CHEMISTRY AND TECHNOLOGY OF MEDICINAL COMPOUNDS AND BIOLOGICALLY ACTIVE SUBSTANCES

ХИМИЯ И ТЕХНОЛОГИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ И БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ

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RESEARCH ARTICLE

Cationic amphiphiles based on malonic acid amides as transfection mediators

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Objectives. *The aim of this work is to synthesize cationic amphiphiles based on malonic acid amides. The target compounds should contain saturated and unsaturated alkyl chains in the hydrophobic portion, and one or two positive charges in the polar head as created by ethylenediamine and amino acid L-ornithine. For such cationic amphiphiles, we determined physicochemical properties and transfection efficiency of liposomes based on them.*

Methods. *The initial compound in the synthesis is diethylmalonate. We used C-alkylation to add the first hydrophobic chain (with octylbromide, dodecylbromide, or octadecylbromide). N-oleylamine was used as the second hydrophobic chain, which was attached at the carboxyl group of the malonic acid via amide bond formation. The polar head was represented by ethylenediamine, which was then attached at the second carboxyl group of the malonic acid. Further, L-ornithine was attached to ethylenediamine to produce cationic lipids with two positive charges in the head group. The structures of the compounds were characterized by infrared spectroscopy, nuclear magnetic resonance spectroscopy, and elemental analysis. Particle size distribution was evaluated by photon correlation spectroscopy. The luciferase test was used to determine transfection efficiency using HeLa cells.*

Results. *We have developed a synthesis scheme to produce new cationic amphiphiles with an asymmetric hydrophobic part. The obtained liposomal particles are approximately 120 nm in size and have a relatively high zeta potential of 29–30 mV.*

Conclusions. *The size of these liposomes allows them to penetrate into cells, which makes it possible to use these compositions for transfection. The high zeta potential shows that the particles are stable. Our results demonstrate that the transfection efficiency of our liposomes (mixed with cholesterol) is comparable to a commercial formulation. Cationic amphiphiles based on malonic acid amides have great potential for liposome development for transfection.*

Keywords: *cationic lipids, malonic acid amides, cationic liposomes, transfection efficiency, targeted delivery, cationic amphiphiles.*

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НАУЧНАЯ СТАТЬЯ

Катионные амфифилы на основе амидов малоновой кислоты в качестве медиаторов трансфекции

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Цели. *Задача данной работы заключалась в получении катионных амфифилов, являющихся амидами малоновой кислоты. Целевые амфифилы должны содержать насыщенную и ненасыщенную алкильные цепи в гидрофобном блоке, а также один или два положительных заряда в полярной головной части за счет этилендиамина и аминокислоты L-орнитина. Для таких катионных амфифилов должны быть определены физико-химические свойства и трансфекционная активность липосомальных композиций на их основе.*
Методы. *Исходным соединением в синтезе был диэтиловый эфир малоновой кислоты. С ним проводили реакцию С-алкилирования для присоединения первой гидрофобной цепи (с использованием октилбромида, додецилбромида и октадецилбромида). В качестве второй гидрофобной цепи использовали N-олеиламин, который присоединяли по карбоксильной группе малоновой кислоты путем образования амидной связи. Полярная головная группа была представлена этилендиамином, который присоединяли по оставшейся карбоксильной группе малоновой кислоты. Далее к этилендиамину присоединяли L-орнитин для получения катионных липидов с двумя положительными зарядами в головной группе. Структуры соединений характеризовали с помощью инфракрасной спектроскопии, спектроскопии ядерного магнитного резонанса и элементного анализа. Методом фотонно-корреляционной спектроскопии оценивали распределение частиц по размерам. С помощью люциферазного теста определяли эффективность трансфекции на клеточной линии HeLa.*
Результаты. *Разработана схема синтеза новых катионных амифифилов с несимметричным гидрофобным блоком. Полученные на их основе липосомальные частицы имеют размер около 120 нм и обладают достаточно высоким дзета-потенциалом (29–30 мВ).*
Выводы. *Размер полученных липосом позволяет им проникать в клетки, что делает возможным использование таких композиций для трансфекции. Высокий дзета-потенциал свидетельствует об их стабильности. Наши результаты показали, что эффективность трансфекции полученными липосомами в смеси с холестерином сопоставима с коммерческим препаратом. Катионные амфифилы на основе амидов малоновой кислоты являются перспективными для разработки трансфекционных липосомальных композиций на их основе.*

Ключевые слова: *катионные липиды, амиды малоновой кислоты, катионные липосомы, трансфекционная активность, направленная доставка, катионные амфифилы*

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INTRODUCTION

Gene therapy is a promising approach for many genetic disease treatments, which introduces missing genes or replaces defective genes with healthy ones [1]. Since the early stages of the development of the concept of gene therapy in medicine, effective methods of transferring nucleic acids to cells have been proposed [2]. These methods provide the delivery of polynucleotides to biological tissue or cells—the so-called gene transfection. Due to nuclease degradation, "pure" DNA or RNA cannot penetrate the cell membrane without using physical methods such as injections or "gene guns," so carriers able to transport DNA through the bloodstream and effectively release genetic material near the cell nucleus are needed [3].

Polynucleotide delivery systems are divided into two main classes: viral and non-viral vectors [4–5]. Despite high transfection efficiencies, viral systems have disadvantages as they can cause immune responses to a greater or lesser extent [6].

Nucleic acid carrier systems based on cationic lipids are a promising pharmaceutical tool for gene therapy strategy implementation [7]. Unlike viral systems, these complexes do not have immunogenic potential or size restrictions of delivered genetic fragments [8]. However, cationic lipids, as well as other non-viral vectors such as polymers and peptides, exhibit low transfection efficiency and pronounced toxicity [9].

