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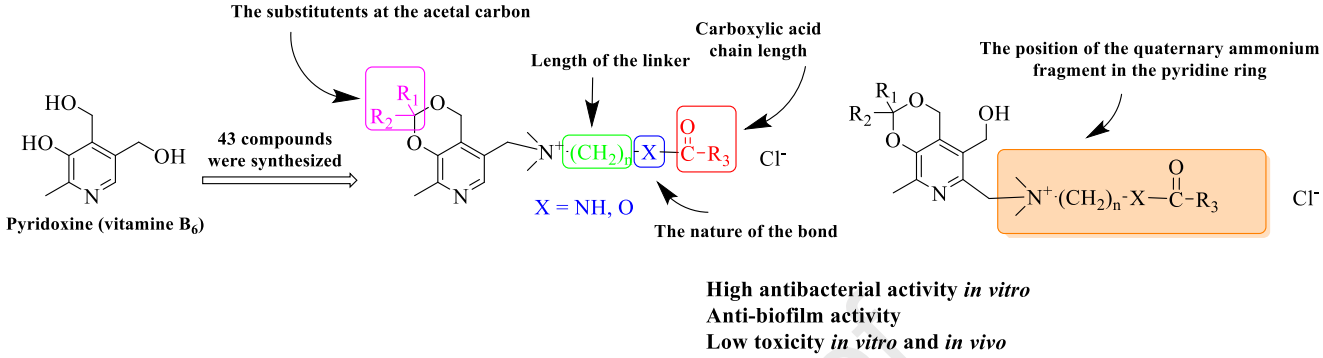

High antibacterial activity *in vitro*
Anti-biofilm activity
Low toxicity *in vitro* and *in vivo*

Graphical Abstract

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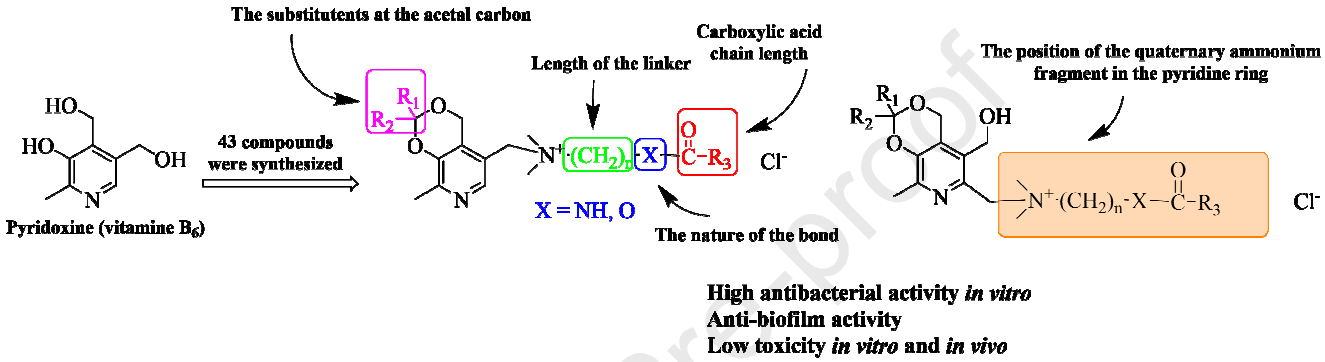

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Design, synthesis, antibacterial activity and toxicity of novel quaternary ammonium compounds based on pyridoxine and fatty acids

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ABSTRACT

A diverse series of 43 novel "soft antimicrobials" based on quaternary ammonium pyridoxine derivatives which include six-membered acetals and ketals of pyridoxine bound via cleavable linker moieties (amide, ester) with a fragment of fatty carboxylic acid was designed. Nine compounds exhibited *in vitro* promising antibacterial activity against Gram-positive and Gram-negative bacterial strains with MIC values comparable with reference antiseptics miramistin, benzalkonium chloride and chlorohexidine. On various clinical isolates, the lead compounds **6i** and **12a** exhibited antibacterial activity comparable with that of benzalkonium chloride while higher than that of miramistin. Moreover, **6i** and **12a** were able to kill bacteria embedded into the matrix of mono- and dual species biofilms. The treatment of bacterial cells by either **6i** and **12a** lead to fast depolarization of the membrane suggesting that the membrane is an apparent molecular target of compounds. **6i** and **12a** were non mutagenic neither in SOS-chromotest nor in Ames test and non-toxic *in vivo* at acute oral (LD_{50} > 2000 mg/kg) and cutaneous administration (LD_{50} >2500 mg/kg) on mice. Taken together, our data allow suggesting the described active compounds as promising starting point for the new antibacterial agents development.

1. Introduction

The bacterial resistance to antimicrobials is one of serious challenges of healthcare worldwide. The multicenter monitoring notices the continuous spread of bacterial strains resistant to many external factors, and pathogenic bacteria quickly become resistant to existing commercially available antibiotics and antiseptics [1, 2]. A similar increase is observed for both nosocomial hospital infections arising due to the direct spread of bacteria from patient to patient and for community-acquired infections [3, 4].

Since the 1930s, quaternary ammonium compounds (QACs) are widely used as antiseptics and disinfectants [5]. A number of QACs such as the benzalkonium chloride [5], dequalinium chloride [6], cetylpyridinium chloride [7] have been widely used for a variety of

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clinical purposes (e.g., preoperative disinfection of intact skin, application to mucous membranes, disinfection of noncritical surfaces etc.). According to literature [7], the general mechanism of the antibacterial activity of QACs is the damage of the cytoplasmic and outer membrane lipid bilayers via association of the positively charged quaternary nitrogen with the anionic head groups of acidic phospholipids and interaction of the lipophilic tail with the hydrophobic membrane core. Because of universal basic architecture of cellular membranes, these properties of QACs have disadvantage such as high toxicity [8]. Moreover, many classical representatives of these compounds has a long half-life in environment, increasing the frequency of bacterial resistance developing to QACs [9, 10]. One of possible ways to solve this problem could be the strategy of soft drugs, i.e. substances that are readily degraded into nontoxic and biologically inactive products both *in vivo* and in the environment. The inclusion of a metabolically sensitive fragment into the structure of designed products allows predicting the main metabolic pathway for degradation and avoiding the toxic products formation. The most suitable for that are ester and amide bonds, which do not affect significantly the hydrophilic-lipophilic balance of molecules while keeping their antibacterial activity [11]. The concept of soft antibacterial agents was proposed by Bodor in 1980 [8] who synthesized isosteric analogues of cetylpyridinium chloride with comparable antimicrobial activity, significantly less toxic and undergo facile hydrolytic cleavage, leading to their deactivation (Fig. 1). Since then, a series of soft QAC antimicrobials have been synthesized [12–15]. Among them, the QAC Miramistin occupies leading position in Russian antiseptics market structurally related to soft drugs.

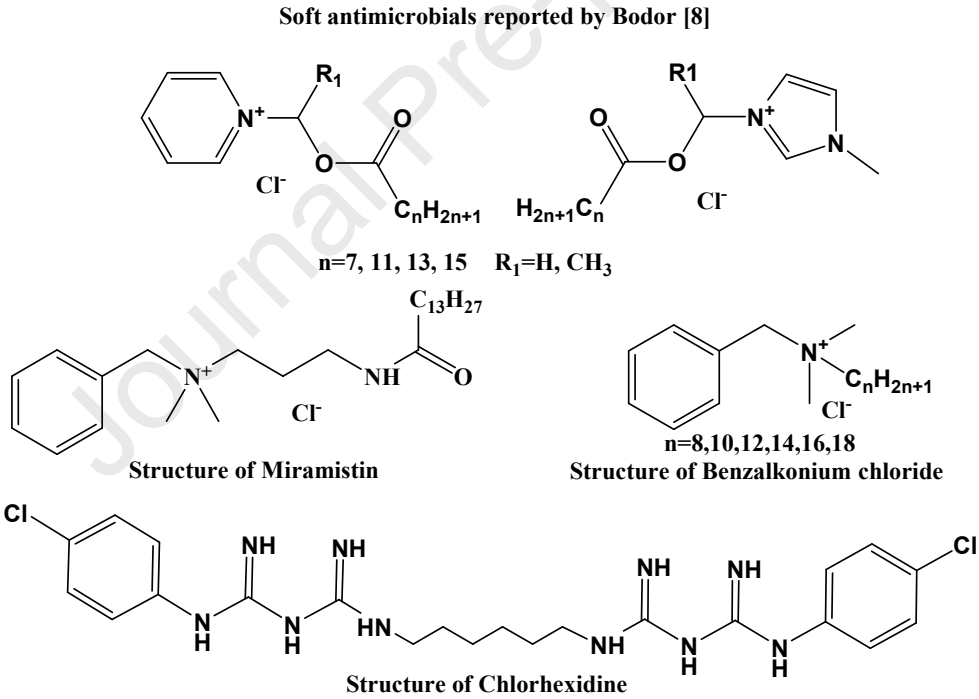


Fig.1. Structures of quaternary ammonium compounds.

In our group, we have systematically studied chemistry and biological activity of pyridoxine (vitamin B_6) derivatives including a series of QAC based on pyridoxine derivatives [16–20] (Fig.2). Some of them possess high antibacterial activity against various clinical Gram-positive and Gram-negative pathogenic bacteria (minimal inhibitory concentration (MIC) 0.5-16 μg/ml) and low toxicity *in vitro*.

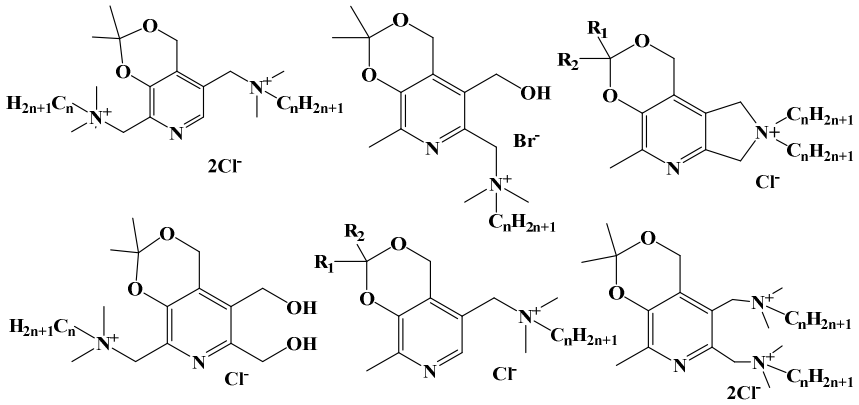


Fig.2. Structures of the QAC based on pyridoxine derivatives obtained previously [17–20].

In this work, we present a series of novel "soft antimicrobials" based on quaternary ammonium pyridoxine derivatives which include cleavable moieties (amide, ester) and a six-membered acetals and ketals of pyridoxine bound via linker moiety with a fragment of fatty carboxylic acid (Fig. 3). The relationship between the structure of synthesized compounds and their antibacterial activity *in vitro* were investigated by variations of the substitutents at the acetal carbon atom, the length of the linker, the nature of the bond between linker and carboxylic acid fragment, the length of the carboxylic acid and the position of the quaternary ammonium fragment in the pyridine ring of pyridoxine. Identified lead compounds inhibited growth of Gram-positive and Gram-negative pathogens, including biofilm-embedded bacteria, while exhibiting low toxicity and resistance development risk.

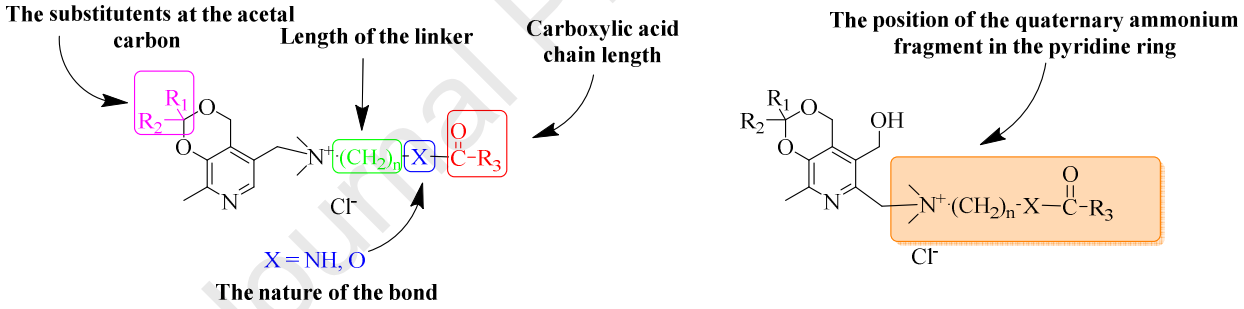
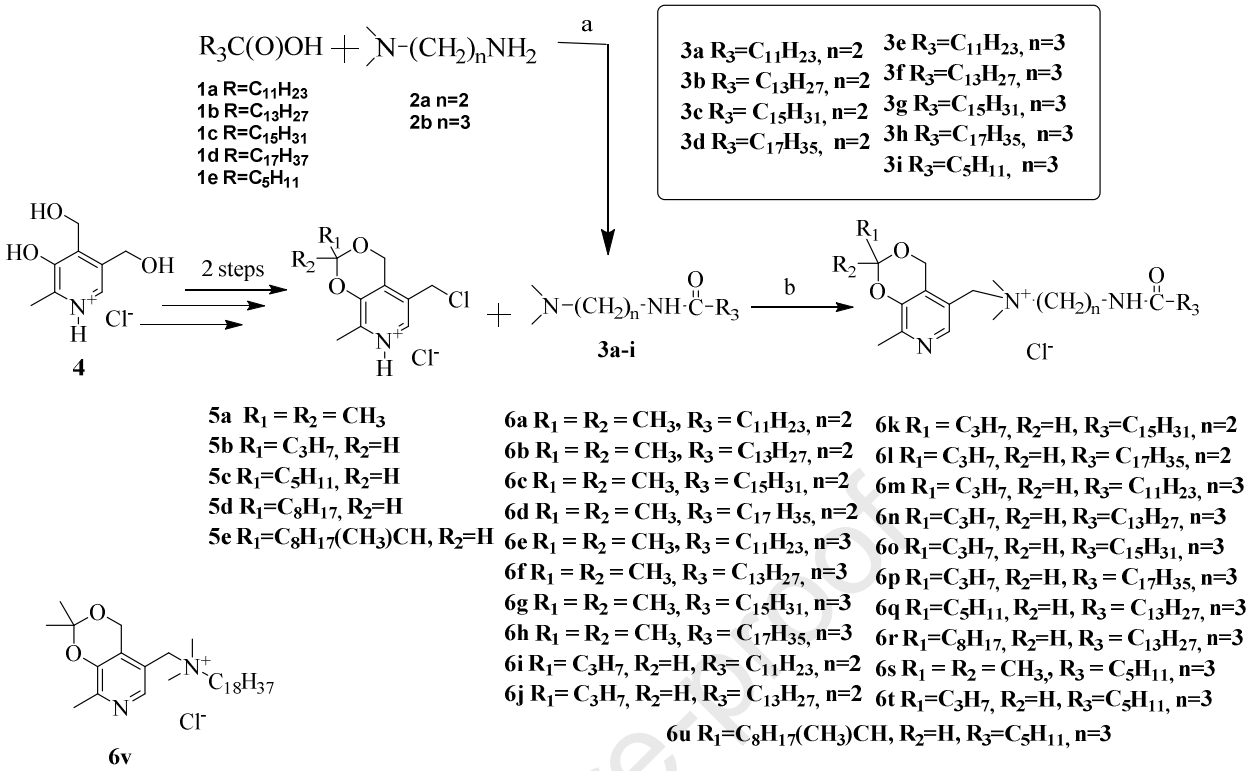


Fig.3. Design of the soft antimicrobials based on quaternary ammonium pyridoxine derivatives.

2. Results and discussion

2.1. Chemistry and primary screening of antibacterial activity in vitro

At first, a series of amides **3a–i** with different lipophilicity were obtained by reaction of different fatty carboxylic acids (caproic acid, lauric acid, myristic acid, palmitic acid and stearic acid) with N,N-dimethylethylenediamine or N,N-dimethylpropylene-1,3-diamine in toluene in the presence of *p*-toluenesulfonic acid [21]. Then, in the reaction of the corresponding amides **3a-i** with chlorine derivatives of pyridoxine **5a-d** containing various substitutents at the acetal carbon atom [19, 22] in ethanol at 70 °C the ammonium salts **6a-u** were obtained (Scheme 1).



Sheme 1. Synthesis of QACs **6a-u**. (a) toluene, reflux (Dean-Stark trap), 48h, 40-74%; (b) $NaHCO_3$, C_2H_5OH, 70 ^oC, 8h, 40-76%.

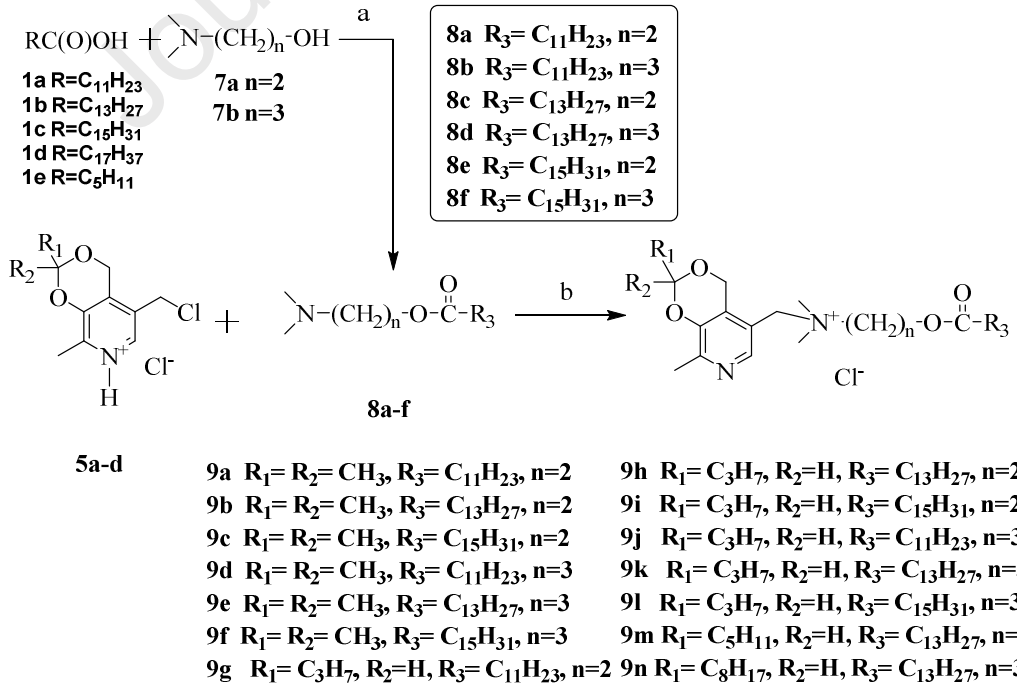
The antibacterial activity of compounds **6a-u** was evaluated on various strains of Gram-positive and Gram-negative bacteria. Table 1 shows the MIC of compounds **6a-u** in comparison with benzalkonium chloride, miramistin, chlorhexidine and their structural analog **6v** without amide moiety described in our recent paper [19]. The lipophilicity of the synthesized ammonium salts was expressed in terms of their partition coefficient values (logP) calculated using online platform Chemicalize (ChemAxon) [23]. For derivatives containing fragments of stearic and caproic acids a sharp decrease in antibacterial activity was observed. For compounds containing identical fragments of carboxylic acid and linker, the antibacterial activity were decreased with the increasing of alkyl chain length at acetal carbon atom ($2CH_3$~C_3H_7 > C_5H_{11} > C_8H_{17}). The number of methylene fragments in the linker between the quaternary and amide nitrogen atoms did not affect the antibacterial activity. The most active compounds **6b, 6f, 6i** and **6j** contain the myristic and lauric acids residues in the amide fragment. Their activity against Gram-positive and Gram-negative bacteria, in general, was comparable with the reference drugs and more than previously reported ammonium salt **6v** without amide moiety**.**

Table 1
Antibacterial activity of quaternary ammonium salts **6a-u**

Compound	MICs (μg/ml)					
	Gram-positive bacteria			Gram-negative bacteria		
	S. aureus ATCC 29213	*B. subtilis* 168	*M. luteus*	*E. coli* MG1655	*P. aeruginosa* ATCC 27853	LogP
6a	1	8	4	32	32	0.52
6b	2	4	4	8	2	1.41
6c	1	4	1	32	2	2.93
6d	4	8	2	>64	8	3.19

6e	8	8	16	64	64	0.58
6f	0.5	2	1	8	8	1.47
6g	4	2	2	>64	8	2.36
6h	4	8	4	>64	8	3.25
6i	1	2	2	4	32	1.35
6j	1	2	1	32	8	2.24
6k	8	16	16	>64	>64	3.13
6l	>64	>64	>64	>64	>64	4.02
6m	1	4	4	8	32	1.41
6n	2	2	2	2	>64	2.30
6o	2	4	2	2	>64	3.19
6p	>64	>64	>64	>64	>64	4.08
6q	2	1	2	>64	>64	3.19
6r	>64	>64	>64	>64	>64	4.53
6s	>64	>64	>64	>64	>64	-2.09
6t	>64	>64	>64	>64	>64	-1.25
6u	>64	>64	>64	>64	>64	2.34
6v	4	>64	16	>64	>64	4.35
Benzalkonium chloride	1	4	0.5	8	1	-
Miramistin	2	4	2	8	1	2.43
Chlorhexidine	2	1	0.5	1	4	4.51

In the second experimental series, we have obtained bioisostere analogues of compounds **6a-u** where the amide fragment was replaced by ester. The synthesis of derivatives based on stearic and caproic acids isn't performed, because these compouds contain an amide fragment (**6d**, **6h**, **6l**, **6p**) exhibiting low antibacterial activity. Esters **8a-f** were prepared by Steglich esterification with amine **7a-b** and fatty carbonic acids [24]. Then, in the reaction of the corresponding esters **8a-f** with chlorine derivatives of pyridoxine **5a-d** in ethanol at 70 °C the ammonium salts **9a-n** were performed (Scheme 2).



