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Multifunctional Lipoamino Acid Derivatives with Potential Biological Activity

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Abstract—This work aims to develop a number of multifunctional derivatives of lipoamino acids, which potentially have different biological activities. To achieve this aim, the hydrophilic-lipophilic balance for a number of cationic amphiphile structures based on L-glutamic acid and short diamines is theoretically calculated. The developed schemes and performed synthesis allowed us to produce preparative sample quantities for further physicochemical and biological tests. The performed calculations, measurements, and experiments on the liposomal dispersions of the synthesized compounds demonstrate the possibility of designing the same type of structures of an amphiphilic nature based on aliphatic amino acid derivatives and diamines, which can be used both in antibacterial therapy and as the delivery vehicles for genetic material.

Keywords: lipoamino acids, diamines, aliphatic derivatives of L-glutamic acid, cationic liposomes, hydrophilic-lipophilic balance, cationic amphiphiles

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INTRODUCTION

Lipids belong to the most important class of biologically active compounds. Due to their unique amphiphilic structure, they can form bilayer aggregates in an aqueous medium. The biological membranes, for which lipids are the main building element, perform the most important functions in living organisms (metabolite transport, energy generation, cell interaction and division, nerve impulse transmission, reception of environmental signals, etc.) [1, 2].

Artificial membranes, which are represented by various aggregates (liposomes, micelles, etc.), formed by natural or artificial lipids were constructed to solve many medicinal problems of a therapeutic character, such as developing slightly toxic targeted antitumor agents, treating hereditary diseases, overcoming biological incompatibility during organ and tissue transplantation, carrying out therapy for certain cardiovascular diseases, and preventing allergies to drugs. With their help, medicinal substances, which are unable to independently overcome the plasma membrane for a number of reasons, are delivered to the cell. They can be used as transfection agents, as well as antibacterial agents [3, 4].

Since drug delivery systems based on liposomes should reach certain organs and tissues, their interaction with cells should also be considered. Despite the complexities in the selection of ideal liposomal constructs for the safe and efficient delivery of medicinal agents, many drugs, which successfully passed preclinical and clinical trials and are used in practice in various fields of medicine, have been developed [5].

The binding efficiency of transport systems to the genetic material and its transfer to the cell is important in gene therapy. Delivery systems should have a positive charge on their surface, allowing them to interact with the negatively charged phosphate residues of nucleosides. In liposomes, this is achieved by cationic amphiphiles in the composition of the bilayer with a relatively large positive charge of the polar head group. In addition, it is possible to change the physicochemical characteristics of the liposome surface, such as the phase transition's temperature, charge, and stability by controlling the composition of liposome constructs.

The aim of this work was to develop a number of the same type of compounds based on the aliphatic derivatives of amino acids and diamines and simulate the direction of their application in different fields of medicine (antimicrobial therapy, targeted drug delivery, and gene therapy). Such a variety of compounds will further allow us to form a library of structures that differ in the size of the polar head groups and in the variations of the hydrophobic fragments. This will make it possible to expand the spectrum of physicochemical and biological studies of this type of cationic amphiphiles.

