 3:1 hexane–dioxane (B), and 65:25:4 chloroform–methanol–water (C). The melting points were measured in a sealed capillary at a heating rate of 1 deg/min. The urea hydrogen peroxide adduct was prepared according to [10], 2-chloro1,3,2-dioxaphospholane, according to [11], 2-chloro-1,3,2-dioxaphosphorinane, according to [12], and propanediyl prosphorochloridite, according to [13].

2-Bromoethyl cholesteryl methylphosphonate (III). A sealed ampule with a solution of ethanediyl phosphite (**I**) (prepared according to [5] from 2 g of cholesterol, 0.64 g of ethanediyl phosphorochloridite, and 0.5 g of triethylamine) and 2.95 g of bromomethane in 20 ml of anhydrous benzene was kept for 12 h at 110°C. The solvent was removed in a vacuum, and ethanediyl phosphonate **III** was isolated on a column of silica gel (20 g), filled with hexane. Compound **III** was eluted with 50 ml of a 5:1 hexane–dioxane mixture. The solvents were removed in a vacuum, and the residue was kept for 2 h at 40°C (1 mm). Yield 1.2 g (40%), n_D^{20} 1.5057, R_f 0.55 (A), 0.27 (B). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.68–2.47 (cholesterol H),[2] 1.47 d (3H, PCH_3, $^2J_{HP}$ 17.50 Hz), 3.48 t (2H, CH_2Br), 4.24 m (3H, $POCH_2$, cholesterol OPC^{3}H), 5.33 d (1H, cholesterol C^{6}H=, $^3J_{HH}$ 3.40 Hz). ^{31}P NMR spectrum (chloroform), δ_P, ppm, δ_P: 30.34 s. Found,%: C 63.10; H 9.25; P 5.50. $C_{30}H_{52}BrO_3P$. Calculated,%: C 63.03, H 9.17, P 5.42.

[2] Here and hereafter, the region at δ 0.68–2.47 ppm, characteristic of protons on the C^{1,2}, C^{4,5}, and C$^{7–27}$ atoms of the phosphocholesterol moiety [8], is labeled (cholesterol H).

3-Bromopropyl cholesteryl methylphosphonate (IV). A sealed ampule with a solution of propanediyl phosphite (**II**) (prepared according to [5] from 2.5 g of cholesterol, 0.73 g of propanediyl phosphorochloridite, and 0.62 g of triethylamine), and 3 g of bromomethane in 20 ml of anhydrous benzene was kept at 120°C for 15 h. The solvent was removed in a vacuum, and phosphonate **IV** was isolated on a column of silica gel (30 g), filled with hexane. Compound **IV** was eluted with 50 ml of a 3:1 hexane–dioxane mixture. The solvents were removed in a vacuum, and the residue was kept for 2 h at 40°C (1 mm). Yield 2.5 g (65%), n_D^{20} 1.4981. R_f 0.60 (A), 0.30 (B). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.67–2.47 (cholesterol H), 1.47 d (3H, PCH_3, $^2J_{PH}$ 17.04 Hz), 2.61 m (2H, $POCH_2$–C*H*$_2$–CH_2, $^3J_{HH}$ 7.15 Hz), 3.49 t (2H, CH_2Br, $^3J_{HH}$ 6.6 Hz), 4.14 m (2H, $POCH_2$), 4.38 m (chlesterol OPC^{3}H), 5.38 d (1H, cholesterol C^{6}H=, $^3J_{HH}$ 3.80 Hz). ^{31}P NMR spectrum (chloroform), δ_P, ppm: 30.02 s. Found,%: C 63.61; H 9.51; P 5.43. $C_{31}H_{54}BrO_3P$. Calculated,%: C 63.57; H 9.29, P 5.29.

Cholesteryl 2-(dimethylamino)ethyl methylphosphonate bromomethylate (V). A sealed ampule with a solution of 1 g of phosphonate **III** and 0.36 g of trimethylamine in 10 ml of anhydrous benzene was kept at room temperature for 12 h. To remove the solvent, the gel-like reaction mixture was kept for 0.5 h at 40°C (1 mm). Yield 0.6 g (55%), mp 210–212°C (begins to melt at 185°C), R_f 0.60 (C). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.67-2.38 (cholesterol H), 1.56 d (3H, PCH_3, $^2J_{PH}$ 17.05 Hz), 3.54 s (9H, N^+Me_3), 4.22 br.m (2H, $POCH_2CH_2N^+$), 4.44 br.m (2H, $POCH_2CH_2N^+$), 4.54 br.m (1H, cholesterol OPC^{3}H), 5.39 br.m (1H, cholesterol C^{6}H=). ^{31}P NMR spectrum (chloroform), δ_P, ppm: 31.00 s. Found, %: C 63.01; H 9.98; P 5.01. $C_{33}H_{61}\cdot BrNO_3P$. Calculated, %: C 62.84; H 9.75; P 4.91.