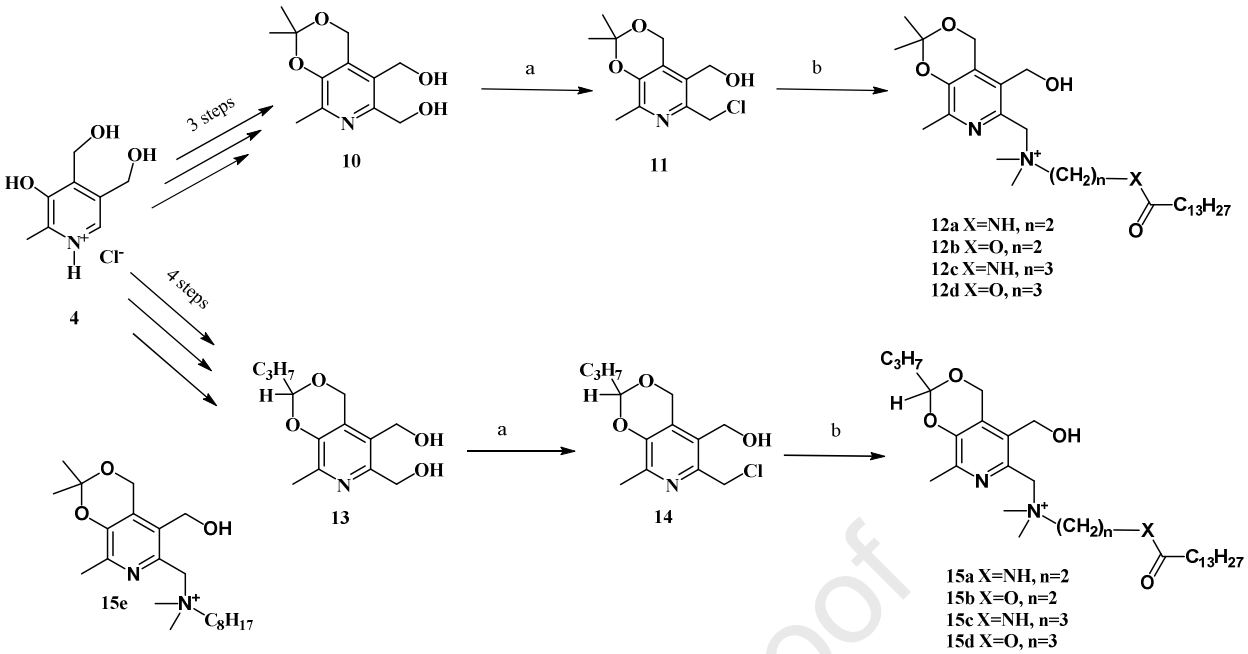
Sheme 2. Synthesis of QACs **9a-n**. (a) DCC, DMAP, CH_2Cl_2, rt, 4h, 47-61%; (b) $NaHCO_3$, C_2H_5OH, 70 °C, 8h, 39-73%.

The antibacterial activity of compounds **9a-n** is shown in Table 2. In general, the effect of obtained bioisostere esters was comparable with a series of amides **6a-u** and references drugs. The highest influence was observed for compounds **9b, 9h** and **9j** containing fragments of myristic and lauric acids.

Table 2
Antibacterial activity of quaternary ammonium salts **9a-n**

Compound	MICs (μg/ml)					
	Gram-positive bacteria			Gram-negative bacteria		
	S. aureus ATCC 29213	*B. subtilis* 168	*M. luteus*	*E. coli* MG1655	*P. aeruginosa* ATCC 27853	LogP
9a	4	4	4	16	64	1.25
9b	0.5	1	0.5	8	1	2.14
9c	0.5	1	0.5	>64	4	3.03
9d	2	1	2	8	32	1.31
9e	2	2	2	16	16	2.20
9f	16	8	2	>64	32	3.09
9g	1	2	2	4	32	2.08
9h	0.5	4	0.5	16	1	2.97
9i	2	4	1	>64	8	3.86
9j	2	2	1	4	32	2.14
9k	2	2	1	>64	4	3.03
9l	32	32	8	>64	32	3.92
9m	8	4	8	>64	>64	3.92
9n	>64	>64	>64	>64	>64	5.25
6v	4	>64	16	>64	>64	4.35
Benzalkonium chloride	1	4	0.5	8	1	-
Miramistin	2	4	2	8	1	2.43
Chlorhexidine	2	1	0.5	1	4	4.51

Synthesis of compounds **12a-d** and **15a-d** in which quaternary ammonium fragment is located in the sixth position of the pyridine ring was carried according to the Scheme 3. Qaternary ammonium salts **12a-d** and **15a-d** were obtained from mono-chlorine derivates **11** and **14** in the reaction with myristic acid amides **3b,f** or esters **8b,e**. Chlorides **11** and **14** were obtained from acetal **10** [25] and ketal **13** [26] by selective chlorination by equimolar quantities of N-chlorocuccinimide in dichloromethane. The regioselective chlorination proceeded similarly to the previously described regioselective bromination of diol **10** under the action of N-bromosuccinimide [26] and also confirmed by ^{13}C chemical shift of CH_2Cl group in the ortho-position of pyridine ring (~45 ppm). The minor products (~15-20 %) of the reactions were dichlorides. The traces of the second regio-isomers were not observed.



Sheme 3. Synthesis of QACs **12a-d**, **15a-d** (a) NCS (1 eqviv.), Ph_3P, CH_2Cl_2, rt, 0.5h, 44-54%; (b) C_2H_5OH, 70 °C, 8h, 44-83%.

Table 3
Antibacterial activity of quaternary ammonium salts **12a-d, 15a-d**

Compound	MICs (μg/ml)					
	Gram-positive bacteria			Gram-negative bacteria		
	S. aureus ATCC 29213	*B. subtilis* 168	*M. luteus*	*E. coli* MG1655	*P. aeruginosa* ATCC 27853	LogP
12a	1	1	2	8	>64	0.73
12b	1	0.5	1	32	64	1.46
12c	2	2	1	16	>64	0.79
12d	1	0.5	2	64	>64	1.52
15a	2	2	4	64	>64	1.56
15b	2	2	2	64	>64	2.29
15c	2	1	1	64	>64	1.62
15d	2	2	2	64	>64	2.35
15e	64	64	64	64	>64	-0.78
Benzalkonium chloride	1	4	0.5	8	1	-
Miramistin	2	4	2	8	1	2.43
Chlorhexidine	2	1	0.5	1	4	4.51

The antibacterial activity of esters and amides of pyridoxine derivatives **12a-d** and **15a-d** on Gram-positive bacteria was comparable with derivatives containing ammonium fragments in the fifth position of pyridoxine and reference drugs. However, their effect on Gram-negative bacteria *P. aeruginosa* and *E. coli* was significantly lower. It should be noted that the previously obtained compound **15e** [18] without ether or amide fragments was practically inactive.

Thus compounds **6b, 6f, 6i, 6g, 9b, 9h, 9j, 12a** and **12b** with promising activities in the primary assay were selected for further in-depth investigation *in vitro*.

2.2. Structure-Activity Relationship (SAR)

To identify quantitative relationships "structure–antibacterial activity" in a series of synthesized QACs based on pyridoxine and fatty acids derivatives, the dependences of antibacterial activity on calculated in ChemAxon lipophilicity [23] were evaluated (Figure 4, for *S. aureus*). MIC values are shown in μg/ml for practical reasons. A strong correlation between the lipophilicity and antimicrobial activity of quaternary ammonium pyridoxine derivatives on *S. aureus* was observed. Thus, all the most active compounds had logP values in the range of 1–3, while compounds with logP > 5 and logP < 0 were almost inactive. Similar relationships were found for the other studied bacteria. Apparently, relationships reflect the important features of the active compounds essential for their effective interaction with the hydrophobic membrane core of bacterial cells.

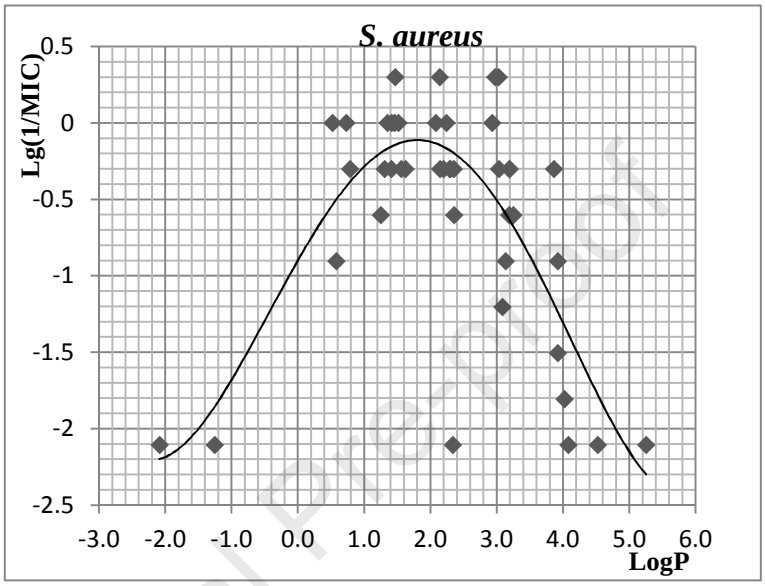


Fig 4. Lg(1/MIC) versus calculated logP relationship for QAS based on pyridoxine and fatty acids. The parabolic line shows the polynomial approximation by using a fifth order model.

2.3. Cytotoxicity

Cytotoxicity of compounds **6b, 6f, 6i, 6j, 9b, 9h, 9j, 12a** and **12b** was evaluated on human embryonic kidney cells (HEK-293), human mesenchymal stem cells (MSK) and primary human skin fibroblasts (HSF) in comparison with benzalkonium chloride, miramistin and chlorhexidine (Table 4). Compounds **6b**, **6f** and **9j** demonstrated the lowest cytotoxicity on all cell lines among the studied molecules. However, their toxicity was greater than that of chlorhexidine and miramistin. The most toxic were compounds **6i** and **9b** whose toxicity exceeded benzalkonium chloride on HEK-293 cells. All other compounds were less toxic than benzalkonium chloride.

Table 4
Cytotoxicity (CC_{50}, μg/mL mean ± SD) of novel pyridoxine-based QACs

Compound	CC_{50}, μg/mL		
	HEK-293	MSK	HSF
6b	1.18 ± 0.33	2.16 ± 0.80	3.03 ± 0.91
6f	1.61 ± 0.40	2.21 ± 0.71	3.14 ± 0.80
6i	0.15 ± 0.01	1.15 ± 0.52	1.79 ± 0.80
6j	1.16 ± 0.92	1.38 ± 0.74	2.15 ± 0.42
9b	0.17 ± 0.04	1.08 ± 0.54	1.94 ± 0.42
9h	1.00 ± 0.13	1.28 ± 0.52	2.18 ± 0.50

9j	2.15 ± 0.67	2.42 ± 0.74	4.32 ± 0.74
12a	1.32 ± 0.75	1.74 ± 0.44	3.21 ± 0.70
12b	0.88 ± 0.14	1.02 ± 0.17	2.24 ± 0.52
Miramistin	6.08 ± 2.14	4.16 ± 0.86	7.21 ± 1.92
Benzalkonium chloride	0.59 ± 0.21	0.82 ± 0.17	1.14 ± 0.40
Chlorhexidine	7.28 ± 1.50	5.12 ± 1.20	6.32 ± 1.24

2.4.Genotoxicity

Genotoxicity of compounds **6b, 6f, 6i, 6j, 9b, 9h, 9j, 12a, 12b** and benzalkonium chloride was evaluated in SOS-chromotest and Ames test. In SOS-chromotest, the β-galactosidase activity was normalized to the amount of cells estimated from the OD_{600} values and SOS induction factor was calculated as a ratio of β-galactosidase activity in the presence of compounds and the solvent control. Mitomycin C (1 μg/ml) was used as positive control. No significant dose-dependent increase more than 2-fold was observed in SOS-chromotest (Table 5) thus indicating the lack of DNA-damage action for all compounds under concentrations tested.

Table 5
DNA-damage activity of novel pyridoxine-based QACs (ratio, fold increase over the solvent control), mean ± SD

Compound	QACs concentration, μg/mL				
	750	150	75	15	7.5
6b	0.4±0.03	0.5±0.06	0.7±0.08	1.5±0.10	1.5±0.06
6f	1.0±0.17	0.5±0.04	0.7±0.18	1.5±0.20	1.5±0.16
6i	0.3±0.02	0.5±0.02	0.7±0.13	1.6±0.10	1.6±0.04
6j	0.3±0.03	0.5±0.04	0.9±0.11	1.6±0.04	1.5±0.09
9b	0.4±0.03	0.6±0.10	1.2±0.11	1.6±0.14	1.6±0.27
9h	0.3±0.02	1.3±0.51	1.5±0.06	1.5±0.17	1.4±0.10
9j	0.5±0.04	1.1±0.15	1.5±0.13	1.4±0.14	1.4±0.19
12a	0.4±0.04	0.7±0.10	0.7±0.15	1.8±0.19	1.6±0.30
12b	0.3±0.02	0.9±0.07	1.2±0.33	1.6±0.13	1.8±0.14
Benzalkonium chloride	0.8±0.17	0.7±0.04	1.1±0.11	1.5±0.10	1.5±0.25
Mitomicin C	12.9±2.41 (1 μg/mL)				

Since compounds demonstrated antibacterial acitivity on *S. typhymuriym* strains (data not shown), the spot-test modification of the Ames test has been used instead of classic technique. For that, 50 μg of compound in water was dropped onto 5-mm whatmann disk placed onto agar surface (see Fig. S1). While almost all compounds led to increase of revertants amount on at least one strain in the Ames test. Only compounds **6b**, **6i** and **12a** could be considered as non-mutagenic in Ames test (Table 6).

Table 6
Mutagenicity of novel pyridoxine-based QACs (ratio, fold increase over the solvent control in Ames spot-test)

Compound	*S. typhimurium* strain				
	TA1535	TA1537	TA98	TA100	TA102
6b	0.8	0.06	1.7	1.3	0.1
6f	0.4	0.03	2.6	2.5	0.2
6i	0.5	0.03	1.9	1.1	0.1
6j	0.5	0.03	4.4	0.7	0.7
9b	0.9	0.02	4.6	1.4	4.8
9h	0.4	0.04	4.7	1.2	0.2

9j	0.5	0.03	1.8	2.4	0.1
12a	1.1	0.01	1.9	1.7	0.4
12b	0.4	0.05	2.5	2.1	0.4
Positive control	Methyl methanesulfonate	4-Nitro-o-phenylenediamine	4-Nitro-o-phenylenediamine	Methyl methanesulfonate	Mitomycin C
	30	3.7	4.2	2.7	1.9

2.5. In vitro antibacterial activity on clinical strains

The antibacterial activity of **6b, 6f, 6i, 6j, 9b, 9h, 9j, 12a** and **12b** was further studied on various Gram-positive and Gram-negative clinical isolates (see Table S2 for their antibiotic resistance profile), miramistin and benzalkonium chloride were used as reference drugs (Table **7**).

The most active compounds **6i** and **12a** exhibited antibacterial activity comparable with benzalkonium chloride on Gram-positive bacteria, while being less active on Gram-negative ones. At the same time, **6i** and **12a** were more active in comparison with miramistin on both Gram-positive and Gram-negative bacteria.

Table 7
In vitro antimicrobial activity of **6b, 6f, 6i, 6j, 9b, 9h, 9j, 12a, 12b** on an extended panel of clinical bacterial pathogens

Strain	MICs (μg/ml)										
	6b	**6f**	**6i**	**6j**	**9b**	**9h**	**9j**	**12a**	**12b**	**Benzalkonium chloride**	**Miramistin**
Gram-positive bacteria											
S. aureus 713 MRSA	4	16	4	2	8	2	16	4	2	8	64
S. aureus 983 MRSA	64	64	16	16	64	64	32	32	32	16	>64
S. aureus MRSA 1053	4	8	2	1	8	2	8	4	2	4	32
S. intermedius MRSI 1061	4	8	4	2	8	2	16	4	4	4	64
S. aureus MRSA 1065	8	8	2	2	8	2	16	2	4	4	32
S. aureus MRSA 1131	16	16	4	2	16	4	16	4	4	4	32
S. intermedius MRSI 1143	4	16	4	2	8	4	16	4	4	4	64
S. aureus 25	4	8	2	2	8	2	8	2	2	2	16
E. faecalis 23	2	4	2	1	4	4	4	1	2	1	4
E. faecalis 3063	2	4	2	1	4	4	4	1	2	2	16
Gram-negative bacteria											
Acinetobacter spp. 3	64	64	32	32	64	64	64	32	64	16	>64
Pseudomonas spp. 5	64	64	32	32	64	64	32	32	64	16	>64
Klebsiella spp. 11	64	64	16	32	64	32	32	32	64	16	>64
Proteus spp. 17	>64	>64	>64	>64	>64	>64	>64	>64	>64	64	>64
E. coli 718	32	>64	8	8	32	32	8	16	32	8	64
Moraxella sp. 765	64	>64	16	16	64	64	64	64	64	16	>64
K. pneumoniae 1813	4	>64	2	1	4	1	8	2	2	1	16
P. aeruginosa 1945	64	>64	16	8	64	64	>64	32	64	32	64
S. marcescens 1966	64	>64	64	64	64	>64	>64	>64	>64	64	>64
S. ureilytica 1972	64	>64	64	>64	>64	>64	>64	>64	>64	>64	>64

Thus based on *in vitro* data of antibacterial activity and toxicity of 43 synthesized quaternary ammonium pyridoxine derivatives compounds **6i** and **12a** were selected as lead compounds for further investigations.