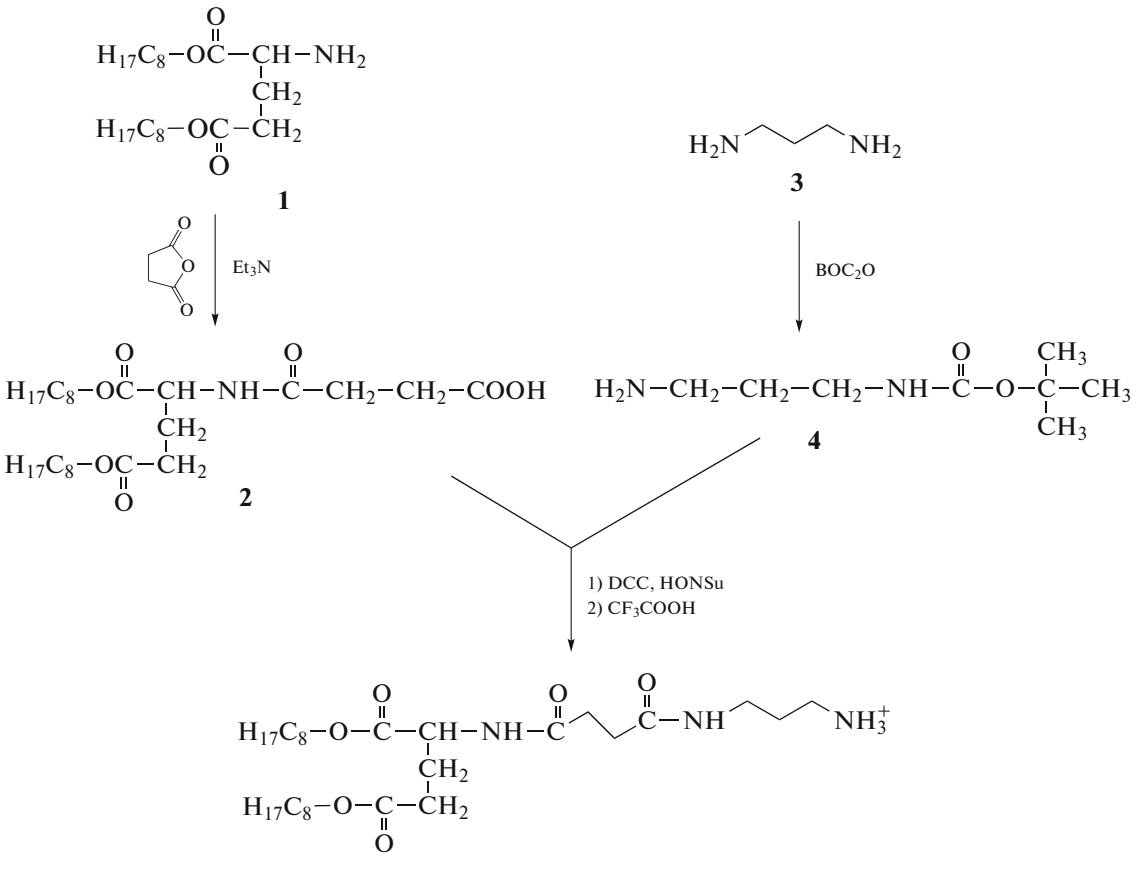
RESULTS AND DISCUSSION

The first stage of the work consisted in the theoretical calculation of the hydrophilic-lipophilic balance (HLB) for diesters of L-glutamic acid and diamines using the software processing of a number of structures and subsequent analysis of the structure–biological activity relationship. The calculation [6] was carried out using the ACD LogP program for 60 structures of cationic amphiphiles differing in the length of the aliphatic chains in the hydrophobic block, as well as in the number and nature of charged groups in the hydrophilic fragment, which enabled us to determine the most promising samples for subsequent studies. The calculated HLB values of the designed library of structures varied from 4.26 to 16.74.

The obtained data served as the basis for the development of schemes and synthesis of compounds **5** (HLB 5.78), **10** (HLB 15.0), which potentially may possess antibacterial activity (HLB 4–6) and be used as transport systems in gene therapy (HLB 14–16).

Dioctyl L-glutamate $Glu(C_8)_2$ (**1**), obtained from L-glutamic acid and octyl alcohol in the presence of *p*-toluenesulfonic acid, was treated with succinic anhydride in an Et_3N medium. As a result, dioctyl-L-glutamyl succinate $Glu(C_8)_2Suc$ (**2**) was obtained [7].

To create a positive charge in the structure of the target amphiphiles (**5**, **10**), it was proposed to use 1,3-propanediamine (**3**) (Scheme 1) and aminoethylethanolamine (**8**) (Scheme 2). According to Scheme 1, compound **3** was initially treated with di-*tert*-butyl pyrocarbonate (Boc_2O) in dioxane as a solvent and with the diamine-Boc_2O reagent ratio of 1 : 0.13 for 6 h. This allowed the production of the mono derivative **4** with a yield of 64%.