During the development of optimal transfection systems, new lipids are constantly being synthesized and studied. To increase the rate of transfection, the structures of cationic lipids are changed, the ratios with uncharged auxiliary lipids are changed, and varying amounts of DNA are added [10–12]. In this regard, it is important to study the physical and chemical properties of lipids to better understand the structure-efficiency relationship for gene transfer [7]. Therefore, numerous studies with multidisciplinary approaches are needed [13].

The advantage of cationic lipids is that they can be constructed in accordance with the "modular principle"—it is possible to perform structural changes separately in the head group, the linker, and the lipophilic region [11, 14, 15]. The structure of the hydrophobic domain determines the phase transition temperature and bilayer fluidity, which further affects the liposome stability, protection of DNA from nucleases, endosomal output, release of DNA from the lipoplex, and penetration into the nucleus [3, 16]. Oleic unsaturated chains promote endosomal release by increasing the membrane fluidity of transfection complexes [17]. The effectiveness of transfection of cationic lipids is also affected by asymmetry [3]. In addition, multivalent lipids are more active than monovalent lipids [11].

A promising class of cationic lipids is malonic acid diamides [13]. Malonic acid-based cationic lipids were first described in the literature (as nucleic acid carriers) about 10 years ago. Malonic acid diethyl ether is the central building block of synthesis. The chemical properties of malonic acid make it possible to attach alkyl chains via acyl functions in the form of amides or esters [11]. Malonic acid diamides with two long hydrophobic alkyl chains and a polar head group, used as a new class of non-viral gene-transferring agent, have shown high transfection efficiency and moderate toxicity. In addition, amide bonds showed better hydrolytic stability than ester bonds. It was shown that an increase in the cationic head group by combining with two lysine molecules, rather than just one, leads to an increased transfection efficiency [10, 15]. Thus, the characterization of the biocompatibility of effective lipid compositions and the study of therapeutic concepts for the medical use of highly effective cationic lipids derived from malonic acid are an urgent task [11].

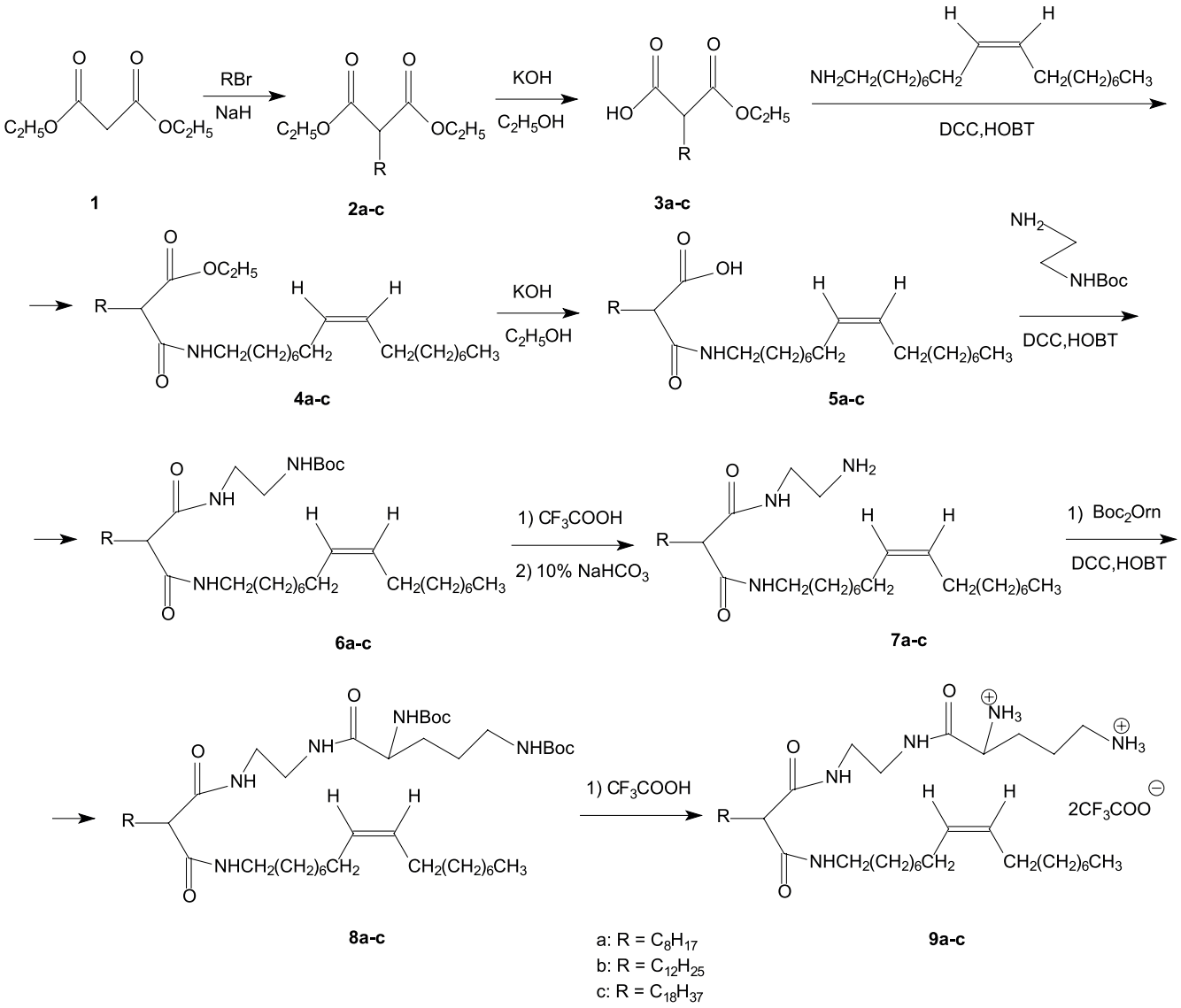
The aim of this work is to obtain cationic amphiphiles based on malonic acid amides containing saturated and unsaturated alkyl chains in a hydrophobic block (scheme 1), as well as one or two positive charges in the polar head region created by ethylenediamine and L-ornithine, and study the properties and transfection activities of the liposomal compositions based on them.