Cholesteryl 3-(dimethylamino)propyl methylphosphonate bromomethylate (VI) was obtained analogously to phosphonate **V** from 1.5 g of compound **IV** and 0.62 g of trimethylamine in 15 ml of anhydrous benzene. Yield 1.02 g (62%), mp 255–257°C (begins to melt at 210°C), R_f 0.63 (C). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.63–2.40 (cholesterol H), 1.49 d (3H, PCH_3, $^3J_{PH}$ 17.18 Hz), 2.23 br.s (2H, $POCH_2$–C*H*$_2$–CH_2N^+), 4.17 br.m (1H, cholesterol OPC^{3}H), 5.35 br.m (1H, cholesterol C^{6}H=). ^{31}P NMR spectrum (chloroform), δ_P, ppm: 30.69 s. Found, %: C 63.51; H 10.01; P 5.05. $C_{34}H_{63}BrNO_3P$. Calculated, %: C 63.33; H 9.85; P 4.80.

2-Cholesteryloxy-1,3,2λ^5-dioxaphospholane 2-oxide (VII). To a solution of ester **I** (prepared according to [5] from 1 g of cholesterol and 0.32 g of ethanediyl phosphorochloridite in the presence of 0.3 g of triethylamine) in 20 ml of anhydrous benzene, 0.2 g of urea hydrogen peroxide adduct was added at room temperature, and the reaction mixture left to stand at that temperature for 5 h. Excess adduct was filtered off, and the solvent was removed in a vacuum. Compound **VII** was purified on a column of silica gel (10 g), filled with hexane, eluent 5:1 hexane–dioxane (40 ml). The solvents were removed in a vacuum, and the residue was kept for 2 h at 40°C (1 mm). Yield 0.38 g (40%), mp 145–148°C, R_f 0.70 (A), 0.45 (B). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.68-2.47 (cholesterol H), 4.40 m (4H, OCH_2CH_2O), 4.47 m (1H, cholesterol OPC^{3}H), 5.39 d (1H, cholesterol C^{6}H=, $^3J_{HH}$ 4.30 Hz). ^{31}P NMR spectrum (chloroform), δ_P, ppm: 15.98 s. Found, %: C 36.91, H 9.89, P 6.03. $C_{29}H_{49}O_4P$. Calculated, %: C 36.56, H 10.02, P 6.29.

2-Cholesteryloxy-1,3,2λ^5-dioxaphosphorinane 2-oxide (IX). To a solition of a mixture of 1 g of cholesterol (**VIII**) and 0.25 g of triethylamine in 30 ml of anhydrous benzene, a solution of 0.4 g of propanediyl phosphorochloridate in 5 ml of the same solvent was added dropwise with stirring at 0°C. The temperature of the reaction mixture was gradually raised to 80°C and maintained for 8 h. Then triethylamine hydrochloride was filtered off, and benzene was removed in a vacuum. Compound **IX** was purified on a column of silica gel (15 g), filled with hexane, eluent 3:1 hexane–dioxane (50 ml). The solvent was removed in a vacuum, and the residue was kept for 2 h at 40°C (1 mm). Yield 0.45 g (35%), mp 190–192°C, R_f 0.70 (A), 0.50 (B). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.67-2.46 (cholesterol H), 2.20 m (2H, OCH_2–C*H*$_2$–CH_2O), 4.46 m (5H, $POCH_2CH_2\cdot CH_2O$, cholesterol OPC^{3}H), 5.41 d (1H, cholesterol C^{6}H=, $^3J_{HH}$ 4.85 Hz). ^{31}P NMR spectrum (chloroform), δ_P, ppm: –8.76 s. Found,%: C 71.38,H 10.31, P 6.23. $C_{30}H_{51}O_4P$. Calculated,%: C 71.11; H 10.14; P 6.11.

Cholesterol phosphocholine X. A sealed ampule with a solution of 0.4 g of phosphate **VII** and 0.15 g of trimethylamine in 5 ml of anhydrous benzene was heated at 90–95°C for 30 h. The solvent was removed in a vacuum, and the oily residue was washed with hexane (2 × 5 ml) and dried for 2 h at 40°C (1 mm). Yield 0.3 g (30%), mp 285–286°C (begins to melt at 250°C), R_f 0.00 (A), 0.30 (C). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.67–2.42 (cholesterol H), 3.40 s (9H, N^+Me_3), 3.65 br.s (2H, $POCH_2CH_2N^+$), 4.38 br.m (1H, cholesterol OPC^{3}H), 5.40 br.m (1H, cholesterol C^{6}H=). ^{31}P NMR spectrum (chloroform), δ_P, ppm: –0.2 s. Found, %: C 69.72; H 10.49, P 5.43.