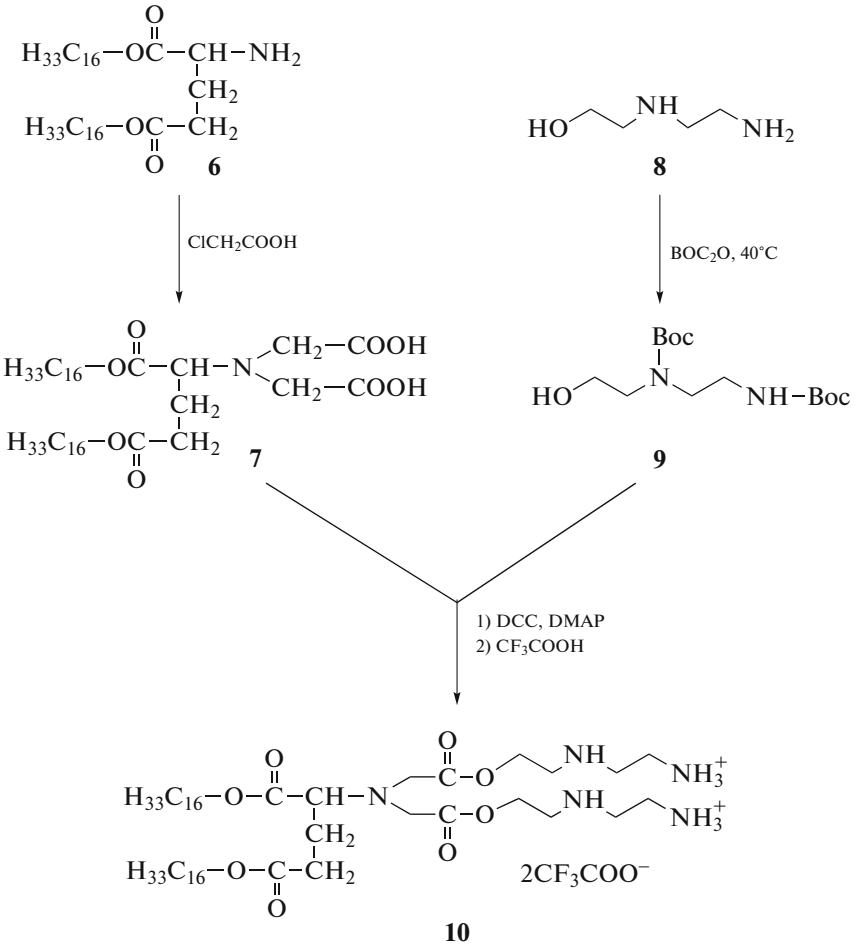


Scheme 1.

The conjugation of the polar part and the hydrophobic block was carried out by the activated ester method using *N*,*N*'-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HONSu), followed by the removal of the Boc protecting groups by exposure to trifluoroacetic acid in methylene chloride. The structure of the obtained product **5** was confirmed by the data of IR, ^1H NMR spectroscopy and mass spectrometry.

According to Scheme 2, dihexadecyl-L-glutamate $Glu(C_{16})_2$ (**6**) was treated with a 6-fold excess of 2-chlo-

roacetic acid in the presence of pyridine [8, 9]. This operation enabled us to obtain a branched construct of amphiphile **7** with a yield of 58.9%. The reaction for the preparation of a Boc derivative of aminoethylethanolamine (AEEA) (**8**) was carried out in isopropanol at 40°C under stirring for 8 h. The yield of product **9** was 98.3%. The reaction of the synthesized compounds **7** and **9** was carried out by the treatment with DCC and 4-dimethylaminopyridine (DMAP) as an activating agent. After the removal of the protecting groups, two-antenna cationic amphiphile **10** was obtained with a yield of 31.2%. The structure of all the intermediate and final compounds was confirmed by ^{1}H NMR spectroscopy. The peak of the molecular ion was present in the MALDI mass spectrum of sample **10**.



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Scheme 2.