2.6. Anti-biofilm activity

The anti-biofilm activity of compounds **6i** and **12a** has been tested on mono- and dual species biofilms. Firstly, 48h-old biofilms of *S. aureus*, *M. luteus*, *E. coli* or *P. aeruginosa* were prepared in 24-well plates in basal medium (BM) broth, washed with sterile phosphate-buffered saline (PBS) and filled with 1 mL of fresh BM broth containing **6i, 12a** or benzalkonium chloride in concentrations of their respective 1-32 × minimum bactericidal concentrations (MBCs) (see Table S1). After 24 hours of incubation, the viability of biofilm-embedded cells was evaluated by colony-forming units (CFUs) counting. No statistically significant difference in the activity of **6i, 12a** and benzalkonium chloride has been observed. All studied antimicrobials provided a 3-log drop of CFUs of all biofilm-embedded bacteria at nearly the same relative concentration (expressed as fold excess of respective MBC), which varied by only one dilution step (Fig. 5). Thus, both **6i** and **12a** demonstrated anti-biofilm activity comparable with that of the benzalkonium chloride.

Next, the action of **6i** and **12a** against bacterial dual-species biofilms was studied in a similar experimental design. A 48-h old biofilms formed by *S. aureus* and *E. coli* or *S. aureus* and *P. aeruginosa* were incubated with different concentrations of antimicrobials for 24 h and then quantified by differential CFUs counting. In these experiments, effects of both **6i** and **12a** against both *S. aureus* and *E. coli* in dual-species culture remained more or less similar to benzalkonium chloride (Fig. 6). By contrast, the influence of both compounds worsened against *S. aureus* - *P. aeruginosa* dual-species biofilms, apparently because of changed metabolism of these bacteria in their consortium.

These data allow suggesting **6i** and **12a** as a promising topical antimicrobials with action comparable with benzalkonium chloride.

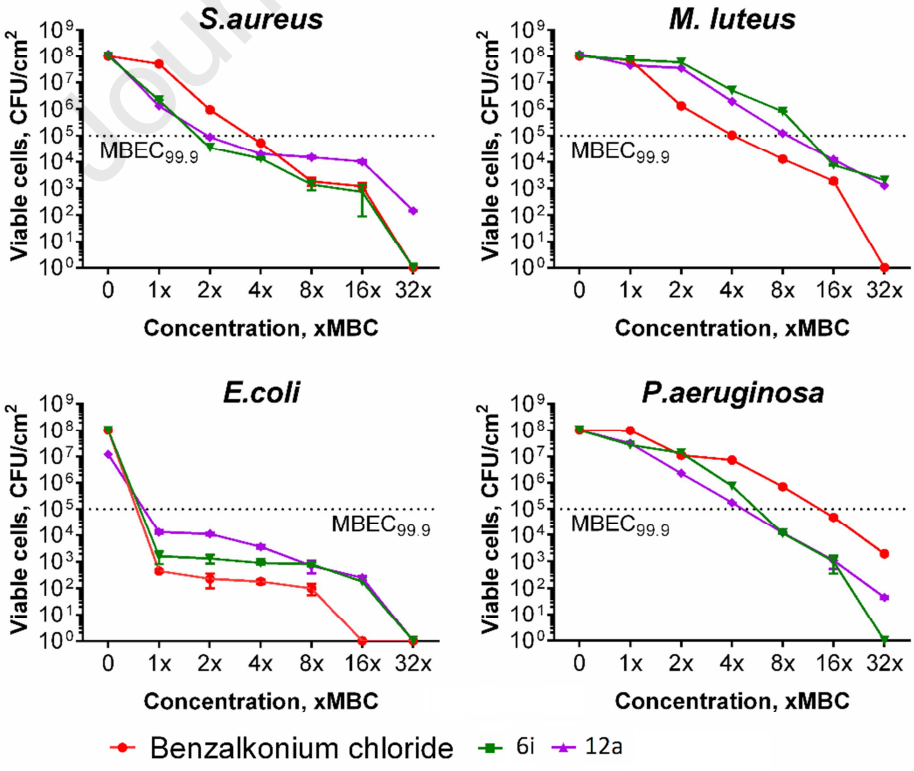


Fig. 5. Antimicrobial effect of novel pyridoxine-based QACs **6i** and **12a** against biofilm-embedded bacteria. Benzalkonium chloride served as reference. Antimicrobials were added to 48 hours-old biofilms. After 24 h

incubation, the biofilms were washed twice with sterile 0.9% NaCl. The adherent cells were scratched, resuspended and CFUs were counted. The median values with (IQRs) from six independent measurements are shown.

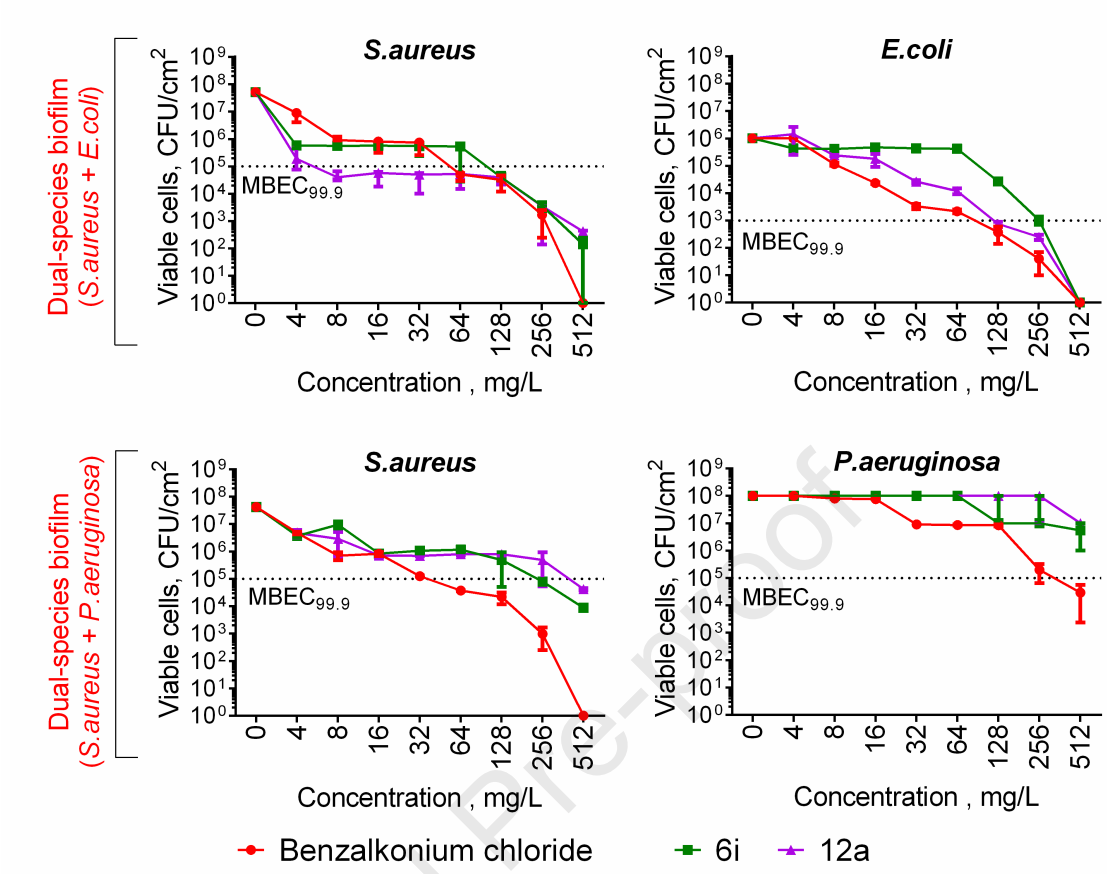


Fig. 6. Antimicrobial effect of novel pyridoxine-based QACs **6i** and **12a** against dual-species biofilms. Benzalkonium chloride served as reference. Antimicrobials were added to 48 hours-old biofilms. After 24 h incubation, the biofilms were washed twice with sterile 0.9% NaCl. The adherent cells were scratched, resuspended and CFUs were differentially counted on Endo-agar, Cetrimide agar and Salt-mannitol agar. The median values with IQRs from six independent measurements are shown.

2.7. In vitro resistance development

Spontaneous development of resistance to **6i** and **12a** in comparison with miramistine and benzalkonium chloride was studied on *S. aureus* and *E. coli* strains. MICs of all antimicrobials increased significantly (up to 32-fold) on the 8th passage on *S. aureus*, and 4-fold on *E. coli* (Fig. 7). After removal of antimicrobials, the MIC values decreased significantly suggesting that observed resistance is rather phenotypic than genetically determined.

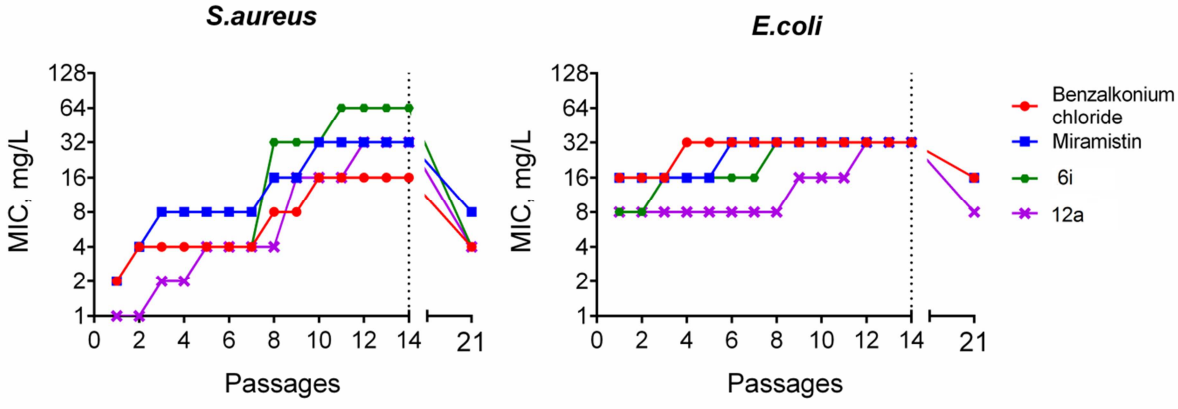


Fig. 7. The development of *S.aureus* (A) and *E.coli* (B) resistance to the novel pyridoxine-based QACs **6i** and **12a**. A series of 2-fold serial dilutions of the test compounds were inoculated with 10^7 CFUs of *S. aureus* or *E. coli*. After 24 h incubation, cultures from the wells with the highest antibiotic concentration permitting growth to the equivalent of a 0.5 McFarland standard were used as inoculum for the next round of MIC determination. This procedure was repeated for 14 passages, and MICs were recorded at the end of every passage. Next 7 passages were performed in absence of antimicrobials and MIC on 21st passage was determined. Benzalkonium chloride and miramistin were served as references.

2.8. In vivo toxicity

For lead compounds **6i** and **12a** *in vivo* acute oral and dermal toxicity on mice were perfomed as described in section 4.5.

In acute oral toxicity study after administration of **6i** and **12a** in dose 2000 mg/kg the death of animals occurred within the first 7 days (Table 8). Necropsy of compound **6i** treated group revealed one case of enlarged spleen, pale liver and kidneys. Autopsy of mice received **12a** revealed a pale liver, pale gastric mucosa in 3 cases (one of them had black contents in the stomach, presumably blood). Despite this, compounds **6i** and **12a** were considerably less toxic (LD_{50} > 2000 mg/kg) than benzalkonium chloride (LD_{50} 180 mg/kg [11]), miramistine (LD_{50} 1000 mg/kg [27]), and chlorhexidine (LD_{50} 1260 mg/kg) [28]).

During 14 days after the single cutaneous administration of compounds **6i** and **12a** at dose 2500 mg/kg no toxicity-related clinical symptoms or mortalities were revealed (Table 8). For compound **12a** a weak skin irritation was observed, which completely disappeared on day 5. At necropsy, no remarkable findings were noted in the control and tested compound treatment groups. Thus, we can conclude that compounds **6i** and **12a** are non-toxic at cutaneous administration.

Based on results of acute oral and dermal *in vivo* studies compounds **6i** and **12a** belong to category 5 of globally harmonized system of classification and labeling of chemicals [29]. It should be noted that the *in vivo* toxicity data did not correlate with the *in vitro* data, according to which compounds **6i** and **12a** were more toxic than chlorhexidine and miramistin.

Table 8
Acute oral and dermal toxicity data of compounds **6**i and **12a** on mice

Compound	Acute oral toxicity		Acute dermal toxicity	
	Dead/total animals	LD_{50}, mg/kg	Dead/total animals	LD_{50}, mg/kg
6i	2/12	> 2000	0/12	> 2500
12a	5/12		0/12	

*2.9. Unraveling the mechanism of antibacterial activity of **6i** and **12a***

The cell membrane damage causing changes in the membrane potential is a well-known mechanism of antimicrobial activity exhibited by various quaternary ammonium salts [7]. To test whether **6i** and **12a** can damage the cell membrane thereby leading to the cells death, the membrane potential of bacterial cells was measured by detection of $DioC_2(3)$ fluorescence. *S. aureus* and *E. coli* were grown for 18 h, harvested and washed with PBS. Cells were re-

suspended until reaching the final density of 10^5 CFU/mL in PBS supplemented with $DioC_2(3)$ (10 µM). After 30 min preincubation at 25 °C, antimicrobials have been added at their respective 1×MIC and 1-4×MBCs (see Tables 1,3, S1) and the fluorescence was measured for 35 min with 5-minute intervals. As expected, a significant dose-dependent decrease of fluorescence has been observed in all cells treated with either **6i** or **12a** at their respective 1×MIC and higher, similarly to benzalkonium chloride and miramistine regardless of the cell wall traits, suggesting that the membrane potential decreased due to the membrane damage (Fig. 8). As could be seen from the figure, the absolute drop in RFUs was 1.5-fold higher for Gram positive than on Gram negative strains and was characterized by the time-dependent drop of membrane potential apparently assuming their continuous damage. By contrast, for the Gram negative strains fast drop with no further changes in relative membrane potential was detected allowing speculating that probably the outer membrane becomes broken while the inner membrane remains intact.

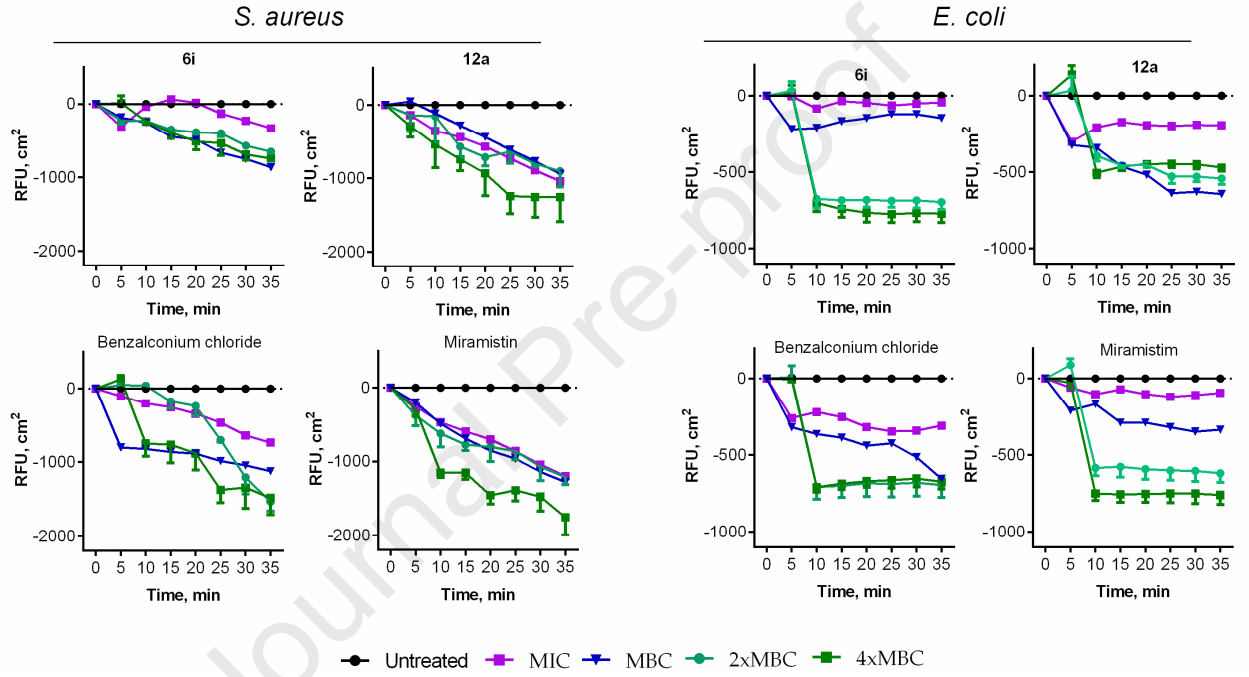


Fig. 8. Relative membrane potential of *S.aureus* (A) and *E.coli* (B) treated with QACs **6i** and **12a** (1×MIC, 1×, 2× or 4× of corresponding MBC, for values see Tables 1, 3 and S1).Benzalkonium chloride and miramistin served as references drugs. Bacteria in the late exponential growth phase were harvested, washed by PBS and resuspended in it, then supplemented by 10 µM of $DioC_2(3)$. After 30 min preincubation, compounds were added as indicated and the fluorescence was measured for 35 min with 5-minute interval. Lines represent the median values with IQRs from 5 independent measurements.

3. Conclusion

In conclusion, a diverse library of 43 novel "soft antimicrobials" based on quaternary ammonium pyridoxine derivatives was designed and their antibacterial activity against six Gram-positive and Gram-negative bacterial strains was evaluated *in vitro*. The structure of obtained compounds include six-membered acetals and ketals of pyridoxine bound via cleavable linker moieties (amide, ester) with a fragment of fatty carboxylic acid. Nine compounds exhibit promising antibacterial activity with MIC values comparable with that of miramistin, benzalkonium chloride and chlorohexidine. SOS-chromotest in *S. typhimurium* showed the lack of DNA-damage activity for all active compounds and they were non-mutagenic in the Ames test. Cytotoxicity studies on HEK-293, MSK and HSF cells demonstrated that some of the active

compounds were less toxic than the benzalkonium chloride, but more toxic than chlorhexidine and miramistin. The investigation of antibacterial activity on various Gram-positive and Gram-negative clinical isolates showed that the most active compounds **6i** and **12a** exhibited antibacterial activity comparable with benzalkonium chloride and higher than miramistin. The further in-depth investigations showed that compounds **6i** and **12a** have comparable with benzalkonium chloride activity against mono- and dual species biofilms. Compounds **6i** and **12a** are less toxic *in vivo* (LD_{50} > 2000 mg/kg) than benzalkonium chloride, miramistine and chlorhexidine at oral administration and non-toxic at dermal administration (LD_{50} > 2500 mg/kg) on mice. The mechanism of antibacterial activity of **6i** and **12a** includes membrane integrity damage. The obtained results make the described active compounds a promising starting point for new antibacterial agents development.