MATERIALS AND METHODS

General method

All chemicals and reagents were used without pretreatment: diethylmalonate, ethylenediamine, octylbromide, dodecylbromide, octadecylbromide (all—*Acros Organics*, Belgium), *N*-oleylamine (*Sigma-Aldrich*, USA), di-*tert*-butyl dicarbonate (Boc_2O) (*Sigma-Aldrich*, USA), L-ornithine monohydrochloride (L-Orn*HCl) (*Acros Organics*, Belgium), *N,N'*-dicyclohexylcarbodiimide (DCC) (*Sigma-Aldrich*, USA), 1-hydroxybenzotriazole (HOBT) (*Sigma-Aldrich,* USA), potassium hydroxide (*CHIMMED*, Russia), sodium sulfate anhydrous (*CHIMMED*, Russia), and trifluoroacetic acid (TFA) (*Biochem*, Russia).

^{1}H NMR nuclear magnetic resonance spectra were recorded in deuterated chloroform using a Bruker WM-400 pulsed NMR spectrometer (*Bruker,* Germany) with an operating frequency of 400 MHz. The internal standard is hexamethyldisiloxane. Infrared spectra were recorded on an EQUINOX 55 (*Bruker,* Germany) Fourier transform-infrared spectrometer. Elemental analysis was performed using



Scheme 1. Malonic acid amides.

a CHNS analyzer FLASH EA 1112 (*Thermo Finnigan Italia S.p.A,* Italia). Thin-layer chromatography (TLC) was performed on Sorbfil plates (*IMID*, Russia), and column chromatography was performed on silica gel with a size of 0.040–0.063 mm (*Merck*, Germany). To detect spots in TLC, heating over the flame of an alcohol lamp was used. To detect substances containing amino groups, a 5% solution of ninhydrin was used, followed by heating to 50°C.

EXPERIMENTAL

Mono-*N*-*tert*-butoxicarbonylethylenediamine (1). 8.01 g (133.5 mmol) of 1,2-ethylenediamine was dissolved in dioxane in an inert argon atmosphere, and a solution of 3.78 g (17.34 mmol) Boc_2O in dioxane was added dropwise for 3 h. The mixture was stirred at room temperature for 24 h. The solvent was distilled in a vacuum, and the remaining solid was dissolved in water. The product was isolated by dichloromethane extraction (3 × 50 mL). The product yield was 2.18 g (78.5%).

IR spectrum (ν_{max}, cm^{-1}): 3355 (NH), 2978, 2934 (CH_2), 1693 (C=O, "I amide band"), 1526 (NH, "II amide band"), 1392, 1367, 1278, 1253 (C–O), 1173 (C–N), 1045 (CH_2), 966 (CH_3).

Diethyl ether of octylmalonic acid (2a). 0.49 g (20.42 mmol) sodium hydride was gradually added to a solution of diethylmalonate 3 g (18.75 mmol) in anhydrous tetrahydrofuran and mixed for 1 h at room temperature. After that, a solution of 3.29 g (17.05 mmol) of octylbromide in anhydrous tetrahydrofuran was added to the protonated diethylmalonate drop by drop and mixed for 24 h. The solvent was distilled on a rotary evaporator. Ice water was added to the reaction mass, the products were extracted with ethyl acetate, dried

with sodium sulfate, and the solvent was distilled on a rotary evaporator. We obtained 4.23 g (82.9%) of the product.

^{1}H NMR spectrum of compound **2a** ($CDCl_3$, δ, ppm): 0.86 (t, 3H, $-CH_3$), 1.27 (m, 18H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.89 (m, 2H, $-CH\underline{CH_2}-$), 3.35 (t, 1H, –CH–), 4.18 (m, 4H, $-O\underline{CH_2}CH_3$).

Diethyl ether of dodecylmalonic acid (2b). Similarly, from 3 g (18.75 mmol) of diethylmalonate, 0.49 g (20.42 mmol) of sodium hydride, and 4.24 g (17.03 mmol) of dodecylbromide. The product yield was 5.16 g (83.9%).

^{1}H NMR spectrum of compound **2b** ($CDCl_3$, δ, ppm): 0.87 (t, 3H, $-CH_3$), 1.28 (m, 26H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.78 (m, 2H, $-CH\underline{CH_2}-$), 3.30 (t, 1H, –CH–), 4.20 (m, 4H, $-O\underline{CH_2}CH_3$).

Diethyl ether of octadecylmalonic acid (2c). Similarly: from 2 g (12.50 mmol) of diethylmalonate, 0.33 g (13.75 mmol) of sodium hydride and 3.79 g (11.37 mmol) of octadecylbromide. The product yield was 3.91 g (83.5%).

^{1}H NMR spectrum of compound **2c** ($CDCl_3$, δ, ppm): 0.90 (t, 3H, $-CH_3$), 1.27 (m, 38H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.84 (m, 2H, $-CH\underline{CH_2}-$), 3.37 (t, 1H, –CH–), 4.21 (m, 4H, $-O\underline{CH_2}CH_3$).

Monoethyl ether of octylmalonic acid (3a). To a solution of 8.37 g (30.77 mmol) of compound **2a** in ethanol, a solution of 2.07 g (36.96 mmol) KOH in ethanol was added dropwise for 1 h. Then the mixture was mixed for 3 h and left overnight without stirring. After that, ethanol was distilled to half the volume and the solution was acidified with an equimolar amount of 0.1 M hydrochloric acid. The target compound was extracted with ethyl acetate, dried with sodium sulfate, and the solvent was distilled on a rotary evaporator. The product was extracted by flash chromatography with hexane elution, then by a 15 : 1 chloroform : methanol system. We obtained 3.69 g (49.2%) of the product.