The advantage of the developed and implemented schemes for the synthesis of cationic amphiphiles based on diesters of L-glutamic amino acid and diamines consists in the universality of the approach that can be used to obtain a number of derivatives and to develop various combinations of products in the preparative amounts required for subsequent biochemical studies.

Preliminary HLB Calculation for Cationic Amphiphiles

We preliminarily assessed the HLB values using the ACD/LogP program for the subsequent determination of the structure–biological activity relationship.

Liposome Production

The classical method of amphiphile thin film hydration followed by treatment in an ultrasonic bath was applied to produce liposomal aggregates from the synthesized compounds **5** and **10** [10].

Determination of Particle Size

One of the main liposome characteristics is the diameter of the formed constructs. It determines the ability of aggregates to penetrate the pores of blood vessels in the experiments in vivo and not be captured by the cells of the immune system [11]. The particle size was determined using the photon correlation

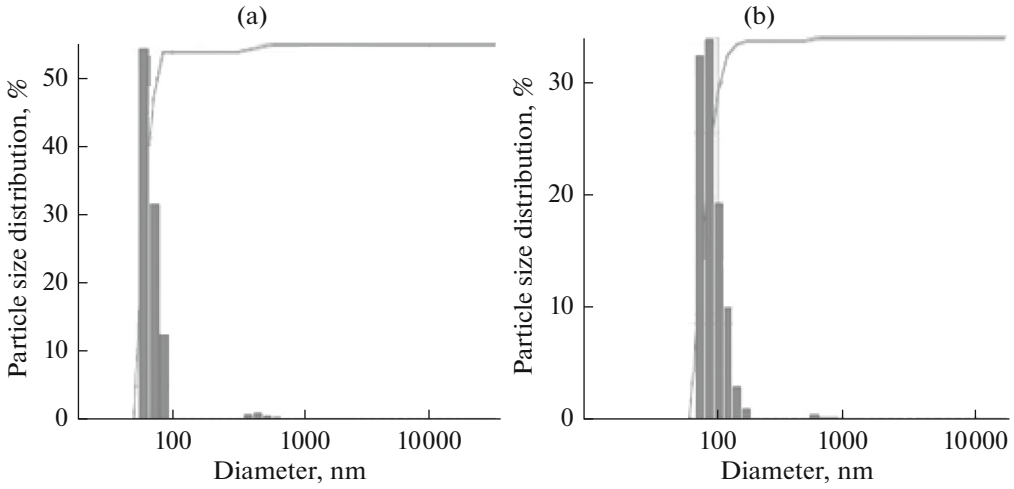

Fig. 1. Average particle size for aqueous dispersions of compounds: (a) *N*-(dioctyl-L-glutamylsuccinyl)-1,3-propanediamine (**5**); (b) dihexadecyl-L-glutamyl-*N*,*N*-di(carboxymethyl)-*O*,*O*'-di(aminoethylethanolamine) (**10**).

spectroscopy method on Beckman Coulter's Delsa Nano Common instrument.

The average particle size was 76 nm for compound **5** and 101 nm for product **10** (Fig. 1). According to the published data [12], the obtained results are potentially promising for compound **5** in antibacterial activity studies and for conjugate **10** when used as a transfection agent.

Determination of Particle Stability

The stability of the liposomal dispersions formed based on compounds **5** and **10** was detected by the change in the absorbance of the aqueous dispersion. For all compounds, it remained constant throughout the month. The total error was not more than 2.7%, which fits into the required parameters and allows considering them convenient for practical use.

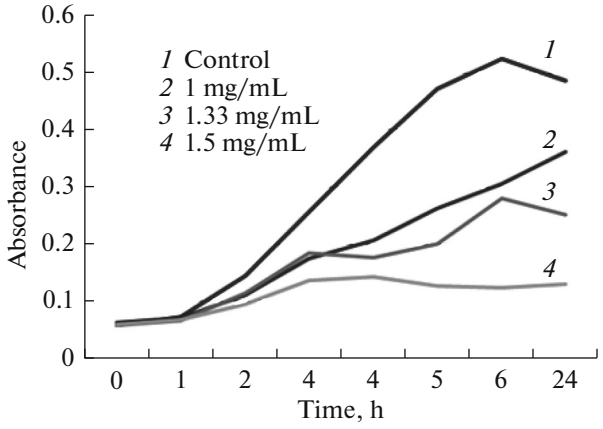

Fig. 2. Effect of aggregate based on compound **5** at different concentrations on *E. coli* JS5 cells. Studies were carried out in group of cross-linking enzymes of Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, under the supervision of Candidate of Chemical Sciences N.B. Pestov.