4. Experimental section

4.1. Chemistry

^{1}H and ^{13}C NMR spectra were recorded on a "Bruker AVANCE 400" at operating frequency 400 and 101.56 MHz, respectively. Chemical shifts were measured with reference to the residual protons of the solvent (DMSO-d_6, ^{1}H, 2.50 ppm, ^{13}C, 39.52 ppm; $CDCl_3$, ^{1}H, 7.26 ppm, ^{13}C, 77.16 ppm). Coupling constants (*J*) are given in Hertz (Hz). The following abbreviations are used to describe coupling: s = singlet; d = doublet; t = triplet; m = multiplet; br s = broad singlet, AB = AB system. Melting points were determined using a Stanford Research Systems MPA-100 OptiMelt melting point apparatus and are uncorrected. For thin layer chromatography analysis, silica gel plates from Sorbfil (Krasnodar, Russia) were used with UV light (254 nm/365 nm) or iron (III) chloride as developing agent. Column chromatography was performed on silica gel (60-200 mesh) from Acros or reversed-phase chromatography on PF-15C18HP column from Interchim.

High-resolution (HRMS) mass spectra were obtained on a quadrupole time-of-flight (qTOF) AB Sciex Triple TOF 5600 mass spectrometer using turbo-ion spray source (nebulizer gas nitrogen, a positive ionization polarity, needle voltage 5500 V). Recording of the spectra was performed in "TOF MS" mode with collision energy 10 eV, declustering potential 100 eV and with resolution more than 30 000 full-width half-maximum. Samples with the analyte concentration 5 μmol/l were prepared by dissolving the test compounds in a mixture of methanol (HPLC-UV Grade, LabScan) and water (LC-MS Grade, Panreac) in 1:1 ratio.

Analytical reversed-phase HPLC was used for determination of uncalibrated purity of the compounds and conducted using a Zorbax RX-SIL column (5μm, 250*4.6 mm); eluent A, 0.77 % solution of ammonium acetate in water; eluent B CH_3CN; isocratic elution A:B = 1:9; flow rate was 1.6 mL/min. HPLC analysis was performed at 40 ^{o}C during 17 min at 285 nm.

*4.1.1. General procedure for preparation of compounds **3a-h***

The amine **2a-b** (1.4 equiv) was added to carboxylic acid **1a-d** (1 equiv) in 50 ml of toluene. The reaction mixture was refluxed with a Dean-Stark trap for 48 h. The progress of reaction was monitored for water formed in the Dean-Stark trap. Then the solvent was evaporated under reduced pressure. The product was recrystallized from acetone.

*4.1.1.1. N-(2-(Dimethylamino)ethyl)laurylamide **(3a)***

The reaction was carried out following the general procedure with compound **1a** (5.00 g, 25.0 mmol) and compound **2a** (3.82 ml, 35.0 mmol). Yield 74% (5.00 g); white solid; mp 48 °C. ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^{3}J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{10}H_{22}$), 1.23-1.29 (m, 16H, $8CH_2$), 1.56-

1.64 (m, 2H, CH_2), 2.15 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.23 (s, 6H, $(CH_3)_2N$), 2.40 (t, 2H, $^3J_{HH}$ = 5.8 Hz, CH_2N), 3.29-3.33 (m, 2H, $\underline{CH_2}NH$), 6.07 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.22 (CH_3), 22.79 (CH_2), 25.91 (CH_2), 29.44 (CH_2), 29.48 (CH_2), 29.62 (CH_2), 29.72 (CH_2), 32.02 (CH_2), 36.71 (CH_2), 36.88 (CH_2NH), 45.21 ($(CH_3)_2N$), 58.03 (CH_2N), 173.42 (C=O). ESI-HRMS m/z: 271.2749 $[M+H]^+$ (calculated for $[C_{16}H_{35}N_2O]^+$ - 271.2744).

*4.1.1.2. N-(2-(Dimethylamino)ethyl)myristylamide **(3b)***

The reaction was carried out following the general procedure with compound **1b** (5.00 g, 21.9 mmol) and compound **2a** (3.35 ml, 30.7 mmol). Yield 69% (4.51 g); white solid; mp 52 °C. ^1H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.24-1.30 (m, 20H, $10CH_2$), 1.57-1.65 (m, 2H, CH_2), 2.17 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.27 (s, 6H, $(CH_3)_2N$), 2.46 (t, 2H, $^3J_{HH}$ = 5.9 Hz, CH_2N), 3.32-3.36 (m, 2H, $\underline{CH_2}NH$), 6.18 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.25 (CH_3), 22.82 (CH_2), 25.92 (CH_2), 29.46 (CH_2), 29.48 (CH_2), 29.50 (CH_2), 29.65 (CH_2), 29.75 (CH_2), 29.78 (CH_2), 29.80 (CH_2), 32.05 (CH_2), 36.56 (CH_2), 36.88 (CH_2), 45.12 ($(CH_3)_2N$), 58.08 (CH_2N), 173.51 (C=O). ESI-HRMS m/z: 299.3062 $[M+H]^+$ (calculated for $[C_{18}H_{39}N_2O]^+$ - 299.3057).

*4.1.1.3 N-(2-(Dimethylamino)ethyl)palmitamide **(3c)***

The reaction was carried out following the general procedure with compound **1c** (5.00 g, 19.5 mmol) and compound **2a** (2.98 ml, 27.3 mmol). Yield 64% (4.08 g); white solid; mp 64 °C. ^1H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{14}H_{28}$), 1.24-1.32 (m, 24H, $12CH_2$), 1.57-1.64 (m, 2H, CH_2), 2.17 (t, 2H, $^3J_{HH}$ = 7.9 Hz, $CH_2C(O)$), 2.31 (s, 6H, $(CH_3)_2N$), 2.52 (t, 2H, $^3J_{HH}$ = 5.7 Hz, CH_2N), 3.37-3.39 (m, 2H, $\underline{CH_2}NH$), 6.45 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.28 (CH_3), 22.83 (CH_2), 25.91 (CH_2), 29.49 (CH_2), 29.52 (CH_2), 29.66 (CH_2), 29.77 (CH_2), 29.80 (CH_2), 29.83 (CH_2), 32.06 (CH_2), 36.33 (CH_2), 36.84 (CH_2), 44.88 ($(CH_3)_2N$), 58.01 (CH_2N), 173.63 (C=O). ESI-HRMS m/z: 327.3375 $[M+H]^+$ (calculated for $[C_{20}H_{43}N_2O]^+$ - 327.3370).

*4.1.1.4. N-(2-(Dimethylamino)ethyl)stearamide **(3d)***

The reaction was carried out following the general procedure with compound **1d** (7.14 g, 25.1 mmol) and compound **2a** (3.83 ml, 35.1 mmol). Yield 73% (6.49 g); white solid; mp 72 °C. ^1H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{16}H_{32}$), 1.24-1.32 (m, 28H, $14CH_2$), 1.57-1.64 (m, 2H, CH_2), 2.17 (t, 2H, $^3J_{HH}$ = 7.9 Hz, $CH_2C(O)$), 2.32 (s, 6H, $(CH_3)_2N$), 2.52 (t, 2H, $^3J_{HH}$ = 5.8 Hz, CH_2N), 3.35-3.39 (m, 2H, $\underline{CH_2}NH$), 6.44 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.28 (CH_3), 22.84 (CH_2), 25.91 (CH_2), 29.49 (CH_2), 29.52 (CH_2), 29.67 (CH_2), 29.77 (CH_2), 29.80 (CH_2), 29.84 (CH_2), 32.07 (CH_2), 36.33 (CH_2), 36.85 (CH_2), 44.89 ($(CH_3)_2N$), 58.02 (CH_2N), 173.63 (C=O). ESI-HRMS m/z: 355.3683 $[M+H]^+$ (calculated for $[C_{22}H_{47}N_2O]^+$ - 355.3683).

*4.1.1.5. N-(3-(Dimethylamino)propyl)laurylamide **(3e)***

The reaction was carried out following the general procedure with compound **1a** (5.00 g, 25.0 mmol) and compound **2b** (4.37 ml, 35.0 mmol). Yield 70% (4.97 g); white solid; mp 34 °C. ^1H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^3J_{HH}$ = 6.0 Hz, $\underline{CH_3}C_{10}H_{22}$), 1.22-1.28 (m, 16H, $8CH_2$), 1.53-1.59 (m, 2H, CH_2), 1.62-1.68 (m, 2H, CH_2), 2.11 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.24 (s, 6H, $(CH_3)_2N$), 2.40 (t, 2H, $^3J_{HH}$ = 6.4 Hz, CH_2N), 3.26-3.31 (m, 2H, $\underline{CH_2}NH$), 6.97 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.17 (CH_3), 22.74 (CH_2), 25.86 (CH_2), 26.08 ($CH_2\underline{C}H_2CH_2NH$), 29.41 ($CH_2$), 29.47 ($CH_2$), 29.58 ($CH_2$), 29.64 ($CH_2$), 29.69 ($CH_2$), 31.97 ($CH_2$), 37.01 ($CH_2$), 38.99 ($CH_2NH$), 45.19 ($(CH_3)_2N$), 58.34 ($CH_2N$), 173.28 (C=O). ESI-HRMS m/z: 285.2906 $[M+H]^+$ (calculated for $[C_{17}H_{37}N_2O]^+$ - 285.2900).

*4.1.1.6. N-(3-(Dimethylamino)propyl)myristylamide **(3f)***

The reaction was carried out following the general procedure with compound **1a** (10.00 g, 43.8 mmol) and compound **2b** (7.66 ml, 61.3 mmol). Yield 65% (8.89 g); white solid; mp 51 °C.

^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^{3}J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.24-1.31 (m, 20H, $10CH_2$), 1.55-1.62 (m, 2H, CH_2), 1.64-1.70 (m, 2H, CH_2), 2.14 (t, 2H, $^{3}J_{HH}$ = 7.7 Hz, $CH_2C(O)$), 2.27 (s, 6H, $(CH_3)_2N$), 2.42 (t, 2H, $^{3}J_{HH}$ = 6.4 Hz, CH_2N), 3.33-3.35 (m, 2H, $\underline{CH_2}NH$), 6.95 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.26 (CH_3), 22.83 (CH_2), 25.90 (CH_2), 26.09 ($CH_2\underline{C}H_2CH_2NH$), 29.48 ($CH_2$), 29.53 ($CH_2$), 29.64 ($CH_2$), 29.78 ($CH_2$), 32.05 ($CH_2$), 37.11 ($CH_2$), 39.21 ($CH_2$), 45.37 ($(CH_3)_2N$), 58.63 ($CH_2N$), 173.27 (C=O). ESI-HRMS m/z: 313.3219 $[M+H]^+$ (calculated for $[C_{19}H_{41}N_2O]^+$ - 313.3213).

4.1.1.7. N-(3-(Dimethylamino)propyl)palmitamide ***(3g)***

The reaction was carried out following the general procedure with compound **1b** (4.00 g, 15.6 mmol) and compound **2b** (2.73 ml, 21.8 mmol). Yield 59% (5.31 g); white solid; mp 57 °C. ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^{3}J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{14}H_{28}$), 1.24-1.32 (m, 24H, $12CH_2$), 1.55-1.62 (m, 2H, CH_2), 1.66-1.73 (m, 2H, CH_2), 2.14 (t, 2H, $^{3}J_{HH}$ = 7.8 Hz, $CH_2C(O)$), 2.30 (s, 6H, $(CH_3)_2N$), 2.47 (t, 2H, $^{3}J_{HH}$ = 6.5 Hz, CH_2N), 3.30-3.35 (m, 2H, $\underline{CH_2}NH$), 6.98 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.27 (CH_3), 22.83 (CH_2), 25.91 (CH_2), 25.94 ($CH_2\underline{C}H_2CH_2NH$), 29.47 ($CH_2$), 29.50 ($CH_2$), 29.54 ($CH_2$), 29.66 ($CH_2$), 29.77 ($CH_2$), 29.80 ($CH_2$), 29.83 ($CH_2$), 32.06 ($CH_2$), 37.08 ($CH_2$), 38.87 ($CH_2$), 45.08 ($(CH_3)_2N$), 58.17 ($CH_2N$), 173.39 (C=O). ESI-HRMS m/z: 341.3532 $[M+H]^+$ (calculated for $[C_{21}H_{45}N_2O]^+$ - 341.3526).

4.1.1.8. N-(3-(Dimethylamino)propyl)stearamide ***(3h)***

The reaction was carried out following the general procedure with compound **1c** (10.00 g, 35.2 mmol) and compound **2b** (6.15 ml, 49.2 mmol). Yield 61% (7.91 g); white solid; mp 64 °C. ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^{3}J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{16}H_{32}$), 1.24-1.32 (m, 28H, $14CH_2$), 1.56-1.63 (m, 2H, CH_2), 1.69-1.75 (m, 2H, CH_2), 2.15 (t, 2H, $^{3}J_{HH}$ = 7.8 Hz, $CH_2C(O)$), 2.33 (s, 6H, $(CH_3)_2N$), 2.51 (t, 2H, $^{3}J_{HH}$ = 6.5 Hz, CH_2N), 3.30-3.35 (m, 2H, $\underline{CH_2}NH$), 6.95 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.26 (CH_3), 22.83 (CH_2), 25.86 (CH_2), 25.92 ($CH_2\underline{C}H_2CH_2NH$), 29.49 ($CH_2$), 29.54 ($CH_2$), 29.66 ($CH_2$), 29.70 ($CH_2$), 29.79 ($CH_2$), 29.84 ($CH_2$), 32.06 ($CH_2$), 37.05 ($CH_2$), 38.57 ($CH_2$), 44.84 ($(CH_3)_2N$), 57.79 ($CH_2N$), 173.48 (C=O). ESI-HRMS m/z: 369.3845 $[M+H]^+$ (calculated for $[C_{23}H_{49}N_2O]^+$ - 369.3839).

4.1.1.9. N-(3-(Dimethylamino)propyl)hexanamide ***(3i)***

The reaction was carried out following the general procedure with compound **1e** (4.50 g, 38.7 mmol) and compound **2b** (5.80 ml, 46.5 mmol). Then the solvent was evaporated under reduced pressure. Oily residue then was refluxed in petroleum ether during 1h and organic layer was decanted. Yield 40% (3.16 g); yellow oil. ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^{3}J_{HH}$ = 6.4 Hz, $\underline{CH_3}C_4H_8$), 1.24-1.31 (m, 4H, $2CH_2$), 1.53-1.60 (m, 2H, CH_2), 1.64-1.71 (m, 2H, $CH_2\underline{C}H_2CH_2NH$), 2.12 (t, 2H, $^{3}J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.28 (s, 6H, $(CH_3)_2N$), 2.45 (t, 2H, $^{3}J_{HH}$ = 6.5 Hz, CH_2N), 3.27-3.31 (m, 2H, $\underline{CH_2}NH$), 7.05 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.00 (CH_3), 22.47 (CH_2), 25.49 (CH_2), 25.91 ($CH_2\underline{C}H_2CH_2NH$), 31.51 ($CH_2$), 36.88 ($CH_2$), 38.75 ($CH_2$), 45.05 ($(CH_3)_2N$), 58.14 ($CH_2N$), 173.40 (C=O). ESI-HRMS m/z: 201.1967 $[M-Cl]^+$ (calculated for $[C_{11}H_{25}N_2O]^+$ - 201.1961).

4.1.2. General procedure for preparation of compound ***8a-f***

The amine **7a-b** (1 equiv), carboxylic acid **1a-c** (1 equiv) and DMAP (0.05 equiv) was dissolved in 30 ml of methylene chloride then DCC was added slowly during 15 min. The reaction mixture was stirred for 4 h at 25 °C. Then the formed precipitate was filtered. Filtrate was concentrated and purified by column chromatography (eluent methylene chloride). Then the eluate was evaporated under reduced pressure. The dry residue was emulsified in water and neutralized with hydrochloric acid (pH=7). Then the water was evaporated under reduced pressure. Then dry residue was refluxed in 30 ml of petroleum ether for 1 h and the precipitate was filtered. The precipitate was dissolved in water and $NaHCO_3$ was added (1 equiv). Then the

solvent was evaporated under reduced pressure. The dry residue was dissolved in methylene chloride and NaCl was filtered. Then the solvent was evaporated under reduced pressure.

*4.1.2.1. 2-(Dimethylamino)ethyl laurylate **(8a)***

The reaction was carried out following the general procedure with compound **1a** (5.00 g, 25.0 mmol), compound **7a** (2.51 ml, 25.0 mmol), DCC (5.15 g, 25.0 mmol), DMAP (0.15 g, 1.3 mmol). Yield 61% (4.15 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^{3}J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{10}H_{22}$), 1.21-1.28 (m, 16H, $8CH_2$), 1.54 -1.61 (m, 2H, CH_2), 2.24 (s, 6H, $(CH_3)_2N$), 2.28 (t, 2H, $^{3}J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.52 (t, 2H, $^{3}J_{HH}$ = 5.8 Hz, CH_2N), 4.13 (t, 2H, $^{3}J_{HH}$ = 5.8 Hz, CH_2O). ^{13}C NMR ($CDCl_3$) δ: 14.19 (CH_3), 22.75 (CH_2), 25.00 (CH_2), 29.20 (CH_2), 29.34 (CH_2), 29.41 (CH_2), 29.53 (CH_2), 29.67 (CH_2), 31.98 (CH_2), 34.32 (CH_2), 45.77 ($(CH_3)_2N$), 57.92 (CH_2N), 62.05 (CH_2O), 174.03 (C=O). ESI-HRMS m/z: 272.2590 $[M+H]^+$ (calculated for $[C_{16}H_{34}N_2O]^+$ - 272.2584).

4.1.2.2. 2-(Dimethylamino)ethyl myristate **(8b)**

The reaction was carried out following the general procedure with compound **1b** (5.00 g, 21.9 mmol), compound **7a** (2.20 ml, 21.9 mmol), DCC (4.52 g, 21.9 mmol), DMAP (0.13 g, 1.1 mmol). Yield 52% (3.42 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.81 (t, 3H, $^{3}J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.19-1.26 (m, 20H, $10CH_2$), 1.51-1.59 (m, 2H, CH_2), 2.22 (s, 6H, $(CH_3)_2N$), 2.26 (t, 2H, $^{3}J_{HH}$ = 7.3 Hz, CH_2CO), 2.50 (t, 2H, $^{3}J_{HH}$ = 5.7 Hz, CH_2N), 4.11 (t, 2H, $^{3}J_{HH}$ = 5.7 Hz, CH_2O). ^{13}C NMR ($CDCl_3$) δ: 14.15 (CH_3), 22.72 (CH_2), 24.96 (CH_2), 29.17 (CH_2), 29.31 (CH_2), 29.39 (CH_2), 29.50 (CH_2), 29.63 (CH_2), 29.68 (CH_2), 29.71 (CH_2), 31.96 (CH_2), 34.27 (CH_2), 45.68 ($(CH_3)_2N$), 57.85 (CH_2N), 61.95 (CH_2O), 173.90 (C=O). ESI-HRMS m/z: 300.2903 $[M+H]^+$ (calculated for $[C_{18}H_{38}NO_2]^+$ - 300.2897).