$C_{32}H_{58}NO_4P$. Calculated, %: C 69.65; H 10.60, P 5.61.

Cholesterol phosphohomocholine (XI) was prepared analogously from 0.5 g of phosphate **IX** and 0.25 g of trimethylamine in 10 ml of anhydrous benzene at 90–100°C for 35 h. Yield 0.3 g (42%), mp 300–301°C (decomp.) (begins to melt at 285°C), R_f 0.00 (A), 0.25 (B). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.67–2.48 (cholesterol H), 2.38 br.m (2H, $POCH_2CH_2CH_2N^+$), 2.85 br.m (2H, $POCH_2CH_2 \cdot CH_2N^+$), 3.32 s (9H, N^+Me_3), 3.75 br.m (2H, $POCH_2 \cdot CH_2CH_2N^+$) 4.20 br.m (1H, cholesterol OPC^3H), 5.35 br.m (1H, cholesterol C^6H=). ^{31}P NMR spectrum (chloroform), δ_P, ppm: 0.27 s. Found, %: C 70.15; H 10.81, P 5.58. $C_{33}H_{60}NO_4P$. Calculated, %: C 70.04; H 10.69; P 5.47.

Cholesteryl *N*-(2-bromoethyl)-*N*,*P*-dimethylphosphonamidate (XIII). A sealed ampule with a solution of 2-cholesteryloxy-3-methyl-1,3,2-oxazaphospholane (**XIII**) (prepared according to [5] from 2 g of cholesterol and 2.56 g of hexaethylphosphorous triamide and subsequent reaction of the intermediate cholesterol tetraethylphosophorodiamidite with 0.4 g of *N*-methylethanolamine) and 3.5 g of bromomethane in 25 ml of anhydrous benzene was kept at 90°C for 8 h. The solvent was removed in a vacuum, and phosphonate **XIII** was isolated on a column of silica gel (15 g), filled with hexane, eluent 1 : 1 hexane–dioxane (50 ml). The solvent was removed in a vacuum, and the residue was kept for 2 h at 40°C (1 mm). Yield 1.4 g (45%), n_D^{20} 1.4785, R_f 0.45 (A), 0.19 (B). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.65–2.47 (cholesterol H), 1.45 d (3H, PCH_3, $^2J_{PH}$ 12.02 Hz), 2.06 m and 2.66 m (2H, $PNCH_2CH_2$, $^3J_{PH}$ 9.35 Hz), 2.99 t and 3.02 t (2H, CH_2Br, $^3J_{HH}$ 7.14 Hz), 3.43 d (3H, $PNCH_3$, $^3J_{HP}$ 2.20 Hz), 4.30 m (1H, cholesterol OPC^3H), 5.36 d (1H, cholesterol C^6H=, $^3J_{HH}$ 5.50 Hz). ^{31}P (chloroform), δ_P, ppm: 33.12 s and 33.42 s, intensity ratio 10:8. Found, %: C 63.72; H 9.63. $C_{31}H_{55}BrNO_2P$. Calculated, %: C 63.68, H 9.48, P 5.30.

Cholesteryl *N*-(2-bromoethyl)-*N*,*P*-dimethylphosphonamidate bromomethylate (XIV) was obtained similarly to phosphonate **V** from 1 g of compound **XIII** and 0.45 g of triethylamine in 10 ml of anhydrous benzene. Yield 0.46 g (42°C), mp 225–227°C (begins to melt at 205°C), R_f 0.69 (C). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.66–2.37 (cholesterol H), 1.46 d (3H, PCH_3, $^2J_{PH}$ 15.79 Hz), 1.46 d (3H, PCH_3, $^2J_{PH}$ 15.79 Hz), 2.75 d (3H, $PNCH_3$, $^3J_{PH}$ 9.81 Hz), 3.50 s (9H, N^+Me_3), 3.56 br.m (2H, $PNCH_2 \cdot CH_2$), 4.03 br,m (3H, $PNCH_2CH_2$ and cholesterol OPC^3H), 5.36 br.m (1H, cholesterol C^6H=). ^{31}P NMR spectrum (chloroform), δ_P, ppm: 34.39 and 34.69, intensity ratio 10:8. Found, %: C 63.53; H 10.21, P 4.95. $C_{34}H_{64}BrN_2O_2P$. Calculated, %: C 63.43; H 10.02, P 4.81.

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