Biochemical Studies

The antibacterial properties of the liposome dispersions based on compound **5** were preliminarily estimated in the cells of the *E. coli* JS5 strain at different concentrations (from 0.05 to 1.5 mg/mL) in increments of 0.05 to 0.3. The results obtained (Fig. 2), which show a decrease in cell growth under the action of sample **5**, were observed starting from a concentration of 1 mg/mL. The ability of the aqueous dispersion of the bivalent conjugate **10** to deliver the genetic material into the cells of the HEK 293T line was at the level of the OrnOrnGlu$(C_{16})_2$ lipotripeptide, which was previously synthesized and studied in our laboratory [13]. The studies were carried out at the Department of Molecular Immunology of the State Scientific Center Institute of Immunology under the supervision of Candidate of Biological Sciences O.O. Koloskova.

EXPERIMENTAL

The ACD LogP software was used for the HLB calculation. ^{1}H NMR spectra were taken in deuterated chloroform on a BrukerWM-400 pulse NMR spectrometer at an operating frequency of 400 MHz. Hexamethyldisiloxane was used as the internal standard. IR spectra were recorded on an EQUINOX 55 FTIR spectrometer (Bruker). Mass spectra were taken on a VISION 2000 time-of-flight mass spectrometer by the MALDI method using dihydroxybenzene (DHB) as the template. Thin layer chromatography was carried out on Sorbfil plates, preparative thin layer chromatography was carried out on a Sigma Aldrich TLC

standard grade silica gel, and column chromatography was performed on Acros silica gel 0.060–0.200 mm; 60 A. Spots of the substances during TLC were detected in iodine vapor or when heated above the flame of an alcohol lamp. Substances containing free amino groups were detected using a 5% solution of ninhydrin with subsequent heating to 50–80°C.

Dioctyl-L-glutamyl succinate Glu(C_8)$_2$Suc (2). 0.42 g (4.2 mmol) of succinic anhydride was added to a solution of 1.53 g (2.8 mmol) diester **1** [7] in 20 mL of methylene chloride and stirred for 12 h at room temperature in the presence of a catalytic amount of triethylamine. The solvent was evaporated in vacuo; the residue was dissolved in 50 mL of chloroform, washed with 0.1 N HCl then with water to pH 7, and dried; and the solvent was removed in vacuo. The yield of product **2** was 0.85 g (64.3%). ^{1}H NMR spectrum ($CDCl_3$, δ, ppm): 0.97 (m, 6H, CH_3); 1.26 (s, 20H, CH2); 1.57 (m, 4H, $COOCH_2CH_2$); 2.3 (m, 4H, CH_2CH_2 (Glu)); 2.55 (m, 2H, CH_2CH_2COOH (Suc)); 4.04 (m, 2H, $COOCH_2$).

1-*N*-*tert*-butoxycarbonyl-1,3-propanediamine (4). A solution of 2.48 g (11 mmol) di-*tert*-butyl pyrocarbonate in 30 mL dioxane was added dropwise over 1 h to a solution of 5 g (67.4 mmol) 1,3-propanediamine **3** in 60 mL of dioxane in an inert argon atmosphere. The mixture was stirred at room temperature for 24 h, then the solvent was removed in vacuo, the product was isolated by extraction with methylene chloride, dried with Na_2SO_4, and evaporated in vacuo. Mono Boc-protected 1,3-propanediamine **4** (1.2 g, 64%) was obtained as an oil. IR spectrum (ν_{max}, cm^{-1}): 3356 (NH), 2933 (CH_2), 1630 (C=O, I amide band), 1550 (N–H, II amide band), 1480 (C–O), 1296, 1161 (C–N), and 1046 (CH_2).