4.1.2.3. 2-(Dimethylamino)ethyl palmitate **(8c)**

The reaction was carried out following the general procedure with compound **1c** (5.00 g, 19.5 mmol), compound **7a** (1.96 ml, 19.5 mmol), DCC (4.02 g, 19.5 mmol), DMAP (0.12 g, 0.9 mmol). Yield 52% (3.32 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^{3}J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.24-1.30 (m, 24H, $12CH_2$), 1.57-1.64 (m, 2H, CH_2), 2.31 (s, 6H, $(CH_3)_2N$), 2.32 (t, 2H, $^{3}J_{HH}$ = 7.7 Hz, $CH_2C(O)$), 2.59 (t, 2H, $^{3}J_{HH}$ = 5.7 Hz, CH_2N), 4.18 (t, 2H, $^{3}J_{HH}$ = 5.7 Hz, CH_2O). ^{13}C NMR ($CDCl_3$) δ: 14.27 (CH_3), 22.83 (CH_2), 25.07 (CH_2), 29.27 (CH_2), 29.41 (CH_2), 29.50 (CH_2), 29.60 (CH_2), 29.74 (CH_2), 29.79 (CH_2), 29.83 (CH_2), 32.06 (CH_2), 34.40 (CH_2), 45.72 ($(CH_3)_2N$), 57.89 (CH_2N), 61.90 (CH_2O), 174.06 (C=O). ESI-HRMS m/z: 328.3216 $[M+H]^+$ (calculated for $[C_{20}H_{42}NO_2]^+$ - 328.3210).

4.1.2.4. 3-(Dimethylamino)propyl laurylate **(8d)**

The reaction was carried out following the general procedure with compound **1a** (5.00 g, 25.0 mmol), compound **7b** (2.95 ml, 25.0 mmol), DCC (5.15 g, 25.0 mmol), DMAP (0.15 g, 1.3 mmol). Yield 51% (3.64 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^{3}J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{10}H_{22}$), 1.23-1.31 (m, 16H, $8CH_2$), 1.55-1.62 (m, 2H, CH_2), 1.73-1.80 (m, 2H, $CH_2\underline{CH_2}CH_2O$), 2.20 (s, 6H, $(CH_3)_2N$), 2.26 (t, 2H, $^{3}J_{HH}$ = 7.5 Hz, $CH_2C(O)$), 2.30 (t, 2H, $^{3}J_{HH}$ = 7.0 Hz, CH_2N), 4.09 (t, 2H, $^{3}J_{HH}$ = 6.1 Hz, CH_2O). ^{13}C NMR ($CDCl_3$) δ: 14.23 (CH_3), 22.79 (CH_2), 25.09 (CH_2), 27.12 (c, $CH_2\underline{C}H_2CH_2O$), 29.26 ($CH_2$), 29.38 ($CH_2$), 29.44 ($CH_2$), 29.57 ($CH_2$), 29.71 ($CH_2$), 32.01 ($CH_2$), 34.45 ($CH_2$), 45.59 ($(CH_3)_2N$), 56.37 ($CH_2N$), 62.68 ($CH_2O$), 174.02 (C=O). ESI-HRMS m/z: 286.2746 $[M+H]^+$ (calculated for $[C_{17}H_{36}N_2O]^+$ - 286.2741).

4.1.2.5. 3-(Dimethylamino)propyl myristate **(8e)**

The reaction was carried out following the general procedure with compound **1b** (5.00 g, 21.9 mmol), compound **7b** (2.59 ml, 21.9 mmol), DCC (4.52 g, 21.9 mmol), DMAP (0.13 g, 1.1 mmol). Yield 47% (3.28 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^{3}J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.24-1.32 (m, 20H, $10CH_2$), 1.57-1.63 (m, 2H, CH_2), 1.77-1.84 (m, 2H, CH_2), 2.24

(s, 6H, $(CH_3)_2N$), 2.28 (t, 6H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.35 (t, 2H, $^3J_{HH}$ = 7.5 Hz, CH_2N), 4.10 (t, 2H, $^3J_{HH}$ = 6.5 Hz, CH_2O). ^{13}C NMR ($CDCl_3$) δ: 14.26 (CH_3), 22.82 (CH_2), 25.12 (CH_2), 27.00 ($CH_2\underline{C}H_2CH_2O$), 29.29 ($CH_2$), 29.41 ($CH_2$), 29.49 ($CH_2$), 29.60 ($CH_2$), 29.77 ($CH_2$), 32.05 ($CH_2$), 34.47 ($CH_2$), 45.48 ($(CH_3)_2N$), 56.35 ($CH_2N$), 62.63 ($CH_2O$), 174.03 (C=O). ESI-HRMS m/z: 314.3059 $[M+H]^+$ (calculated for $[C_{19}H_{40}NO_2]^+$ - 314.3054).

4.1.2.6. 3-(Dimethylamino)propyl palmitate **(8f)**

The reaction was carried out following the general procedure with compound **1c** (5.00 g, 19.5 mmol), compound **7b** (2.31 ml, 19.5 mmol), DCC (4.02 g, 19.5 mmol), DMAP (0.12 g, 0.9 mmol). Yield 51% (3.42 g); colorless oil. ^1H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{14}H_{28}$), 1.24-1.35 (m, 24H, $12CH_2$), 1.56-1.64 (m, 2H, CH_2), 1.78-1.85 (m, 2H, CH_2), 2.26 (s, 6H, $(CH_3)_2N$), 2.28 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.37 (t, 2H, $^3J_{HH}$ = 7.3 Hz, CH_2N), 4.11 (t, 2H, $^3J_{HH}$ = 6.5 Hz, CH_2O). ^{13}C NMR ($CDCl_3$) δ: 14.27 (CH_3), 22.83 (CH_2), 25.12 (CH_2), 26.94 ($CH_2\underline{C}H_2CH_2O$), 29.30 ($CH_2$), 29.41 ($CH_2$), 29.50 ($CH_2$), 29.61 ($CH_2$), 29.75 ($CH_2$), 29.79 ($CH_2$), 29.83 ($CH_2$), 32.06 ($CH_2$), 34.47 ($CH_2$), 45.44 ($(CH_3)_2N$), 56.34 ($CH_2N$), 62.59 ($CH_2O$), 174.02 (C=O). ESI-HRMS m/z: 342.3372 $[M+H]^+$ (calculated for $[C_{21}H_{44}NO_2]^+$ - 342.3367).

4.1.3. General procedure for preparation of quaternary ammonium salts ***6a-u***, ***9a-n***

A solution of the corresponding chloride **5a–e** (1 equiv) in 20 ml of ethanol was neutralized with aqueous solution of $NaHCO_3$ (1 equiv). The solvent was evaporated under reduced pressure and the residue was dissolved in ethanol (20 ml), and the formed NaCl was filtered off. Compounds **3a-i, 8a-f** (1 equiv) were added to the filtrate. The reaction mixture was heated at 70 °C for 8 h, then the solvent was evaporated under reduced pressure. The product was recrystallized from acetone (compounds **6a-u, 9b-g, 9i, 9k-n**) or purified by column chromatography (compound **9a**, **9h**, **9j**) on reversed-fase column (eluent isopropanol/water = from 0:100 to 100:0).

4.1.3.1. 5-(Methylene(N,N-dimethyl-N-(2-lauroylaminoethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6a)***

The reaction was carried out following the general procedure with compound **5a** (0.66 g, 2.5 mmol), $NaHCO_3$ (0.21 g, 2.5 mmol) and compound **3a** (0.68 g, 2.5 mmol). Yield 71% (0.89 g); white solid; mp 140-142 °C (dec). ^1H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{10}H_{20}$), 1.18-1.28 (m, 16H, $8CH_2$), 1.51 (s, 6H, $(CH_3)_2C$), 1.52-1.58 (m, 2H, CH_2), 2.23 (t, 4H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.42 (s, 3H, CH_{3Pyr}), 3.34 (s, 6H, $(CH_3)_2N^+$), 3.80-3.83 (m, 2H, CH_2), 3.99-4.02 (m, 2H, CH_2), 4.95 (s, 2H, CH_2), 5.10 (s, 2H, CH_2), 8.26 (s, 1H, CH_{Pyr}), 8.71 (t, 1H, $^3J_{HH}$ = 5.4 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 14.23 (CH_3), 18.60 (CH_{3Pyr}), 22.78 (CH_2), 24.95 ($C(\underline{C}H_3)_2$), 25.61 (CH_2), 29.44 (CH_2), 29.50 (CH_2), 29.55 (CH_2), 29.65 (CH_2), 29.72 (CH_2), 29.75 (CH_2), 32.00 (CH_2), 34.34 (CH_2), 36.39 (CH_2), 49.99 (CH_3N^+), 59.43 (CH_2), 62.82 (CH_2), 64.30 (CH_2), 100.78 ($(CH_3)_2\underline{C}$), 117.27 (C_{Pyr}), 129.16 (C_{Pyr}), 143.53 (C_{Pyr}), 147.06 (C_{Pyr}), 150.88 (C_{Pyr}), 174.98 (C=O). ESI-HRMS m/z: 462.3696 $[M-Cl]^+$ (calculated for $[C_{27}H_{48}N_3O_3]^+$ - 462.3690).

4.1.3.2. 5-(Methylene(N,N-dimethyl-N-(2-myristoylaminoethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6b)***

The reaction was carried out following the general procedure with compound **5a** (0.37 g, 1.4 mmol), $NaHCO_3$ (0.12 g, 1.4 mmol) and compound **3b** (0.41 g, 1.4 mmol). Yield 53% (0.59 g); beige solid; mp 132-135 °C (dec). ^1H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 7.0 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.21-1.28 (m, 20H, $10CH_2$), 1.52 (s, 6H, $(CH_3)_2C$), 1.55-1.59 (m, 2H, CH_2), 2.25 (t, 2H, $^3J_{HH}$ = 7.5 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.29 (s, 6H, $(CH_3)_2N^+$), 3.82-3.86 (m, 2H, CH_2), 4.01 (br s, 2H, CH_2), 4.84 (s, 2H, CH_2), 5.07 (s, 2H, CH_2), 8.18 (s, 1H, CH_{Pyr}), 8.63 (t, 1H, $^3J_{HH}$ = 5.0 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 14.22 (CH_3), 18.75 (CH_{3Pyr}), 22.78 (CH_2), 24.94 ($(\underline{C}H_3)_2C$), 25.61 (CH_2), 29.45 (CH_2), 29.50 (CH_2), 29.56 (CH_2), 29.65 (CH_2), 29.77 (CH_2),

32.01 (CH_2), 34.32 (CH_2), 36.38 (CH_2), 49.97 (CH_3N^+), 59.40 (CH_2), 62.90 (CH_2), 64.30 (CH_2), 100.69 (($CH_3)_2\underline{C}$), 117.08 (C_{Pyr}), 128.84 (C_{Pyr}), 143.82 (C_{Pyr}), 146.95 (C_{Pyr}), 151.07 (C_{Pyr}), 174.98 (C=O). ESI-HRMS m/z: 490.4003 $[M\text{-}Cl]^+$ (calculated for $[C_{29}H_{52}N_3O_3]^+$ - 490.4003).

4.1.3.3. 5-(Methylene(N,N-dimethyl-N-(2-palmitoylaminoethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6c)***

The reaction was carried out following the general procedure with compound **5a** (0.34 g, 1.3 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol) and compound **3c** (0.43 g, 1.3 mmol). Yield 36% (0.26 g); beige solid; mp 135-136 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.6 Hz, $\underline{CH_3}C_{14}H_{28}$), 1.20-1.27 (m, 24H, $12CH_2$), 1.50 (s, 6H, $(CH_3)_2C$), 1.55 (br s, 2H, CH_2), 2.24 (t, 2H, $^3J_{HH}$ = 7.3 Hz, $CH_2C(O)$), 2.40 (s, 3H, CH_{3Pyr}), 3.32 (s, 6H, $(CH_3)_2N^+$), 3.81 (br s, 2H, CH_2), 4.00 (br s, 2H, CH_2), 4.89 (s, 2H, CH_2), 5.08 (s, 2H, CH_2), 8.19 (s, 1H, CH_{Pyr}), 8.72 (t, 1H, $^3J_{HH}$ = 5.4 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 14.18 (CH_3), 18.94 (CH_{3Pyr}), 22.73 (CH_2), 24.89 (($\underline{C}H_3)_2C$), 25.57 (CH_2), 29.41 (CH_2), 29.47 (CH_2), 29.53 (CH_2), 29.62 (CH_2), 29.75 (CH_2), 31.97 (CH_2), 34.26 (CH_2), 36.33 (CH_2), 49.88 (CH_3N^+), 59.32 (CH_2), 62.94 (CH_2), 64.22 (CH_2), 100.47 (($CH_3)_2\underline{C}$), 116.80 (C_{Pyr}), 128.25 (C_{Pyr}), 144.35 (C_{Pyr}), 146.69 (C_{Pyr}), 151.28 (C_{Pyr}), 174.91 (C=O). ESI-HRMS m/z: 518.4316 $[M\text{-}Cl]^+$ (calculated for $[C_{31}H_{56}N_3O_3]^+$ - 518.4316).

4.1.3.4. 5-(Methylene(N,N-dimethyl-N-(2-stearoylaminoethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6d)***

The reaction was carried out following the general procedure with compound **5a** (0.58 g, 2.2 mmol), $NaHCO_3$ (0.18 g, 2.2 mmol) and compound **3d** (0.78 g, 2.2 mmol). Yield 44% (0.56 g); beige solid; mp 143 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.3 Hz, $\underline{CH_3}C_{16}H_{32}$), 1.20-1.30 (m, 28H, $14CH_2$), 1.51 (s, 6H, $(CH_3)_2C$), 1.54 (br s, 2H, CH_2), 2.22 (t, 2H, $^3J_{HH}$ = 7.4 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.30 (s, 6H, $(CH_3)_2N^+$), 3.82 (br s, 2H, CH_2), 3.98 (br s, 2H, CH_2), 4.89 (s, 2H, CH_2), 5.08 (s, 2H, CH_2), 8.24 (s, 1H, CH_{Pyr}), 8.62 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.25 (CH_3), 18.67 (CH_{3Pyr}), 22.80 (CH_2), 24.94 (($\underline{C}H_3)_2C$), 25.62 (CH_2), 29.48 (CH_2), 29.53 (CH_2), 29.60 (CH_2), 29.69 (CH_2), 29.78 (CH_2), 29.83 (CH_2), 32.04 (CH_2), 34.20 (CH_2), 36.38 (CH_2), 49.94 (CH_3N^+), 59.37 (CH_2), 62.86 (CH_2), 64.31 (CH_2), 100.71 (($CH_3)_2\underline{C}$), 117.21 (C_{Pyr}), 129.00 (C_{Pyr}), 143.70 (C_{Pyr}), 146.96 (C_{Pyr}), 150.91 (C_{Pyr}), 175.02 (C=O). ESI-HRMS m/z: 546.4629 $[M\text{-}Cl]^+$ (calculated for $[C_{33}H_{60}N_3O_3]^+$ - 546.4629).

4.1.3.5. 5-(Methylene(N,N-dimethyl-N-(3-lauroylaminopropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6e)***

The reaction was carried out following the general procedure with compound **5a** (0.29 g, 1.1 mmol), $NaHCO_3$ (0.09 g, 1.1 mmol) and compound **3e** (0.31 g, 1.1 mmol). Yield 45% (0.25 g); white solid; mp 109-111 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{10}H_{20}$), 1.17-1.29 (m, 16H, $8CH_2$), 1.52 (s, 6H, $(CH_3)_2C$), 1.52-1.58 (m, 2H, CH_2), 2.11-2.18 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.24 (t, 4H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.43 (s, 3H, CH_{3Pyr}), 3.22 (s, 6H, $(CH_3)_2N^+$), 3.32-3.35 (m, 2H, CH_2), 3.97-4.00 (m, 2H, CH_2), 4.90 (s, 2H, CH_2), 5.11 (s, 2H, CH_2), 8.12 (br s, 1H, NH), 8.23 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.23 (CH_3), 18.53 (CH_{3Pyr}), 22.78 (CH_2), 23.11 ($CH_2\underline{C}H_2CH_2NH$), 24.97 ($C(\underline{C}H_3)_2$), 25.93 ($CH_2$), 29.46 ($CH_2$), 29.56 ($CH_2$), 29.66 ($CH_2$), 29.71 ($CH_2$), 29.74 ($CH_2$), 29.79 ($CH_2$), 32.01 ($CH_2$), 36.36 ($CH_2$), 36.46 ($CH_2$), 49.39 ($CH_3N^+$), 59.41 ($CH_2$), 62.39 ($CH_2$), 63.39 ($CH_2$), 100.85 (($CH_3)_2\underline{C}$), 117.45 ($C_{Pyr}$), 129.40 ($C_{Pyr}$), 143.21 ($C_{Pyr}$), 147.15 ($C_{Pyr}$), 150.72 ($C_{Pyr}$), 174.81 (C=O). ESI-HRMS m/z: 476.3853 $[M\text{-}Cl]^+$ (calculated for $[C_{28}H_{50}N_3O_3]^+$ - 476.3847).

4.1.3.6. 5-(Methylene(N,N-dimethyl-N-(3-myristoylaminopropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6f)***

The reaction was carried out following the general procedure with compound **5a** (1.00 g, 3.8 mmol), $NaHCO_3$ (0.32 g, 3.8 mmol) and compound **3f** (1.18 g, 3.8 mmol). Yield 40% (0.82 g); beige solid; mp 123-125 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.8 Hz,

$\underline{CH_3}C_{12}H_{24}$), 1.21-1.29 (m, 20H, $10CH_2$), 1.52 (s, 6H, $(CH_3)_2C$), 1.56 (br s, 2H, CH_2), 2.13-2.19 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.27 (t, 2H, $^3J_{HH}$ = 7.7 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.20 (s, 6H, $(CH_3)_2N^+$), 3.34-3.38 (m, 2H, CH_2), 4.03-4.06 (m, 2H, CH_2), 4.84 (s, 2H, CH_2), 5.09 (s, 2H, CH_2), 8.11 (t, 1H, $^3J_{HH}$ = 5.4 Hz, NH), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.24 (CH_3), 18.89 (CH_{3Pyr}), 22.80 (CH_2), 23.10 ($CH_2\underline{C}H_2CH_2NH$), 24.96 (($\underline{C}H_3)_2C$), 25.93 ($CH_2$), 29.35 ($CH_2$), 29.48 ($CH_2$), 29.57 ($CH_2$), 29.66 ($CH_2$), 29.74 ($CH_2$), 29.78 ($CH_2$), 29.82 ($CH_2$), 32.04 ($CH_2$), 34.40 ($CH_2$), 36.48 ($CH_2$), 49.39 ($CH_3N^+$), 59.35 ($CH_2$), 62.56 ($CH_2$), 63.58 ($CH_2$), 100.63 (($CH_3)_2\underline{C}$), 116.92 ($C_{Pyr}$), 128.56 ($C_{Pyr}$), 143.93 ($C_{Pyr}$), 146.87 ($C_{Pyr}$), 151.31 ($C_{Pyr}$), 174.99 (C=O). ESI-HRMS m/z: 504.4160 $[M\text{-}Cl]^+$ (calculated for $[C_{30}H_{54}N_3O_3]^+$ - 504.4160).