^{1}H NMR spectrum of compound **3a** ($CDCl_3$, δ, ppm): 0.86 (t, 3H, $-CH_3$), 1.28 (m, 15H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.89 (m, 2H, $-CH\underline{CH_2}-$), 3.37 (t, 1H, –CH–), 4.19 (m, 2H, $-O\underline{CH_2}CH_3$).

Monoethyl ether of dodecylmalonic acid (3b). Similarly: of the 7.83 g (23.87 mmol) compound **2b** and 1.6 g (28.57 mmol) KON. The product yield was 2.9 g (33.6%).

^{1}H NMR spectrum of compound **3b** ($CDCl_3$, δ, ppm): 0.87 (t, 3H, $-CH_3$), 1.24 (m, 23H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.70 (m, 2H, $-CH\underline{CH_2}-$), 3.20 (t, 1H, –CH–), 4.16 (m, 2H, $-O\underline{CH_2}CH_3$).

Monoethyl ether of octadecylmalonic acid (3c). Similarly: from 7.02 g (17.04 mmol) of compound **2c** and 1.15 g (20.54 mmol) KOH. The product yield was 2.6 g (39.8%).

^{1}H NMR spectrum of compound **3c** ($CDCl_3$, δ, ppm): 0.88 (t, 3H, $-CH_3$), 1.28 (m, 33H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.91 (m, 2H, $-CHCH_2\underline{CH_2}-$), 3.38 (t, 1H, –CH–), 3.72 (m, 2H, $-CH\underline{CH_2}-$), 4.18 (m, 2H, $-O\underline{CH_2}CH_3$).

Ethyl ester of 2-{[(9*Z*)-octadec-9-enoylamino]-carbonyl}decanoic acid (4a). To a solution of 217.9 mg (0.89 mmol) of compound **3a** in anhydrous methylene chloride at 0°C, 241 mg (1.79 mmol) HOBT and a solution of 368 mg (1.79 mmol) DCC were added with stirring. After that, 287 mg (1.07 mmol) of oleylamine in anhydrous dichloromethane was added to the solution. The mixture was kept for 2 h when cooled and left for a day while stirring at room temperature, and then the precipitate was filtered out. The solvent was distilled on a rotary evaporator. The product was isolated by column chromatography in a 40 : 1 tetrachloromethane : methanol system. We obtained 299 mg (68.0%) of the product.

^{1}H NMR spectrum of compound **4a** ($CDCl_3$, δ, ppm): 0.86 (t, 6H, $2CH_3$), 1.26 (m, 37H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.47 (m, 2H, $-NHCH_2\underline{CH_2}-$), 1.84 (m, 2H, $-CH\underline{CH_2}-$), 1.97 (m, 4H, $-\underline{CH_2}CH{=}CH\underline{CH_2}-$), 3.23 (m, 3H, –CH–, $-\underline{CH_2}NHCO-$), 4.18 (m, 2H, $-O\underline{CH_2}CH_3$), 5.35 (m, 2H, –CH=CH–), 6.55 (m, 1H, –NH–).

Ethyl ether of 2-{[(9*Z*)-octadec-9-enoylamino]-carbonyl}tetradecanoic acid (4b). Similarly: from 182 mg (0.61 mmol) of compound **3b**, 164 mg (1.21 mmol) of 1-hydroxybenzotriazole, 251 mg (1.22 mmol) of *N,N'*-dicyclohexylcarbodiimide, and 195 mg (0.73 mmol) of oleylamine. The product yield was 226 mg (67.5%).

^{1}H NMR spectrum of compound **4b** ($CDCl_3$, δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.26 (m, 45H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.48 (m, 2H, $-NHCH_2\underline{CH_2}-$), 1.86 (m, 2H, $-CH\underline{CH_2}-$), 1.99 (m, 4H, $-\underline{CH_2}CH{=}CH\underline{CH_2}-$), 3.23 (m, 3H, –CH–, $-\underline{CH_2}NHCO-$), 4.18 (m, 2H, $-O\underline{CH_2}CH_3$), 5.34 (m, 2H, –CH=CH–), 6.58 (m, 1H, –NH–).

Ethyl ether of 2-{[(9*Z*)-octadec-9-enoylamino]-carbonyl}eicosanic acid (4c). Similarly: of the 190 mg (0.49 mmol) compound **3c**, 134 mg (0.99 mmol) HOBT, 204 mg (0.99 mmol) DCC, and 159 mg (0.59 mmol) oleylamine. The product yield was 205 mg (65.5%).

^{1}H NMR spectrum of compound **4c** ($CDCl_3$, δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.27 (m, 57H, $-\underline{CH_2}CH_3$, $-OCH_2\underline{CH_3}$), 1.49 (m, 2H, $-NHCH_2\underline{CH_2}-$), 1.83 (m, 2H, $-CH\underline{CH_2}-$), 2.00 (m, 4H, $-\underline{CH_2}CH{=}CH\underline{CH_2}-$), 3.23 (m, 3H, –CH–, $-\underline{CH_2}NHCO-$), 4.18 (m, 2H, $-O\underline{CH_2}CH_3$), 5.34 (m, 2H, –CH=CH–), 6.57 (m, 1H, –NH–).