***N*-(dioctyl-L-glutamylsuccinyl)-1,3-propanediamine (5).** A solution of 0.32 g (1.56 mmol) *N*,*N*'-dicyclohexylcarbodiimide was added under stirring to a solution of 0.60 g (1.30 mmol) dioctyl-L-glutamyl succinate (**2**) and 0.18 g (1.56 mmol) *N*-hydroxysuccinimide in 10 mL of anhydrous methylene chloride, cooled to 0°C. The mixture was kept for 30 min under cooling, then for 2 h at room temperature. The precipitated dicyclohexylurea was filtered off, and 0.12 g (1.56 mmol) of 1-*N*-*tert*-butoxycarbonyl-1,3-propanediamine (**4**) was added to the filtrate and the mixture was kept under vigorous stirring for 24 h. The reaction was monitored by TLC. The reaction product was purified by column chromatography in the toluene–ethyl acetate system (1:5). Further, 0.35 g of the obtained compound was dissolved in 5 mL of anhydrous methylene chloride and a solution of 1 mL trifluoroacetic acid in 5 mL anhydrous methylene chloride was added under stirring. The mixture was kept for 3 and the solvent was evaporated in vacuo. The yield of product **5** was 0.14 g (47.8%). ^{1}H NMR spectrum ($CDCl_3$, δ, ppm): 0.88 (m, 6H, CH_3); 1.10 (m, 2H, $NHCH_2\underline{CH_2}CH_2$); 1.31 (s, 20H, CH_2); 1.62 (m, 4H, β-CH_2); 1.93 (m, 2H, β-CH_2 (Glu)); 2.44 (t, 2H, δ-CH_2 (Glu)); 2.75 (m, 4H, $CO\underline{CH_2CH_2}CO$); 2.82 (m, 2H, $NH\underline{CH_2}CH_2CH_2$); 3.40–3.65 (m, 2H, CH_2NH_3); 4.24 (m, 4H, $COOCH_2$); 4.35 (t, 1H, α-CH2 (Glu)). Mass spectrum, *m*/*z*: 549.68 ($M^+ + Na^+$).

Dihexadecyl-L-glutamyl-*N*,*N*-di(carboxymethane) (7). A solution of 1 g (1.68 mmol) L-glutamic acid dihexadecyl ester (**6**) in 2 mL of THF was added to a solution of 0.33 g (3.43 mmol) monochloroacetic acid in 5 mL of THF and 0.30 mL of pyridine under stirring and at a temperature of 56°C, and stirring was continued for 1 h. Further, a solution of 0.65 g (6.86 mmol) monochloroacetic acid in 10 mL of THF and 0.60 mL of pyridine was added, maintaining a slightly alkaline medium. The course of the reaction was monitored by TLC. When finished the reaction mass was acidified with 0.1 N HCl, the precipitate was recrystallized from water, 0.73 g (61%) of product **7** was obtained. ^{1}H NMR spectrum ($CDCl_3$, δ, ppm): 0.88 (t, 6H, CH_3); 1.32 (s, 52H, CH_2); 1.7 (m, 4H, β-$\underline{CH_2}CH_2OCO$); 2.12 (s, 4H, $N\underline{CH_2}COOH$); 2.38 (m, 2H, β-CH_2); 2.65 (t, 2H, δ-CH_2); 3.62 (t, 1H, CH); 4.09 (t, 2H, α-$\underline{CH_2}OCO$); 4.22 (*t*, 2H, α-$\underline{CH_2}$-OCO). IR spectrum (ν_{max}, cm^{-1}): 3365 (COOH), 2916 (CH), 2848 (OH), 1742 (C=O), 1510, 1466, 1219 (C–O), 1214 (C–N), 1177 (CH).