4.1.3.7. 5-(Methylene(N,N-dimethyl-N-(3-palmitoylaminopropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6g)***

The reaction was carried out following the general procedure with compound **5a** (0.34 g, 1.3 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol) and compound **3g** (0.45 g, 1.3 mmol). Yield 43% (0.32 g); beige solid; mp 114-115 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{14}H_{28}$), 1.21-1.29 (m, 24H, $12CH_2$), 1.51 (s, 6H, $(CH_3)_2C$), 1.56 (br s, 2H, CH_2), 2.13-2.20 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.26 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.19 (s, 6H, $(CH_3)_2N^+$), 3.34-3.38 (m, 2H, CH_2), 4.00-4.04 (m, 2H, CH_2), 4.82 (s, 2H, CH_2), 5.08 (s, 2H, CH_2), 8.11 (t, 1H, $^3J_{HH}$ = 5.5 Hz, NH), 8.15 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.19 (CH_3), 18.81 (CH_{3Pyr}), 22.76 (CH_2), 23.10 ($CH_2\underline{C}H_2CH_2NH$), 24.91 (($\underline{C}H_3)_2C$), 25.89 ($CH_2$), 29.35 ($CH_2$), 29.44 ($CH_2$), 29.55 ($CH_2$), 29.63 ($CH_2$), 29.67 ($CH_2$), 29.70 ($CH_2$), 29.74 ($CH_2$), 29.79 ($CH_2$), 31.99 ($CH_2$), 34.47 ($CH_2$), 36.42 ($CH_2$), 49.39 ($CH_3N^+$), 59.29 ($CH_2$), 62.46 ($CH_2$), 63.36 ($CH_2$), 100.55 (($CH_3)_2\underline{C}$), 117.03 ($C_{Pyr}$), 128.52 ($C_{Pyr}$), 144.00 ($C_{Pyr}$), 146.77 ($C_{Pyr}$), 151.11 ($C_{Pyr}$), 174.89 (C=O). ESI-HRMS m/z: 532.4474 $[M\text{-}Cl]^+$ (calculated for $[C_{32}H_{58}N_3O_3]^+$ - 532.4473).

4.1.3.8. 5-(Methylene(N,N-dimethyl-N-(3-stearoylaminopropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6h)***

The reaction was carried out following the general procedure with compound **5a** (0.34 g, 1.3 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol) and compound **3h** (0.49 g, 1.3 mmol). Yield 49% (0.39 g); beige solid; mp 134-136 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{16}H_{32}$), 1.21-1.23 (m, 28H, $14CH_2$), 1.51 (s, 6H, $(CH_3)_2C$), 1,55 (br s, 2H, $\underline{CH_2}$), 2.17 (br s, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.25 (t, 2H, $^3J_{HH}$ = 7.5 Hz, $CH_2C(O)$), 2.40 (s, 3H, CH_{3Pyr}), 3.19 (s, 6H, $(CH_3)_2N^+$), 3.33-3.37 (m, 2H, CH_2), 3.97-4.01 (m, 2H, CH_2), 4.82 (s, 2H, CH_2), 5.08 (s, 2H, CH_2), 8.11 (t, 1H, $^3J_{HH}$ = 5.4 Hz, NH), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.18 (CH_3), 18.79 (CH_{3Pyr}), 22.74 (CH_2), 23.09 ($CH_2\underline{C}H_2CH_2NH$), 24.90 (($\underline{C}H_3)_2C$), 25.88 ($CH_2$), 29.34 ($CH_2$), 29.42 ($CH_2$), 29.54 ($CH_2$), 29.63 ($CH_2$), 29.72 ($CH_2$), 29.79 ($CH_2$), 31.98 ($CH_2$), 34.45 ($CH_2$), 36.41 ($CH_2$), 49.37 ($CH_3N^+$), 59.29 ($CH_2$), 62.42 ($CH_2$), 63.31 ($CH_2$), 100.53 (($CH_3)_2\underline{C}$), 117.03 ($C_{Pyr}$), 128.52 ($C_{Pyr}$), 143.98 ($C_{Pyr}$), 146.76 ($C_{Pyr}$), 151.08 ($C_{Pyr}$), 174.86 (C=O). ESI-HRMS m/z: 560.4786 $[M\text{-}Cl]^+$ (calculated for $[C_{34}H_{62}N_3O_3]^+$ - 560.4786).

4.1.3.9. 5-(Methylene(N,N-dimethyl-N-(2-lauroylaminoethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6i)***

The reaction was carried out following the general procedure with compound **5b** (0.33 g, 1.2 mmol), $NaHCO_3$ (0.11 g, 1.2 mmol) and compound **3a** (0.34 g, 1.2 mmol). Yield 45% (0.29 g); white solid; mp 125 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{10}H_{20}$), 0.96 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.16-1.25 (m, 16H, $8CH_2$), 1.45-1.54 (m, 4H, $2CH_2$), 1.73-1.85 (m, 2H, CH_2), 2.21 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.42 (s, 3H, CH_{3Pyr}), 3.30 (s, 3H, CH_3N^+), 3.31 (s, 3H, CH_3N^+), 3.79 (m, 2H, CH_2), 3.95 (br s, 2H, CH_2), 4.89, 4.92 (AB, $^2J_{HH}$ = -13.6 Hz, 2H, CH_2), 5.04, 5.34 (AB, $^2J_{HH}$ = -16.4 Hz, 2H, CH_2), 5.09 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2C\underline{H}$), 8.25 (s, 1H, CH_{Pyr}), 8.63 (t, 1H, $^3J_{HH}$ = 4.9 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 13.99 ($\underline{C}H_3CH_2CH_2$), 14.21 (CH_3), 16.90 ($CH_3\underline{C}H_2CH_2$), 18.52 (CH_{3Pyr}), 22.76 (CH_2), 25.59

(CH_2), 29.43 (CH_2), 29.49 (CH_2), 29.55 (CH_2), 29.64 (CH_2), 29.71 (CH_2), 29.74 (CH_2), 31.99 (CH_2), 34.23 (CH_2), 36.25 (CH_2), 36.36 (CH_2), 49.94 (CH_3N^+), 50.09 (CH_3N^+), 62.87 (CH_2), 64.23 (CH_2), 65.28 (CH_2), 100.58 ($CH_3CH_2CH_2\underline{C}H$), 117.53 ($C_{Pyr}$), 130.66 ($C_{Pyr}$), 144.24 ($C_{Pyr}$), 148.46 ($C_{Pyr}$), 150.46 ($C_{Pyr}$), 174.93 (C=O). ESI-HRMS m/z: 476.3852 $[M\text{-}Cl]^+$ (calculated for $[C_{28}H_{50}N_3O_3]^+$ - 476.3847). HPLC analysis: retention time 8.8 min; purity 99.6 %.

4.1.3.10 5-(Methylene(N,N-dimethyl-N-(2-myristoylaminoethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6j)***

The reaction was carried out following the general procedure with compound **5b** (0.33 g, 1.2 mmol), $NaHCO_3$ (0.11 g, 1.2 mmol) and compound **3b** (0.36 g, 1.2 mmol). Yield 58% (0.38 g); beige solid; mp 136-139 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{12}H_{24}$), 0.98 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.21-1.28 (m, 20H, $10CH_2$), 1.48-1.59 (m, 4H, $2CH_2$), 1.74-1.86 (m, 2H, CH_2), 2.24 (t, 3H, $^3J_{HH}$ = 7.7 Hz, $CH_2C(O)$), 2.43 (s, 3H, CH_{3Pyr}), 3.29 (s, 3H, CH_3N^+), 3.30 (s, 3H, CH_3N^+), 3.81-3.84 (m, 2H, CH_2), 3.97-4.00 (m, 2H, CH_2), 4.82, 4.88. (AB, 2H, $^2J_{HH}$ = -13.6 Hz, CH_2), 5.04, 5.32 (AB, 2H, $^2J_{HH}$ = -16.4 Hz, CH_2), 5.07 (t, 3H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 8.19 (s, 1H, CH_{Pyr}), 8.62 (t, 1H, $^3J_{HH}$ = 5.2 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 13.96 ($\underline{C}H_3CH_2CH_2$), 14.17 (CH_3), 16.89 ($CH_3\underline{C}H_2CH_2$), 18.84 (CH_{3Pyr}), 22.73 (CH_2), 25.56 (CH_2), 29.41 (CH_2), 29.47 (CH_2), 29.53 (CH_2), 29.62 (CH_2), 29.74 (CH_2), 31.97 (CH_2), 34.20 (CH_2), 36.26 (CH_2), 36.32 (CH_2), 49.88 (CH_3N^+), 50.01 (CH_3N^+), 62.97 (CH_2), 64.19 (CH_2), 65.21 (CH_2), 100.43 ($CH_3CH_2CH_2\underline{C}H$), 117.11 ($C_{Pyr}$), 129.81 ($C_{Pyr}$), 144.95 ($C_{Pyr}$), 148.18 ($C_{Pyr}$), 150.86 ($C_{Pyr}$), 174.89 (C=O). ESI-HRMS m/z: 504.4160 $[M\text{-}Cl]^+$ (calculated for $[C_{30}H_{54}N_3O_3]^+$ - 504.4160).

4.1.3.11. 5-(Methylene(N,N-dimethyl-N-(2-palmitoylaminoethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6k)***

The reaction was carried out following the general procedure with compound **5b** (0.33 g, 1.2 mmol), $NaHCO_3$ (0.11 g, 1.2 mmol) and compound **3c** (0.41 g, 1.2 mmol). Yield 46% (0.33 g); beige solid; mp 141-142 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.5 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.96 (t, 3H, $^3J_{HH}$ = 7.3 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.19-1.28 (m, 24H, $12CH_2$), 1.47-1.56 (m, 4H, $2CH_2$), 1.73-1.83 (m, 2H, CH_2), 2.21 (t, 3H, $^3J_{HH}$ = 7.6 Hz, $\underline{\ }CH_2C(O)$), 2.39 (s, 3H, CH_{3Pyr}), 3.27 (s, 3H, CH_3N^+), 3.29 (s, 3H, CH_3N^+), 3.80 (br s, 2H, CH_2), 3.95 (br s, 2H, CH_2), 4.82, 4.87 (AB, 2H, $^2J_{HH}$ = -14.0 Hz, CH_2), 5.01, 5.30 (AB, 2H, $^2J_{HH}$ = -16.1 Hz, CH_2), 5.05 (t, 3H, $^3J_{HH}$ = 4.8 Hz, $CH_3CH_2CH_2\underline{CH}$), 8.19 (s, 1H, CH_{Pyr}), 8.63 (t, 1H, $^3J_{HH}$ = 5.0 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 13.97 ($\underline{C}H_3CH_2CH_2$), 14.18 (CH_3), 16.91 ($CH_3\underline{C}H_2CH_2$), 18.82 (CH_{3Pyr}), 22.75 (CH_2), 25.58 (CH_2), 29.43 (CH_2), 29.46 (CH_2), 29.49 (CH_2), 29.56 (CH_2), 29.65 (CH_2), 29.73 (CH_2), 29.78 (CH_2), 31.98 (CH_2), 34.15 (CH_2), 36.27 (CH_2), 36.32 (CH_2), 49.86 (CH_3N^+), 50.00 (CH_3N^+), 62.96 (CH_2), 64.21 (CH_2), 65.18 (CH_2), 100.43 ($CH_3CH_2CH_2\underline{C}H$), 117.18 ($C_{Pyr}$), 129.86 ($C_{Pyr}$), 144.94 ($C_{Pyr}$), 148.19 ($C_{Pyr}$), 150.80 ($C_{Pyr}$), 174.94 (C=O). ESI-HRMS m/z: 532.4437 $[M\text{-}Cl]^+$ (calculated for $[C_{32}H_{58}N_3O_3]^+$ - 532.4437).

4.1.3.12. 5-(Methylene(N,N-dimethyl-N-(2-stearoylaminoethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6l)***

The reaction was carried out following the general procedure with compound **5b** (0.33 g, 1.2 mmol), $NaHCO_3$ (0.11 g, 1.2 mmol) and compound **3d** (0.44 g, 1.2 mmol). Yield 46% (0.34 g); beige solid; mp 143 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{16}H_{32}$), 0.97 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.19-1.27 (m, 28H, $14CH_2$), 1.46-1.59 (m, 4H, $2CH_2$), 1.76-1.83 (m, 2H, CH_2), 2.22 (t, 2H, $^3J_{HH}$ = 7.4 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.30 (s, 3H, CH_3N^+), 3.31 (s, 3H, CH_3N^+), 3.79-3.82 (m, 2H, CH_2), 3.95-3.99 (m, 2H, CH_2), 4.84, 4.90 (AB, 2H, $^2J_{HH}$ = 13.6 Hz, CH_2), 5.03, 5.32 (AB, 2H, $^2J_{HH}$ = 16.4 Hz, CH_2), 5.06 (t, 1H, $^3J_{HH}$ = 5.1 Hz, CH), 8.20 (s, 1H, CH_{Pyr}), 8.64 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 13.99 ($\underline{C}H_3CH_2CH_2$), 14.21 (CH_3), 16.92 ($CH_3\underline{C}H_2CH_2$), 18.84 (CH_{3Pyr}), 22.77 (CH_2), 25.59 (CH_2), 29.44 (CH_2), 29.51 (CH_2), 29.57 (CH_2), 29.67 (CH_2), 29.71 (CH_2), 29.75 (CH_2), 29.80 (CH_2),

32.00 (CH_2), 34.20 (CH_2), 36.29 (CH_2), 36.36 (CH_2), 49.88 (CH_3N^+), 50.04 (CH_3N^+), 63.02 (CH_2), 64.24 (CH_2), 65.22 (CH_2), 100.47 ($CH_3CH_2CH_2\underline{C}H$), 117.14 ($C_{Pyr}$), 129.89 ($C_{Pyr}$), 144.91 ($C_{Pyr}$), 148.24 ($C_{Pyr}$), 150.90 ($C_{Pyr}$), 174.97 (C=O). ESI-HRMS m/z: 560.4786 $[M\text{-}Cl]^+$ (calculated for $[C_{34}H_{62}N_3O_3]^+$ - 560.4786).

4.1.3.13. 5-(Methylene(N,N-dimethyl-N-(3-lauroylaminopropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6m)***

The reaction was carried out following the general procedure with compound **5b** (0.33 g, 1.2 mmol), $NaHCO_3$ (0.11 g, 1.2 mmol) and compound **3e** (0.35 g, 1.2 mmol). Yield 44% (0.29 g); white solid; mp 130-131 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.97 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.20-1.27 (m, 16H, $8CH_2$), 1.46-1.55 (m, 4H, $2CH_2$), 1.73-1.86 (m, 2H, CH_2), 2.11-2.14 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.23 (t, 2H, $^3J_{HH}$ = 7.3 Hz, $CH_2C(O)$), 2.43 (s, 3H, CH_{3Pyr}), 3.20 (s, 3H, CH_3N^+), 3.25 (s, 3H, CH_3N^+), 3.30-3.35 (m, 2H, CH_2), 3.87-3.99 (m, 2H, CH_2), 4.90 (s, 2H, CH_2), 5.07, 5.34 (AB, 2H, $^2J_{HH}$ = -16.4 Hz, CH_2), 5.14 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.02 (s, 2H, CH_2), 8.08 (t, 1H, $^3J_{HH}$ = 5.6 Hz, NH), 8.22 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.02 ($\underline{C}H_3CH_2CH_2$), 14.23 (CH_3), 16.92 ($CH_3\underline{C}H_2CH_2$), 18.53 (CH_{3Pyr}), 22.78 (CH_2), 23.21 ($CH_2\underline{C}H_2CH_2NH$), 25.90 ($CH_2$), 29.46 ($CH_2$), 29.55 ($CH_2$), 29.64 ($CH_2$), 29.71 ($CH_2$), 29.74 ($CH_2$), 29.79 ($CH_2$), 32.01 ($CH_2$), 36.28 ($CH_2$), 36.46 ($CH_2$), 49.22 ($CH_3N^+$), 49.64 ($CH_3N^+$), 62.72 ($CH_2$), 63.11 ($CH_2$), 65.30 ($CH_2$), 100.62 ($CH_3CH_2CH_2\underline{C}H$), 117.63 ($C_{Pyr}$), 130.90 ($C_{Pyr}$), 143.90 ($C_{Pyr}$), 148.56 ($C_{Pyr}$), 150.43 ($C_{Pyr}$), 174.78 (C=O). ESI-HRMS m/z: 490.4009 $[M\text{-}Cl]^+$ (calculated for $[C_{29}H_{52}N_3O_3]^+$ - 490.4003).

4.1.3.14. 5-(Methylene(N,N-dimethyl-N-(3-myristoylaminopropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6n)***

The reaction was carried out following the general procedure with compound **5b** (0.36 g, 1.3 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol) and compound **3f** (0.41 g, 1.3 mmol). Yield 58% (0.42 g); beige solid; mp 130-132 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{12}H_{24}$), 0.95 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.19-1.27 (m, 20H, $10CH_2$), 1.47-1.54 (m, 4H, $2CH_2$), 1.75-1.81 (m, 2H, CH_2), 2.07-2.15 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.21 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.38 (s, 3H, CH_{3Pyr}), 3.18 (s, 3H, CH_3N^+), 3.22 (s, 3H, CH_3N^+), 3.30-3.33 (m, 2H, CH_2), 3.83-3.98 (m, 2H, CH_2), 4.82, 4.86 (AB, 2H, $^2J_{HH}$ = 14.8 Hz, CH_2), 5.02, 5.30 (AB, 2H, $^2J_{HH}$ = 16.2 Hz, CH_2), 5.09 (t, 3H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 8.08 (t, 1H, $^3J_{HH}$ = 5.4 Hz, CH), 8.16 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.02 ($\underline{C}H_3CH_2CH_2$), 14.22 (CH_3), 16.96 ($CH_3\underline{C}H_2CH_2$), 18.79 (CH_{3Pyr}), 22.78 (CH_2), 23.34 ($CH_2\underline{C}H_2CH_2NH$), 25.89 ($CH_2$), 29.47 ($CH_2$), 29.59 ($CH_2$), 29.67 ($CH_2$), 29.77 ($CH_2$), 29.82 ($CH_2$), 32.02 ($CH_2$), 36.32 ($CH_3CH_2\underline{C}H_2$), 36.48 ($CH_2NH$), 49.54 ($CH_3N^+$), 49.94 ($CH_3N^+$), 63.19 ($CH_2$), 65.31 ($CH_2$), 100.47 ($CH_3CH_2CH_2\underline{C}H$), 117.42 ($C_{Pyr}$), 130.13 ($C_{Pyr}$), 144.76 ($C_{Pyr}$), 148.27 ($C_{Pyr}$), 150.69 ($C_{Pyr}$), 174.88 (C=O). ESI-HRMS m/z: 518.4316 $[M\text{-}Cl]^+$ (calculated for $[C_{31}H_{56}N_3O_3]^+$ - 518.4316).

4.1.3.15. 5-(Methylene(N,N-dimethyl-N-(3-palmitoylaminopropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6o)***

The reaction was carried out following the general procedure with compound **5b** (0.33 g, 1.2 mmol), $NaHCO_3$ (0.11 g, 1.2 mmol) and compound **3g** (0.42 g, 1.2 mmol). Yield 57% (0.41 g); beige solid; mp 130-132 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.96 (t, 3H, $^3J_{HH}$ = 7.3 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.20-1.27 (m, 24H, $12CH_2$), 1.48-1.54 (m, 4H, $2CH_2$), 1.75-1.81 (m, 2H, CH_2), 2.12 (br s, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.21 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $CH_2C(O)$), 2.39 (s, 3H, CH_{3Pyr}), 3.17 (s, 3H, CH_3N^+), 3.22 (s, 3H, CH_3N^+), 3.33 (br s, 2H, CH_2), 3.74-3.93 (m, 2H, CH_2), 4,82 (s, 2H, CH_2), 5,01, 5.28 (AB, 2H, $^2J_{HH}$ = - 16.9 Hz, CH_2), 5.09 (t, 1H, $^3J_{HH}$ = 5.3 Hz, $CH_3CH_2CH_2\underline{CH}$), 8.07 (t, 1H, $^3J_{HH}$ = 5.3 Hz, NH), 8.17 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 13.99 ($\underline{C}H_3CH_2CH_2$), 14.19 (CH_3), 16.92 ($CH_3\underline{C}H_2CH_2$), 18.79 (CH_{3Pyr}), 22.75 (CH_2), 23.26 ($CH_2\underline{C}H_2CH_2NH_2$), 25.86 ($CH_2$), 29.43 ($CH_2$), 29.56 ($CH_2$), 29.66 ($CH_2$), 29.73 ($CH_2$), 29.80 ($CH_2$), 31.99 ($CH_2$), 36.29 ($CH_2$), 36.41 ($CH_2$), 49.28 ($CH_3N^+$), 49.73

(CH_3N^+), 62.82 (CH_2), 62.96 (CH_2), 65.13 (CH_2), 100.40 ($CH_3CH_2CH_2\underline{C}H$), 117.42 ($C_{Pyr}$), 130.05 ($C_{Pyr}$), 144.71 ($C_{Pyr}$), 148.19 ($C_{Pyr}$), 150.60 ($C_{Pyr}$), 174.74 (C=O). ESI-HRMS m/z: 546.4630 $[M\text{-}Cl]^+$ (calculated for $[C_{33}H_{64}N_3O_3]^+$ - 546.4629).