2-{[(9*Z*)-octadec-9-enoylamino]carbonyl}-decanoic acid (5a). To a solution of 277 mg (0.56 mmol) of compound **4a** in ethanol, a solution of 63 mg (1.13 mmol) KOH in ethanol was added dropwise

for one hour. Then the solution was mixed at boiling conditions for eight hours. After that, ethanol was distilled to half the volume and the reaction mass was acidified with an equimolar amount of 0.1 M hydrochloric acid. The compound was extracted with ethyl acetate, dried with sodium sulfate, and the solvent was distilled on a rotary evaporator. We obtained 245 mg (94.0%) of the product.

^{1}H NMR spectrum of compound **5a** ($CDCl_3$, δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.27 (m, 34H, –$\underline{CH_2}CH_3$), 1.47 (m, 2H, –NHCH$_2\underline{CH_2}$–), 1.94 (m, 6H, –CH$\underline{CH_2}$–, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 3.19 (t, 1H, –CH–), 3.30 (m, 2H, –$\underline{CH_2}$NHCO–), 5.34 (m, 2H, –CH=CH–), 6.43 (m, 1H, –NH–).

2-{[(9*Z*)-octadec-9-enoylamino]carbonyl}-tetradecanoic acid (5b). Similarly: of 200 mg (0.36 mmol) compound **4b** and 40 mg (0.71 mmol) KON. The product yield was 153 mg (81.8%).

^{1}H NMR spectrum of compound **5b** ($CDCl_3$, δ, ppm): 0.90 (t, 6H, $2CH_3$), 1.27 (m, 42H, –$\underline{CH_2}CH_3$), 1.53 (m 2H, –NHCH$_2\underline{CH_2}$–), 1.95 (m, 6H, –CH$\underline{CH_2}$–, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 3.15 (t, 1H, –H–), 3.31 (m, 2H, –$\underline{CH_2}$NHCO–), 5.35 (m, 2H, –CH=CH–), 6.26 (m, 1H, –NH–).

2-{[(9*Z*)-octadec-9-enoylamino]carbonyl}-eicosanic acid (5c). Similarly: of the 190 mg (0.30 mmol) compound **4c** and 34 mg (0.61 mmol) KON. The product yield was 170 mg (93.6%).

^{1}H NMR spectrum of compound **5c** ($CDCl_3$, δ, ppm): 0.88 (t, 6H, $2CH_3$), 1.27 (m, 54H, –$\underline{CH_2}CH_3$), 1.53 (m, 2H, –NHCH$_2\underline{CH_2}$–), 2.01 (m, 6H, –CH$\underline{CH_2}$–, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 3.16 (t, 1H, –CH–), 3.30 (m, 2H, –$\underline{CH_2}$NHCO–), 5.35 (m, 2H, –CH=CH–), 6.33 (m, 1H, –NH–).

The diamide *N*-2-[(*N-tert*-butoxycarbonyl)-amino]ethyl-2-octyl-N'-[(9*Z*)-octadec-9-enoyl]-propane (6a). To a solution of 255 mg (0.55 mmol) of compound **5a** in anhydrous methylene chloride at 0°C, 149 mg (1.1 mmol) HOBT and a solution of 226 mg (1.1 mmol) DCC were added with stirring. Then 105 mg (0.66 mmol) of Boc-protected ethylenediamine in anhydrous dichloromethane was added to the solution. The mixture was kept for 2 h at 0ºC and then stirred for 24 h at room temperature. The precipitate was then filtered out. The solvent was distilled on a rotary evaporator. The product was isolated by column chromatography in the 40 : 1 tetrachloromethane : methanol system. We obtained 259 mg (77.8%) of the product.

^{1}H NMR spectrum of compound **6a** ($CDCl_3$, δ, ppm): 0.88 (t, 6H, $2CH_3$), 1.23 (m, 34H, –$\underline{CH_2}CH_3$), 1.45 (m, 11H, $3CH_3$, –NHCH$_2\underline{CH_2}$–), 1.65 (m, 2H, –CH$\underline{CH_2}$–), 1.95 (m, 4H, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 2.95 (t, 1H, –CH–), 3.25 (m, 4H, –OCNH$\underline{CH_2}$–$\underline{CH_2}$NHCO–), 3.43 (m, 2H, –$\underline{CH_2}$NHCO–), 5.01 (m, 1H, –NH–), 5.37 (m, 2H, –CH=CH–), 6.82 (m, 1H, –NH–), 7.28 (m, 1H, –NH–).

The diamide *N*-2-[(*N-tert*-butoxycarbonyl)-amino]ethyl-2-dodecyl-*N'*-[(9*Z*)-octadec-9-enoyl]-propane (6b). Similarly: of 66 mg (0.13 mmol) compound **5b**, 35 mg (0.26 mmol) HOBT, 53 mg (0.26 mmol) DCC, and 26 mg (0.16 mmol) Boc-protected ethylenediamine. The product yield was 55.1 mg (65.6%).

^{1}H NMR spectrum of compound **6b** ($CDCl_3$, δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.16 (m, 42H, –$\underline{CH_2}CH_3$), 1.43 (c, 9H, $3CH_3$),1.63 (m, 2H, –NHCH$_2\underline{CH_2}$–), 1.81 (m, 2H, –CH$\underline{CH_2}$–), 1.99 (m, 4H, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 2.94 (t, 1H, –CH–), 3.24 (m, 4H, –OCNH$\underline{CH_2}$–$\underline{CH_2}$NHCO–), 3.43 (m, 2H, –$\underline{CH_2}$NHCO–), 5.00 (m, 1H, –NH–), 5.34 (m, 2H, –CH=CH–), 6.81 (m, 1H, –NH–), 7.26 (m, 1H, –NH–).

The diamide *N*-2-[(*N-tert*-butoxycarbonyl)-amino]ethyl-2-octadecyl-*N'*-[(9*Z*)-octadec-9-enoyl]-propane (6c). Similarly: of 160 mg (0.26 mmol) compound **5c**, 71 mg (0.53 mmol) HOBT, 109 mg (0.53 mmol) DCC, and 51 mg (0.32 mmol) Boc-protected ethylenediamine. The product yield was 179.8 mg (90.8%).