***N*,*N*'-di-*tert*-butoxycarbonylaminoethylethanolamine (9).** A solution of 10.5 g (48.1 mmol) di-*tert*-butyl pyrocarbonate in 30 mL of isopropanol was added dropwise to a solution of 2 g (19.2 mmol) aminoethylethanolamine (**8**) in 5 mL of isopropanol and 5.3 g (38.5 mmol) potassium carbonate in 10 mL of distilled water. The reaction mixture was stirred at 40°C for 8 h while maintaining pH 10. The mass was then evaporated to dryness, diluted with water, extracted with chloroform. The yield of product **9** as an oil was 5.7 g (98.3%). ^{1}H NMR spectrum ($CDCl_3$, δ, ppm): 1.43 (s, 9H, CH_3); 1.46 (s, 9H, CH_3); 3.35 (m, 6H, CH_2); 3.51 (m, 2H, CH_2OH). IR spectrum (ν_{max}, cm^{-1}): 3345 (OH), 2973, 2927 (CH), 1670 (C=O), 1514 (N–H), 1476, 1408 (OH), 1364 (C (CH_3)), 1243 (N–COO), 1158 (C–O), 1049 (CH–OH), 753 (CH_2).

Dihexadecyl-L-glutamyl-*N*,*N*-di(carboxymethyl)-*O*,*O*'-di(aminoethylethanolamine) (10). Analogously to obtaining compound **6**, divalent amphiphile **10** was obtained from 0.50 g (0.70 mmol) of dihexadecyl-L-glutamyl-*N*,*N*-di(carboxymethane) (7) and 0.85 g (2.81 mmol) of *N*,*N*'-di-*tert*-butoxycarbonyl aminoethylethanolamine (**9**). The yield of the product was 0.17 g (31.2%). ^{1}H NMR spectrum ($CDCl_3$, δ, ppm): 0.67 (t, 6H, CH_3); 1.23 (s, 52H, CH_2); 1.55 (m, 4H, β-$\underline{CH_2}$); 1.66 (s, 4H, $N\underline{CH_2}COOH$); 1.87 (m, 2H, β-CH_2 (Glu)); 2.18 (m, 2H, δ-CH_2); 2.45 (t, 1H, CH); 3.34 (m, 2H, $O\underline{CH_2}CH_2NH$); 3.67 (m, 2H, $OCH_2\underline{CH_2}NH$); 3.99 (t,

4H, α-$\underline{CH_2}OCO$); 4.20 (m, 2H, $NH\underline{CH_2}CH_2NH_2$); 4.32 (t, 2H, $NHCH_2\underline{CH_2}NH_3^+$); 5.0 (s, 1H, NH). Mass spectrum, m/z 883.73 (M^+).

The liposome diameter was studied by photon correlation spectroscopy on a Delsa Nano Common instrument (Beckman Coulter).

The stability of the obtained particles was confirmed by measuring the absorbance of the dispersion in time on a Jasco-7800 spectrophotometer at a wavelength of 400–475 nm (permissible error of 3%). Distilled water was used as the reference.

Liposome production. The studied compounds **5** and **10** were selected in the amount necessary to obtain a concentration of 2 mg/mL and dissolved in a minimal amount of chloroform (0.5–1.5 L). The solvent was distilled off in vacuo until a thin film was formed, which was then dried under vacuum for 3 h, the necessary amount of distilled water was added and the film was kept for 1.5 h. The obtained dispersion was then ultrasonicated for 1 h under heating to 50°C.

Transfection study. On the eve of the transfection, the cells were placed on a plate in the amount of 7×10^5 cells per well in 300 μL of a complete DMEM culture medium and incubated at 37°C in a CO_2 incubator up to 75% confluency. A dispersion of the transfection agent in four concentrations with the total volume of 80 μL consisting of 0.2 μg of plasmid (pGFP or pGL3) and liposomal dispersion of the appropriate concentration was prepared in serum-free OPTIMEM medium. A commercial transfection agent Lipofectamine 2000 was used as the positive control. Blank plasmid was used as the negative control. The mixtures were kept for 20 min at room temperature and introduced into wells with a monolayer of cells. The cells were incubated at 37°C in a CO_2 incubator for 24 h, and then luciferase activity was determined by the luciferase test or the number of transfected cells was determined.

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