4.1.3.16. 5-(Methylene(N,N-dimethyl-N-(3-stearoylaminopropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6p)***

The reaction was carried out following the general procedure with compound **5b** (0.33 g, 1.2 mmol), $NaHCO_3$ (0.11 g, 1.2 mmol) and compound **3g** (0.46 g, 1.2 mmol). Yield 46% (0.35 g); beige solid; mp 143 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{16}H_{32}$), 0.97 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.22-1.28 (m, 28H, $14CH_2$), 1.47-1.56 (m, 4H, $2CH_2$), 1.75-1.85 (m, 2H, CH_2), 2.13 (br s, 2H, $CH_2\underline{CH_2}CH_2NH_2$), 2.24 (t, 2H, $^3J_{HH}$ = 7.9 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_3), 3.18 (s, 3H, CH_3N^+), 3.22 (s, 3H, CH_3N^+), 3.32-3.36 (m, 2H, CH_2N^+), 3.84-3.98 (m, 2H, CH_2), 4.82, 4.86 (AB, 2H, $^2J_{HH}$ = -14.4 Hz, CH_2), 5.04, 5.31 (AB, 2H, $^2J_{HH}$ = -16.4 Hz, CH_2), 5.11 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 8.05 (t, 1H, $^3J_{HH}$ = 5.4 Hz, NH), 8.17 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 13.98 ($\underline{C}H_3CH_2CH_2$), 14.17 (CH_3), 16.91 ($CH_3\underline{C}H_2CH_2$), 18.74 (CH_{3Pyr}), 22.74 (CH_2), 23.20 ($CH_2\underline{C}H_2CH_2NH_2$), 25.85 ($CH_2$), 29.42 ($CH_2$), 29.54 ($CH_2$), 29.63 ($CH_2$), 29.72 ($CH_2$), 29.79 ($CH_2$), 31.97 ($CH_2$), 36.28 ($CH_2$), 36.40 ($CH_2$), 49.26 ($CH_3N^+$), 49.67 ($CH_3N^+$), 62.77 ($CH_2$), 62.99 ($CH_2$), 65.15 ($CH_2$), 100.41 ($CH_3CH_2CH_2\underline{CH}$), 117.39 ($C_{Pyr}$), 130.11 ($C_{Pyr}$), 144.61 ($C_{Pyr}$), 148.22 ($C_{Pyr}$), 150.61 ($C_{Pyr}$), 174.76 (C=O). ESI-HRMS m/z: 574.4943 $[M\text{-}Cl]^+$ (calculated for $[C_{35}H_{64}N_3O_3]^+$ - 574.4942).

4.1.3.17. 5-(Methylene(N,N-dimethyl-N-(3-myristoylaminopropyl)ammonium))-2-pentyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6q)***

The reaction was carried out following the general procedure with compound **5c** (0.80 g, 2.6 mmol), $NaHCO_3$ (0.22 g, 2.6 mmol) and compound **3f** (0.82 g, 2.6 mmol). Yield 57% (0.90 g); white solid; mp 122 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.9 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.89 (t, 3H, $^3J_{HH}$ = 6.9 Hz, $\underline{CH_3}(CH_2)_4CH$), 1.20-1.27 (m, 20H, $10CH_2$), 1.30-1.34 (m, 4H, $2CH_2$), 1.44-1.56 (m, 4H, $2CH_2$), 1.75-1.85 (m, 2H, CH_2), 2.12 (br s, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.23 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.40 (s, 3H, CH_{3Pyr}), 3.18 (s, 3H, CH_3N^+), 3.22 (s, 3H, CH_3N^+), 3.31-3.34 (m, 2H, CH_2), 3.84-4.00 (m, 2H, CH_2), 4.82, 4.86 (AB, 2H, $^2J_{HH}$ = -14.0 Hz, CH_2), 5.02, 5.30 (AB, 2H, $^2J_{HH}$ = -16.4 Hz, CH_2), 5.08 (t, 1H, $^3J_{HH}$ = 5.2 Hz, $CH_3(CH_2)_4\underline{CH}$), 8.10 (t, 1H, $^3J_{HH}$ = 5.1 Hz, NH), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.08 (CH_3), 14.23 (CH_3), 18.92 (CH_{3Pyr}), 22.61 (CH_2), 22.78 ($CH_2\underline{C}H_2CH_2NH$), 23.22 ($CH_2$), 25.90 ($CH_2$), 29.47 ($CH_2$), 29.57 ($CH_2$), 29.66 ($CH_2$), 29.73 ($CH_2$), 29.76 ($CH_2$) 29.81 ($CH_2$), 31.61 ($CH_2$), 32.01 ($CH_2$), 34.26 ($CH_2$), 36.27 ($CH_2$), 36.46 ($CH_2$), 49.21 ($CH_3N^+$), 49.57 ($CH_3N^+$), 62.84 ($CH_2$), 63.15 ($CH_2$), 65.24 ($CH_2$), 100.68 ($CH_3(CH_2)_4\underline{C}H$), 117.14 ($C_{Pyr}$), 129.98 ($C_{Pyr}$), 144.65 ($C_{Pyr}$), 148.29 ($C_{Pyr}$), 150.94 ($C_{Pyr}$), 174.79 (C=O). ESI-HRMS m/z: 546.4635 $[M\text{-}Cl]^+$ (calculated for $[C_{33}H_{60}N_3O_3]^+$ - 546.4629).

4.1.3.18. 5-(Methylene(N,N-dimethyl-N-(3-myristoylaminopropyl)ammonium))-2-octyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6r)***

The reaction was carried out following the general procedure with compound **5d** (0.56 g, 1.6 mmol), $NaHCO_3$ (0.13 g, 1.6 mmol) and compound **3f** (0.50 g, 1.6 mmol). Yield 76% (0.76 g); beige solid; mp 162-165 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.9 Hz, CH_3), 0.86 (t, 3H, $^3J_{HH}$ = 5.9 Hz, CH_3), 1.20-1.34 (m, 30H, $15CH_2$), 1.43-1.52 (m, 4H, $2CH_2$), 1.73-1.87 (m, 2H, CH_2), 2.12 (br s, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.23 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.40 (s, 3H, CH_{3Pyr}), 3.18 (s, 3H, CH_3N^+), 3.22 (s, 3H, CH_3N^+), 3.31-3.34 (m, 2H, CH_2), 3.84-4.00 (m, 2H, CH_2), 4.84 (c, 2H, CH_2), 5.02, 5.30 (AB, 2H, $^2J_{HH}$ = -16.4 Hz, CH_2), 5.08 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3(CH_2)_7\underline{CH}$), 8.09 (t, 1H, $^3J_{HH}$ = 5.4 Hz, NH), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.22 (CH_3), 18.91 (CH_{3Pyr}), 22.77 (CH_2), 23.19 ($CH_2\underline{C}H_2CH_2NH$), 23.56 ($CH_2$), 25.90 ($CH_2$), 29.34 ($CH_2$), 29.47 ($CH_2$), 29.50 ($CH_2$), 29.57 ($CH_2$), 29.67 ($CH_2$) 29.74 ($CH_2$), 29.77 ($CH_2$), 29.82 ($CH_2$), 31.96 ($CH_2$), 32.02 ($CH_2$), 34.32 ($CH_2$), 36.26 ($CH_2$), 36.45 ($CH_2$), 49.20 ($CH_3N^+$), 49.57

(CH_3N^+), 62.83 (CH_2), 63.15 (CH_2), 65.23 (CH_2), 100.68 ($CH_3(CH_2)_7\underline{C}H$), 117.15 ($C_{Pyr}$), 129.97 ($C_{Pyr}$), 144.65 ($C_{Pyr}$), 148.29 ($C_{Pyr}$), 150.93 ($C_{Pyr}$), 174.79 (C=O). ESI-HRMS m/z: 588.5104 $[M-Cl]^+$ (calculated for $[C_{36}H_{66}N_3O_3]^+$ - 588.5099).

4.1.3.19. 5-(Methylene(N,N-dimethyl-N-(3-hexanoylaminopropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6s)***

The reaction was carried out following the general procedure with compound **5a** (0.37 g, 1.4 mmol), $NaHCO_3$ (0.12 g, 1.4 mmol) and compound **3i** (0.28 g, 1.4 mmol). Yield 63% (0.38 g); white solid; mp 124-125 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.79 (t, 3H, $^3J_{HH}$ = 6.9 Hz, $\underline{CH_3}C_4H_8$), 1.18-1.25 (m, 4H, $2CH_2$), 1.48 (s, 6H, $(CH_3)_2C$), 1.50-1.56 (m, 2H, CH_2), 2.09-2.17 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.22 (t, 4H, $^3J_{HH}$ = 6.8 Hz, $CH_2C(O)$), 2.36 (s, 3H, CH_{3Pyr}), 3.20 (s, 6H, $(CH_3)_2N^+$), 3.29-3.33 (m, 2H, CH_2), 3.95-3.98 (m, 2H, CH_2), 4.85 (s, 2H, CH_2), 5.06 (s, 2H, CH_2), 8.16 (s, 1H, CH_{Pyr}), 8.20 (t, 1H, $^3J_{HH}$ = 5.3 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 14.06 (CH_3), 18.96 (CH_{3Pyr}), 22.52 (CH_2), 23.10 ($CH_2\underline{C}H_2CH_2NH$), 24.90 ($CH_2$), 25.51 ($CH_2$), 31.55 ($CH_2$), 36.31 ($CH_2$), 49.28 ($CH_3N^+$), 59.31 ($CH_2$), 62.47 ($CH_2$), 63.23 ($CH_2$), 100.51 (($CH_3)_2\underline{C}$), 116.87 ($C_{Pyr}$), 128.32 ($C_{Pyr}$), 144.11 ($C_{Pyr}$), 146.71 ($C_{Pyr}$), 151.24 ($C_{Pyr}$), 174.71 (C=O). ESI-HRMS m/z: 392.2913 $[M-Cl]^+$ (calculated for $[C_{22}H_{38}N_3O_3]^+$ - 392.2908).

4.1.3.20. 5-(Methylene(N,N-dimethyl-N-(3-hexanoylaminopropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6t)***

The reaction was carried out following the general procedure with compound **5b** (0.39 g, 1.4 mmol), $NaHCO_3$ (0.12 g, 1.4 mmol) and compound **3i** (0.27 g, 1.4 mmol). Yield 40% (0.24 g); white solid; mp 99-100 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.80 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_4H_8$), 0.96 (t, 3H, $^3J_{HH}$ = 7.3 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.16-1.25 (m, 4H, $2CH_2$), 1.44-1.54 (m, 2H, CH_2), 1.72-1.80 (m, 2H, $CH_2\underline{CH_2}CH_2CH$), 2.11 (br s, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.17 (t, 4H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.36 (s, 3H, CH_{3Pyr}), 3.17 (s, 3H, CH_3N^+), 3.22 (s, 3H, CH_3N^+), 3.30-3.32 (m, 2H, CH_2), 3.74-3.78 (m, 2H, CH_2), 4.76, 4.82 (AB, 2H, $^2J_{HH}$ = -14.1 Hz, CH_2), 5.00, 5.24 (AB, 2H, $^2J_{HH}$ = -16.2 Hz, CH_2), 5.09 (t, 3H, $^3J_{HH}$ = 4.9 Hz, CH), 8.08 (br s,1H, NH), 8.17 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.03 ($\underline{C}H_3CH_2CH_2$), 14.10 (CH_3), 16.95 ($CH_3\underline{C}H_2CH_2NH$), 18.85 ($CH_{3Pyr}$), 22.55 ($CH_2$), 23.26 ($CH_2\underline{C}H_2CH_2$), 25.51 ($CH_2$), 31.60 ($CH_2$), 36.22 ($CH_2$), 36.31 ($CH_2$), 36.36 ($CH_2$), 49.29 ($CH_3N^+$), 49.72 ($CH_3N^+$), 62.90 ($CH_2$), 63.09 ($CH_2$), 65.16 ($CH_2$), 100.45 ($\underline{C}HCH_2CH_2CH_3$), 117.38 ($C_{Pyr}$), 130.05 ($C_{Pyr}$), 144.73 ($C_{Pyr}$), 148.24 ($C_{Pyr}$), 150.74 ($C_{Pyr}$), 174.82 (C=O). ESI-HRMS m/z: 406.3070 $[M-Cl]^+$ (calculated for $[C_{23}H_{40}N_3O_3]^+$ - 406.3064).

4.1.3.21. 5-(Methylene(N,N-dimethyl-N-(3-hexanoylaminopropyl)ammonium))-2-(undecan-2-yl)-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride ***(6u)***

The reaction was carried out following the general procedure with compound **5e** (0.35 g, 0.9 mmol), $NaHCO_3$ (0.08 g, 0.9 mmol) and compound **3i** (0.18 g, 0.9 mmol). Yield 40% (0.2 g); white solid; mp 137-140 °C (dec). ^{1}H NMR ($CDCl_3$) δ (mixture of diastereomers): 0.83 (t, 3H, $^3J_{HH}$ = 7.4 Hz, CH_3), 0.86 (t, 3H, $^3J_{HH}$ = 6.7 Hz, CH_3), 0.96 (t, 3H, $^3J_{HH}$ = 7.3 Hz, CH_3), 0.98-1.01 (m., 3H, $CH_3(CH_2)_8CH(\underline{CH_3})$), 1.21-1.32 (m, 17H, $9CH_2$), 1.35-1.44 (m, 2H, CH_2), 1.51-1.62 (m, 4H, $2CH_2$), 1.87 (br s, 1H, $CH_3(CH_2)_8C\underline{H}(CH_3)$), 2.09-2.19 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.25 (t, 4H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.18 (s, 3H, CH_3N^+), 3.22 (s, 3H, CH_3N^+), 3.31-3.36 (m, 2H, CH_2), 3.87-4.04 (m, 2H, CH_2), 4.84, 4.87 (AB, 2H, $^2J_{HH}$ = -14.2 Hz, CH_2), 4.93 (t, 3H, $^3J_{HH}$ = 4.9 Hz, CH), 5.03, 5.35 (AB, 2H, $^2J_{HH}$ = 16.2 Hz, CH_2), 8.15 (br s, 1H, NH), 8.18 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ (mixture of diastereomers): 13.49 (CH_3), 13.70 (CH_3), 14.11 (CH_3), 14.23 (CH_3), 18.85 (CH_{3Pyr}), 18.90 (CH_{3Pyr}), 22.57 (CH_2), 22.79 (CH_2), 23.20 (CH_2), 25.55 (CH_2), 27.00 (CH_2), 27.08 (CH_2), 29.45 (CH_2), 29.74 (CH_2), 29.94 (CH_2), 30.92 (CH_2), 31.04 (CH_2), 31.61 (CH_2), 32.02 (CH_2), 36.25 (CH_2), 36.40 (CH_2), 37.34 (CH_2), 49.24 (CH_3N^+), 49.58 (CH_3N^+), 62.91 (CH_2), 63.31 (CH_2), 65.34 (CH_2), 103.24 ($CH_3(CH_2)_8CH(CH_3)C\underline{H}$), 103.39 ($CH_3(CH_2)_8\underline{C}H(CH_3)$), 117.14 ($C_{Pyr}$), 130.03 ($C_{Pyr}$), 144.59

(C_{Pyr}), 148.49 (C_{Pyr}), 150.99 (C_{Pyr}), 174.85 (C=O). ESI-HRMS m/z: 518.4323 $[M\text{-}Cl]^+$ (calculated for $[C_{31}H_{56}N_3O_3]^+$ - 518.4316).

4.1.3.22. 5-(Methylene(N,N-dimethyl-N-(2-lauroyloxyethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9a)**

The reaction was carried out following the general procedure with compound **5a** (0.40 g, 1.5 mmol), $NaHCO_3$ (0.13 g, 1.5 mmol) and compound **8a** (0.42 g, 1.5 mmol). Yield 47% (0.36 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.9 Hz, $\underline{CH_3}C_{10}H_{20}$), 1.18-1.28 (m, 16H, $8CH_2$), 1.50 (s, 6H, $(CH_3)_2C$), 1.52-1.60 (m, 2H, CH_2), 2.30 (t, 4H, $^3J_{HH}$ = 6.5 Hz, $CH_2C(O)$), 2.40 (s, 3H, CH_{3Pyr}), 3.39 (s, 6H, $(CH_3)_2N^+$), 4.29 (br s, 2H, CH_2), 4.61 (br s, 2H, CH_2), 5.16 (s, 4H, $2CH_2$), 8.21 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.20 (CH_3), 18.92 (CH_{3Pyr}), 22.76 (CH_2), 24.73 (($\underline{C}H_3)_2C$), 25.00 (CH_2), 29.19 (CH_2), 29.33 (CH_2), 29.41 (CH_2), 29.53 (CH_2), 29.68 (CH_2), 31.98 (CH_2), 34.12 (CH_2), 50.12 (CH_3N^+), 57.76 (CH_2), 59.76 (CH_2), 62.72 (CH_2), 63.48 (CH_2), 100.62 (($CH_3)_2\underline{C}$), 117.10 (C_{Pyr}), 129.02 (C_{Pyr}), 144.32 (C_{Pyr}), 146.89 (C_{Pyr}), 151.12 (C_{Pyr}), 172.89 (C=O). ESI-HRMS m/z: 463.3538 $[M\text{-}Cl]^+$ (calculated for $[C_{27}H_{47}N_2O_4]^+$ - 463.3530).

*4.1.3.23. 5-(Methylene(N,N-dimethyl-N-(2-myristoyloxyethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chlorid***e (9b)**

The reaction was carried out following the general procedure with compound **5a** (0.34 g, 1.3 mmol) $NaHCO_3$ (0.11 g, 1.3 mmol) and compound **8c** (0.38 g, 1.3 mmol). Yield 49% (0.33 g); beige solid; mp 126-129 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.4 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.22-1.28 (m, 20H, $10CH_2$), 1.50 (s, 6H, $(CH_3)_2C$), 1.52-1.56 (m, 2H, CH_2), 2.30 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.39 (s, 6H, $(CH_3)_2N^+$), 4.28 (br s, 2H, CH_2), 4.61 (br s, 2H, CH_2), 5.17 (s, 4H, $2CH_2$), 8.23 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.22 (CH_3), 19.00 (CH_{3Pyr}), 22.77 (CH_2), 24.72 (($\underline{C}H_3)_2C$), 24.97 (CH_2), 29.19 (CH_2), 29.34 (CH_2), 29.44 (CH_2), 29.55 (CH_2), 29.69 (CH_2), 29.73 (CH_2), 29.76 (CH_2), 32.00 (CH_2), 34.10 (CH_2), 50.07 (CH_3N^+), 57.75 (CH_2), 59.67 (CH_2), 62.67 (CH_2), 63.45 (CH_2), 100.54 (($CH_3)_2\underline{C}$), 117.00 (C_{Pyr}), 128.80 (C_{Pyr}), 144.43 (C_{Pyr}), 146.80 (C_{Pyr}), 151.18 (C_{Pyr}), 172.90 (C=O). ESI-HRMS m/z: 491.3849 $[M\text{-}Cl]^+$ (calculated for $[C_{29}H_{51}N_2O_4]^+$ - 491.3843).