^{1}H NMR spectrum of compound **6c** ($CDCl_3$, δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.20 (m, 54H, –$\underline{CH_2}CH_3$), 1.43 (c, 9H, $3CH_3$),1.60 (m, 2H, –NHCH$_2\underline{CH_2}$–), 1.72 (m, 2H, –CH$\underline{CH_2}$–), 1.95 (m, 4H, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 2.93 (t, 1H, –CH–), 3.23 (m, 4H, –OCNH$\underline{CH_2}$–$\underline{CH_2}$NHCO–), 3.42 (m, 2H, –$\underline{CH_2}$NHCO–), 4.98 (m, 1H, –NH–), 5.32 (m, 2H, –CH=CH–), 6.76 (m, 1H, –NH–), 7.21 (m, 1H, –NH–).

Diamide *N*-(2-aminoethyl)-2-octyl-*N'*-[(9*Z*)-octadec-9-enoyl]propane (7a). To 248 mg (0.41 mmol) of compound **6a**, 70 mg (6.14 mmol) of TFA was added in 3 mL of methylene chloride, and stirred for 3 h at 0°C. Then the reaction mass was washed with a 10% solution of sodium bicarbonate and water. The organic residue was dried with sodium sulfate, and the solvent was distilled in a vacuum. We obtained 201.8 mg (97.5%) of the product.

Diamide *N*-(2-aminoethyl)-2-dodecyl-*N'*-[(9*Z*)-octadec-9-enoyl]propane (7b). Similarly: from 135 mg (0.20 mmol) of compound **6b** and 35 mg (3.07 mmol) of TFA. The product yield was 110.4 mg (96.0%).

Diamide *N*-(2-aminoethyl)-2-octadecyl-*N'*-[(9*Z*)-octadec-9-enoyl]propane (7c). Similarly: from 169 mg (0.23 mmol) of compound **6c** and 39 mg (3.42 mmol) of TFA. The product yield was 142.6 mg (97.0%).

Diamide *N'*-2-[(2,5-di(*N-tert*-butoxycarbonyl)-amino-1-oxopentyl)amino]ethyl-2-octyl-N-[(9*Z*)-octadec-9-enoyl]propane (8a). To a 250 mg (0.49 mmol) solution of compound **7a** in anhydrous methylene chloride at 0°C, 133 mg (0.99 mmol) HOBT and a

203 mg (0.99 mmol) DCC solution were added during mixing. Then 196 mg (0.59 mmol) of Boc-protected ornithine in anhydrous dichloromethane was added to the solution. The mixture was kept for 2 h when cooled and stirred for 24 h at room temperature, and the precipitate was filtered out. The solvent was distilled on a rotary evaporator. The product was isolated by column chromatography in the 40 : 1 tetrachloromethane : methanol system. We obtained 219 mg (54.1%) of the product.

^{1}H NMR spectrum of compound **8a** ($CDCl_3$, δ, ppm): 0.85 (t, 6H, $2CH_3$), 1.21 (m, 34H, –$\underline{CH_2}CH_3$), 1.43 (s, 18H, $6CH_3$), 1.54 (m, 2H, –NHC$H_2\underline{CH_2}$–), 1.69 (m, 4H, –CH$\underline{CH_2}\underline{CH_2}CH_2$NHCO–), 1.93 (m, 6H, –CH$\underline{CH_2}$–, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 3.08 (m, 2H, –$\underline{CH_2}$NHCO), 3.20 (m, 3H, –CH–, –$\underline{CH_2}$NHCO–), 3.46 (m, 5H, –CH–, –OCNH$\underline{CH_2}$–$\underline{CH_2}$NHCO–), 4.10 (m, 1H, –NH–), 4.89 (m, 1H, –NH–), 5.23 (m, 1H, –NH–), 5.33 (m, 2H, –CH=CH–), 6.96 (m, 1H, –NH–), 7.37 (m, 1H, –NH–).

Diamide *N*'-2-[(2,5-di(*N*-*tert*-butoxycarbonyl)-amino-1-oxopentyl)amino]ethyl-2-dodecyl-*N*-[(9*Z*)-octadec-9-enoyl]propane (8b). Similarly: of 141 mg (0.25 mmol) compound **7b**, 68 mg (0.5 mmol) HOBT, 103 mg (0.5 mmol) DCC, and 100 mg (0.3 mmol) Boc-protected ornithine. The product yield was 166 mg (75.5%).

^{1}H NMR spectrum of compound **8b** ($CDCl_3$, δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.24 (m, 42H, –$\underline{CH_2}CH_3$), 1.43 (s, 18H, $6CH_3$), 1.54 (m, 2H, –NHC$H_2\underline{CH_2}$–), 1.69 (m, 4H, –CH$\underline{CH_2}\underline{CH_2}CH_2$NHCO–), 1.92 (m, 6H, –CH$\underline{CH_2}$–, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 3.06 (m, 2H, –$\underline{CH_2}$NHCO), 3.19 (m, 3H, –CH–, –$\underline{CH_2}$NHCO–), 3.44 (m, 5H, –CH–, –OCNH$\underline{CH_2}$–$\underline{CH_2}$NHCO–), 4.10 (m, 1H, –NH–), 4.87 (m, 1H, –NH–), 5.20 (m, 1H, –NH–), 5.33 (m, 2H, –CH=CH–), 6.94 (m, 1H, –NH–), 7.36 (m, 1H, –NH–).

Diamide *N*'-2-[(2,5-di(*N*-*tert*-butoxycarbonyl)-amino-1-oxopentyl)amino]ethyl-2-octadecyl-*N*-[(9*Z*)-octadec-9-enoyl]propane (8c). Similarly: of the 159 mg (0.25 mmol) compound **7c**, 66 mg (0.49 mmol) HOBT, 101 mg (0.49 mmol) DCC, and 97 mg (0.29 mmol) Boc-protected ornithine. The product yield was 158.8 mg (67.3%).

^{1}H NMR spectrum of compound **8c** ($CDCl_3$, δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.20 (m, 54H, –$\underline{CH_2}CH_3$), 1.46 (s, 18H, $6CH_3$), 1.59 (m, 2H, –NHC$H_2\underline{CH_2}$–), 1.70 (m, 4H, –CH$\underline{CH_2}\underline{CH_2}CH_2$NHCO–), 1.92 (m, 6H, –CH$\underline{CH_2}$–, –$\underline{CH_2}$CH=CH$\underline{CH_2}$–), 3.07 (m, 2H, –$\underline{CH_2}$NHCO), 3.26 (m, 3H, –CH–, –$\underline{CH_2}$NHCO–), 3.45 (m, 5H, –CH–, –OCNH$\underline{CH_2}$–$\underline{CH_2}$NHCO–), 4.15 (m, 1H, –NH–), 4.88 (m, 1H, –NH–), 5.25 (m, 1H, –NH–), 5.33 (m, 2H, –CH=CH–), 6.94 (m, 1H, –NH–), 7.38 (m, 1H, –NH–).

Diamide *N*'-2-[(2,5-diamino-1-oxopentyl)amino]-ethyl-2-octyl-*N*-[(9*Z*)-octadec-9-enoyl]propane (9a). To 5 mL of a 208 mg (0.25 mmol) solution of compound **8a** in methylene chloride, 1.59 g (13.95 mmol) of TFA in 1 mL of methylene chloride was added and stirred for three hours at 0°C. The solvent and excess acid were distilled on a vacuum rotary evaporator. We obtained 213 mg (99.1%) of the product. Found, %: C 56.19; H 8.25; N 7.95. $C_{40}H_{73}N_5O_7F_6$. Calculated, %: C 56.54; H 8.60; N 8.25.

Diamide *N*'-2-[(2,5-diamino-1-oxopentyl)amino]-ethyl-2-dodecyl-*N*-[(9*Z*)-octadec-9-enoyl]propane (9b). Similarly: from 64 mg (0.07 mmol) of compound **8b** and 915 mg (8.03 mmol) of TFA. The product yield was 65 mg (98.5%). Found, %: C 57.89; H 8.43; N 7.83. $C_{44}H_{81}N_5O_7F_6$. Calculated, %: C 58.34; H 8.95; N 7.74.

Diamide *N*'-2-[(2,5-diamino-1-oxopentyl)amino]-ethyl-2-octadecyl-*N*-[(9*Z*)-octadec-9-enoyl]-propane (9c). Similarly: from 58 mg (0.06 mmol) of compound **8c** and 1.1 g (9.65 mmol) of TFA. The product yield was 59 mg (98.3%). Found, %: C 60.32; H 9.14; N 6.82. $C_{50}H_{93}N_5O_7F_6$. Calculated, %: C 60.67; H 9.40; N 7.08.

Preparation of liposomal dispersions

Two types of liposomes were obtained: from pure cationic lipid and from a mixture of cationic lipid and cholesterol in a ratio of 7 : 3. Four milligrams of each type of lipid composition were dissolved in chloroform. The solvent was distilled on a vacuum rotary evaporator to form a thin lipid film. The resulting films were dried in vacuum for three hours and then hydrated with 2 mL of distilled water at room temperature. The hydrated films were shaken and processed in an ultrasonic bath (2 × 30 min) at 40°C. The particle size distribution was estimated using photon correlation spectroscopy, which is based on the principles of dynamic light scattering, using a Photocor Compact-Z particle size analyzer (Russia).

Plasmid DNA transfection

Cells were planted in a tablet in the amount of 7×10^5 cells per well in 300 μL of full DMEM culture medium and incubated for 24 h at 37°C in a CO_2 incubator until a monolayer was reached the day before transfection. A dispersion of the transfection agent with a total volume of 80 μL, consisting of 1.177 μL of pGL3 plasmid and 9 μL of liposomal dispersion, was prepared in a serum-free OPTIMEM medium (ratio N : P = 16 : 1). The commercial transfection agent Lipofectamine 2000 (*Thermo Fisher Scientific,* USA) was used as a positive control. A "naked" plasmid was used as a negative control. The prepared mixtures were kept for 20 min at room temperature and applied to a monolayer

of cells in wells. The cells were incubated at 37°C in a CO_2 incubator for 24 h, and then activity was determined using a luciferase test.

Luciferase test

The test was performed using the Luciferase Assay System (*Promega Corporation*, USA) commercial Suite. To do this, growth medium was removed from the wells and then 70 μL of Glo Lysis Buffer, 1X (*Promega Corporation*, USA) was added. The cells were maintained at 37°C for 15 min in a CO_2 incubator to achieve complete lysis. Then they were taken from the bottom of the cells and transferred to Eppendorf plastic tubes (Germany). To precipitate cellular debris, the resulting lysate was centrifuged for three minutes at 10000 rpm. A 50 μL aliquot of the supernatant was selected, and a luciferase substrate was added in a ratio of 1 : 1. The transfection efficiency was assessed by the luminescence level on a GloMax 20/20 Luminometer (USA).

RESULTS AND DISCUSSION

In this work, malonic acid amides were synthesized (Scheme 1), where the initial compound in the synthesis was malonic acid diethyl ether. The mobility of the α-methylene hydrogen link and its increased acidity in the molecule enable the *C*-alkylation reaction, which result in the attachment of the first hydrophobic chain. For this purpose, $C_8H_{17}Br$, $C_{12}H_{25}Br$, and $C_{18}H_{37}Br$ bromides were used, thereby obtaining three corresponding cationic lipids that differ in hydrophobic block length.

The asymmetry effect and presence of unsaturated higher fatty acid residues in the hydrophobic block on the efficiency of transfection is known from literature [3]. Therefore, as the second hydrophobic chain, *N*-oleylamine was used, which was attached to the carboxyl group of malonic acid by forming an amide bond. The polar head group was first represented by ethylenediamine, which was then attached to the remaining carboxyl group of malonic acid by forming an amide bond. Then the natural amino acid L-ornithine was added to ethylenediamine to produce cationic lipids with two positive charges in the head group.

The physicochemical properties of the obtained one- and two-component liposomal dispersions (cationic lipid and a mixture of cationic lipid and cholesterol in a ratio of 7 : 3) were studied, and the particle sizes, zeta potentials, and transfection efficiencies were determined. The particle sizes were practically unchanged when cholesterol was added to the lipid and were within 100 nm, which is necessary for effective transfection (Fig. 1). The zeta potentials of the compositions were 29–30 mV. The values of this parameter at the level of 30 mV refer to the stability of the obtained particles, that is, their stability in relation to aggregation [18].

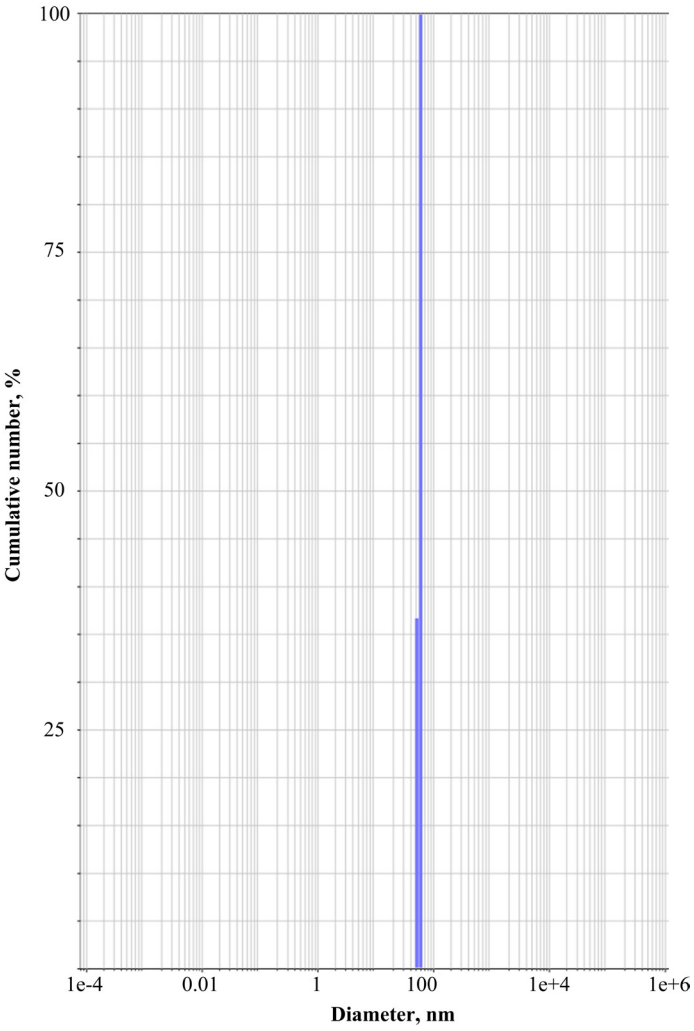


Fig. 1. Size distribution of dispersion particles based on compound **7b**.

Transfection effectiveness of the obtained liposomes was studied on the HeLa cell line (cervical cancer cells) by assessing the level of luminescence in the cell supernatants of the studied samples after delivery of the pGL3 plasmid encoding the luciferase gene. A commercially available transfection agent Lipofectamine 2000 was used as a positive control and a "naked" plasmid was used as a negative control. It was found that sample **7b**, in which cholesterol was added to the cationic lipid, showed a good result, almost reaching the level of transfection activity of Lipofectamine 2000 (Fig. 2).

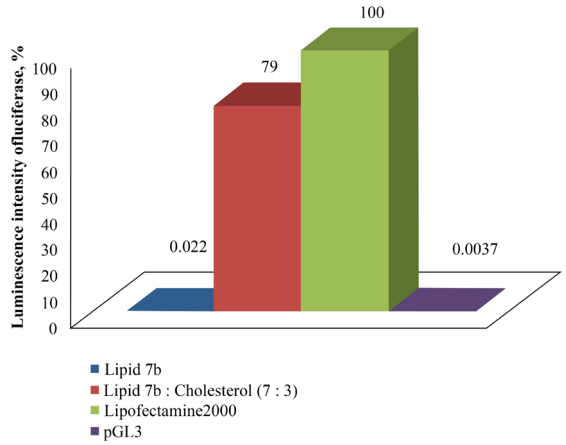


Fig. 2. Transfection efficiency for obtained liposomes.

CONCLUSIONS

A synthesis scheme was developed and new cationic amphiphiles with an asymmetric hydrophobic block were generated. Liposomal dispersions were formed, and their physical and chemical properties and transfection activity were studied. The obtained results indicate good potential of malonic acid amides for creating a new class of cationic amphiphiles used as transfection agents.

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Authors' contribution

N.A. Romanova – carrying out the study, collection and provision of the material, writing the article;

U.A. Budanova – consultation on conducting individual stages of the study, scientific editing;

Yu.L. Sebyakin – development of the research idea, literature analysis.

The authors declare no conflicts of interest.

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