4.1.3.24. 5-(Methylene(N,N-dimethyl-N-(2-palmitoyloxyethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9c)**

The reaction was carried out following the general procedure with compound **5a** (0.32 g, 1.2 mmol), $NaHCO_3$ (0.10 g, 1.4 mmol) and compound **8e** (0.40 g, 1.2 mmol). Yield 41% (0.28 g); white solid; mp 125-128 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.6 Hz, $\underline{CH_3}C_{14}H_{28}$), 1.24-1.29 (m, 24H, $12CH_2$), 1.56 (s, 6H, $(\underline{CH_3})_2C$), 1.56-1.60 (m, 2H, CH_2), 2.33 (t, 2H, $^3J_{HH}$ = 7.3 Hz, $CH_2C(O)$), 2.52 (s, 3H, CH_{3Pyr}), 3.42 (s, 6H, $(CH_3)_2N^+$), 4.32 (br s, 2H, CH_2), 4.63 (br s, 2H, CH_2), 5.27 (s, 2H, CH_2), 5.28 (s, 2H, CH_2), 8.39 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.27 (CH_3), 17.70 (CH_{3Pyr}), 22.83 (CH_2), 24.79 (($\underline{C}H_3)_2C$), 25.10 (CH_2), 29.26 (CH_2), 29.40 (CH_2), 29.50 (CH_2), 29.62 (CH_2), 29.76 (CH_2), 29.80 (CH_2), 29.84 (CH_2), 32.06 (CH_2), 34.19 (CH_2), 50.38 (CH_3N^+), 57.77 (CH_2), 59.96 (CH_2), 63.11 (CH_2), 63.17 (CH_2), 101.47 (($CH_3)_2\underline{C}$), 118.55 (C_{Pyr}), 131.88 (C_{Pyr}), 141.74 (C_{Pyr}), 147.90 (C_{Pyr}), 149.68 (C_{Pyr}), 172.98 (C=O). ESI-HRMS m/z: 519.4162 $[M\text{-}Cl]^+$ (calculated for $[C_{31}H_{55}N_2O_4]^+$ - 519.4156).

4.1.3.25. 5-(Methylene(N,N-dimethyl-N-(3-lauroyloxypropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9d)**

The reaction was carried out following the general procedure with compound **5a** (0.42 g, 1.6 mmol), $NaHCO_3$ (0.13 g, 1.6 mmol) and compound **8b** (0.46 g, 1.6 mmol). Yield 46% (0.38 g); beige solid; mp 159-160 °C (dec). ^{1}H NMR (DMSO-d_6) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{10}H_{20}$), 1.23-1.28 (m, 16H, $8CH_2$), 1.50-1.56 (m, 2H, CH_2), 1.58 (s, 6H, $(CH_3)_2C$), 2.10-2.17 (m, 2H, $CH_2\underline{CH_2}CH_2O$), 2.32 (t, 4H, $^3J_{HH}$ = 7.5 Hz, $CH_2C(O)$), 2.55 (s, 3H, CH_{3Pyr}), 3.09 (s,

6H, $(CH_3)_2N^+$), 3.53-3.57 (m, 2H, CH_2), 4.10 (t, 3H, $^3J_{HH}$ = 5.9 Hz, CH_2), 4.70 (s, 2H, CH_2), 5.15 (s, 2H, CH_2), 8.47 (s, 1H, CH_{Pyr}). ^{13}C NMR (DMSO-d_6) δ: 14.00 (CH_3), 15.27 (CH_{3Pyr}), 21.98 (CH_2), 22.14 ($CH_2\underline{C}H_2CH_2O$), 24.38 ($CH_2$), 24.57 (($\underline{C}H_3)_2C$), 28.54 ($CH_2$), 28.76 ($CH_2$), 28.77 ($CH_2$), 28.95 ($CH_2$), 29.04 ($CH_2$), 31.33 ($CH_2$), 33.40 ($CH_2$), 48.95 ($CH_3N^+$), 59.10 ($CH_2$), 60.16 ($CH_2$), 60.85 ($CH_2$), 61.63 ($CH_2$), 101.60 (($CH_3)_2\underline{C}$), 121.13 ($C_{Pyr}$), 135.18 ($C_{Pyr}$), 138.21 ($C_{Pyr}$), 145.44 ($C_{Pyr}$), 147.77 ($C_{Pyr}$), 172.93 (C=O). ESI-HRMS m/z: 477.3695 [M-*Cl*]$^+$ (calculated for $[C_{28}H_{49}N_2O_4]^+$ - 477.3687).

4.1.3.26. 5-(Methylene(N,N-dimethyl-N-(3-myristoyloxypropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9e)**

The reaction was carried out following the general procedure with compound **5a** (0.34 g, 1.3 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol) and compound **8d** (0.41 g, 1.3 mmol). Yield 73% (0.52 g); beige solid; mp 148-150 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.24-1.30 (s, 20H, $10CH_2$), 1.51 (s, 6H, ($\underline{CH_3}$)$_2$C), 1.54-1.57 (m, 2H, CH_2), 2.24 (br s, 2H, $CH_2\underline{CH_2}CH_2O$), 2.31 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.38 (s, 6H, $(CH_3)_2N^+$), 3.75-3.79 (m, 2H, CH_2), 4.20 (t, 2H, $^3J_{HH}$ = 5.4 Hz, CH_2), 5.02 (s, 2H, CH_2), 5.17 (s, 2H, CH_2), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.26 (CH_3), 19.03 (CH_{3Pyr}), 22.81 (CH_2), 22.95 ($CH_2\underline{C}H_2CH_2$), 24.92 (($\underline{C}H_3)_2C$), 25.00 (CH_2), 29.27 (CH_2), 29.41 (CH_2), 29.48 (CH_2), 29.62 (CH_2), 29.78 (CH_2), 32.04 (CH_2), 34.13 (CH_2), 49.88 (CH_3N^+), 59.70 (CH_2), 60.55 (CH_2), 61.14 (CH_2), 62.62 (CH_2), 100.61 (($CH_3)_2\underline{C}$), 117.02 (C_{Pyr}), 128.87 (C_{Pyr}), 144.07 (C_{Pyr}), 146.90 (C_{Pyr}), 151.27 (C_{Pyr}), 173.78 (C=O). ESI-HRMS m/z: 505.4005 [M-Cl]$^+$ (calculated for $[C_{30}H_{53}N_2O_4]^+$ - 505.4000).

4.1.3.27. *5-(Methylene(N,N-dimethyl-N-(3-palmitoyloxypropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride* ***(9f)***

The reaction was carried out following the general procedure with compound **5a** (0.32 g, 1.2 mmol), $NaHCO_3$ (0.12 g, 1.4 mmol) and compound **8f** (0.40 g, 1.2 mmol). Yield 40% (0.27 g); beige solid; mp 141-145 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.6 Hz, $\underline{CH_3}C_{14}H_{28}$), 1.21-1.31 (m, 24H, $12CH_2$), 1.52 (s, 6H, $(CH_3)_2C$), 1.52 - 1.57 (m, 2H, CH_2), 2.24 (br s, 2H, $CH_2\underline{CH_2}CH_2O$), 2.31 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.38 (s, 6H, $(CH_3)_2N^+$), 3.76-3.80 (m, 2H, CH_2), 4.20 (t, 2H, $^3J_{HH}$ = 5.3 Hz, CH_2), 5.02 (s, 2H, CH_2), 5.17 (s, 2H, CH_2), 8.15 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.27 (CH_3), 19.04 (CH_{3Pyr}), 22.82 (CH_3), 22.93 ($CH_2\underline{C}H_2CH_2O$), 24.92 (($\underline{C}H_3)_2C$), 24.99 ($CH_2$), 29.28 ($CH_2$), 29.43 ($CH_2$), 29.49 ($CH_2$), 29.64 ($CH_2$), 29.83 ($CH_2$), 32.05 ($CH_2$), 34.13 ($CH_2$), 49.91 ($CH_3N^+$), 59.64 ($CH_2$), 60.58 ($CH_2$), 61.16 ($CH_2$), 62.65 ($CH_2$), 100.59 (($CH_3)_2\underline{C}$), 117.01 ($C_{Pyr}$), 128.81 ($C_{Pyr}$), 144.09 ($C_{Pyr}$), 146.88 ($C_{Pyr}$), 151.29 ($C_{Pyr}$), 173.81 (C=O). ESI-HRMS m/z: 533.4318 [M-Cl]$^+$ (calculated for $[C_{32}H_{57}N_2O_4]^+$ - 533.4313).

4.1.3.28. 5-(Methylene(N,N-dimethyl-N-(2-lauroyloxyethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9g)**

The reaction was carried out following the general procedure with compound **5b** (0.44 g, 1.6 mmol), $NaHCO_3$ (0.13 g, 1.4 mmol) and compound **8a** (0.44 g, 1.6 mmol). Yield 45% (0.37 g); beige solid; mp 123-125 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.95 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.21-1.27 (m, 16H, $8CH_2$), 1.44-1.55 (m, 4H, $2CH_2$), 1.74-1.78 (m, 2H, CH_2), 2.28 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.36 (s, 3H, CH_3N^+), 3.39 (s, 3H, CH_3N^+), 4.24-4.26 (m, 2H, CH_2), 4.58-4.60 (m, 2H, CH_2N^+), 5.10 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.12, 5.43 (AB, 2H, $^2J_{HH}$ = -16.8 Hz, CH_2), 5.15, 5.22 (AB, 2H, $^2J_{HH}$ = -13.6 Hz, CH_2), 8.24 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.00 ($\underline{C}H_3CH_2CH_2$), 14.19 (CH_3), 16.89 ($CH_3\underline{C}H_2CH_2$), 18.59 (CH_{3Pyr}), 22.74 (CH_2), 24.69 (CH_2), 29.17 (CH_2), 29.31 (CH_2), 29.40 (CH_2), 29.52 (CH_2), 29.66 (CH_2), 31.96 (CH_2), 34.07 (CH_2), 36.28 ($CH_3CH_2\underline{C}H_2$), 50.07 (CH_3N^+), 50.18 (CH_3N^+), 57.65 (CH_2), 62.67 (CH_2), 63.38 (CH_2), 65.63 (CH_2), 100.53 ($CH_3CH_2CH_2\underline{C}H$), 117.61 ($C_{Pyr}$), 130.98 ($C_{Pyr}$), 144.45 ($C_{Pyr}$),

148.43 (C_{Pyr}), 150.36 (C_{Pyr}), 172.85 (C=O). ESI-HRMS m/z: 477.3692 $[M\text{-}Cl]^+$ (calculated for $[C_{28}H_{49}N_2O_4]^+$ - 477.3687).

4.1.3.29. 5-(Methylene(N,N-dimethyl-N-(2-myristoyloxyethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9h)**

The reaction was carried out following the general procedure with compound **5b** (0.28 g, 1.0 mmol), $NaHCO_3$ (0.08 g, 1.4 mmol) and compound **8c** (0.31 g, 1.0 mmol). Yield 39% (0.22 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{12}H_{24}$), 0.98 (t, 3H, $^3J_{HH}$ = 7.3 Hz, $\underline{CH_3}CH_2CH_2$), 1.23-1.30 (m, 20H, $10CH_2$), 1.47-1.59 (m, 4H, $2CH_2$), 1.78-1.82 (m, 2H, CH_2), 2.31 (t, 2H, $^3J_{HH}$ = 7.5 Hz, $CH_2C(O)$), 2.42 (s, 3H, CH_{3Pyr}), 3.35 (br s, 6H, $(CH_3)_2N^+$), 4.25 (br s, 2H, CH_2), 4.61 (br s, 2H, CH_2), 5.09-5.24 (m, 4H, $2CH_2$+CH), 5.40-5.43 (m, 1H, CH_2), 8.19 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.06 ($\underline{C}H_3CH_2CH_2$), 14.25 (CH_3), 16.97 ($CH_3\underline{C}H_2CH_2$), 18.94 (CH_{3Pyr}), 22.82 (CH_2), 24.76 (CH_2), 29.23 (CH_2), 29.37 (CH_2), 29.48 (CH_2), 29.59 (CH_2), 29.73 (CH_2), 29.77 (CH_2), 32.04 (CH_2), 34.14 (CH_2), 36.37 ($CH_3CH_2\underline{C}H_2$), 50.18 (CH_3N^+), 50.32 (CH_3N^+), 57.73 (CH_2), 62.89 (CH_2), 63.77 (CH_2), 65.69 (CH_2), 100.56 ($CH_3CH_2CH_2\underline{C}H$), 117.20 ($C_{Pyr}$), 130.42 ($C_{Pyr}$), 144.96 ($C_{Pyr}$), 148.38 ($C_{Pyr}$), 150.94 ($C_{Pyr}$), 172.92 (C=O). ESI-HRMS m/z: 505.4005 $[M\text{-}Cl]^+$ (calculated for $[C_{30}H_{53}N_2O_4]^+$ - 505.4000).

4.1.3.30. 5-(Methylene(N,N-dimethyl-N-(2-palmitoyloxyethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9i)**

The reaction was carried out following the general procedure with compound **5b** (0.22 g, 0.8 mmol), $NaHCO_3$ (0.07 g, 0.8 mmol) and compound **8e** (0.26 g, 0.8 mmol). Yield 53% (0.24 g); beige solid; mp 122-127 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.98 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2$), 1.22-1.30 (m, 24H, $12CH_2$), 1.50-1.60 (m, 4H, $2CH_2$), 1.79-1.84 (m, 2H, CH_2), 2.32 (t, 2H, $^3J_{HH}$ = 7.4 Hz, $CH_2C(O)$), 2.43 (s, 3H, CH_{3Pyr}), 3.35 (s, 3H, CH_3N^+), 3.38 (s, 3H, CH_3N^+), 4.27 (br s, 2H, CH_2), 4.61 (br s, 2H, CH_2), 5.03-5.19 (m, 4H, $2CH_2$+CH), 5.41-5.45 (m, 1H, CH_2), 8.19 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.07 ($\underline{C}H_3CH_2CH_2$), 14.27 (CH_3), 16.98 ($CH_3\underline{C}H_2CH_2$), 18.93 (CH_{3Pyr}), 22.83 (CH_2), 24.77 (CH_2), 29.24 (CH_2), 29.38 (CH_2), 29.50 (CH_2), 29.60 (CH_2), 29.75 (CH_2), 29.79 (CH_2), 29.83 (CH_2), 32.06 (CH_2), 34.16 (CH_2), 36.37 ($CH_3CH_2\underline{C}H_2$), 50.20 (CH_3N^+), 50.35 (CH_3N^+), 57.69 (CH_2), 62.94 (CH_2), 63.80 (CH_2), 65.71 (CH_2), 100.61 ($CH_3CH_2CH_2\underline{C}H$), 117.17 ($C_{Pyr}$), 130.52 ($C_{Pyr}$), 144.85 ($C_{Pyr}$), 148.44 ($C_{Pyr}$), 150.98 ($C_{Pyr}$), 172.91 (C=O). ESI-HRMS m/z: 533.4318 $[M\text{-}Cl]^+$ (calculated for $[C_{32}H_{57}N_2O_4]^+$- 533.4313).

4.1.3.31. 5-(Methylene(N,N-dimethyl-N-(3-lauroyloxypropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9j)**

The reaction was carried out following the general procedure with compound **5b** (0.39 g, 1.4 mmol), $NaHCO_3$ (0.12 g, 1.4 mmol) and compound **8a** (0.40 g, 1.4 mmol). Yield 42% (0.31 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.97 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.23-1.29 (m, 16H, $8CH_2$), 1.46-1.55 (m, 4H, $2CH_2$), 1.74 - 1.82 (m, 2H, CH_2), 2.18-2.26 (m, 2H, $CH_2\underline{CH_2}CH_2O$), 2.29 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.44 (s, 3H, CH_{3Pyr}), 3.36 (s, 3H, CH_3N^+), 3.40 (s, 3H, CH_3N^+), 3.73-3.79 (m, 2H, CH_2), 4.14-4.19 (m, 2H, CH_2N^+), 5.14, 5.46 (AB, $^2J_{HH}$ = -16.8 Hz, 2H, CH_2), 5.07, 5.12 (AB, $^2J_{HH}$ = -13.4 Hz, 2H, CH_2), 5.12 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 8.25 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.03 ($\underline{C}H_3CH_2CH_2$), 14.23 (CH_3), 16.92 ($CH_3\underline{C}H_2CH_2$), 18.38 (CH_{3Pyr}), 22.78 (CH_2), 22.94 ($CH_2\underline{C}H_2CH_2O$), 24.90 ($CH_2$), 29.25 ($CH_2$), 29.40 ($CH_2$), 29.44 ($CH_2$), 29.60 ($CH_2$), 29.72 ($CH_2$), 32.00 ($CH_2$), 34.12 ($CH_2$), 36.30 ($CH_2$), 49.85 ($CH_3N^+$), 49.97 ($CH_3N^+$), 60.52 ($CH_2$), 61.22 ($CH_2$), 62.41 ($CH_2$), 65.64 ($CH_2$), 100.66 ($CH_3CH_2CH_2\underline{C}H$), 117.97 ($C_{Pyr}$), 131.52 ($C_{Pyr}$), 143.74 ($C_{Pyr}$), 148.64 ($C_{Pyr}$), 150.12 ($C_{Pyr}$), 173.80 (C=O). ESI-HRMS m/z: 491.3849 $[M\text{-}Cl]^+$ (calculated for $[C_{29}H_{51}N_2O_4]^+$ - 491.3843).

4.1.3.32. 5-(Methylene(N,N-dimethyl-N-(3-myristoyloxypropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9k)**

The reaction was carried out following the general procedure with compound **5b** (0.47 g, 1.7 mmol), $NaHCO_3$ (0.14 g, 1.7 mmol) and compound **8d** (0.52 g, 1.7 mmol). Yield 60% (0.55 g); beige solid; mp 150-153 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{12}H_{24}$), 0.98 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.24-1.29 (m, 20H, $10CH_2$), 1.47-1.59 (m, 4H, $2CH_2$), 1.77-1.83 (m, 2H, CH_2), 2.23 (br s, 2H, $CH_2\underline{CH_2}CH_2O$), 2.30 (t, 2H, , $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.45 (s, 3H, CH_{3Pyr}), 3.36 (s, 3H, CH_3N^+), 3.40 (s, 3H, CH_3N^+), 3.69-3.82 (m, 2H, CH_2), 4.18 (br s, 2H, CH_2), 5.05, 5.13 (AB, 2H, $^2J_{HH}$ = -12.9 Hz, CH_2), 5.11 (t, 1H, $^3J_{HH}$ = 5.2 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.13, 5.46 (AB, 2H, $^2J_{HH}$ = -16.6 Hz, CH_2), 8.23 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.05 ($\underline{C}H_3CH_2CH_2$), 14.25 (CH_3), 16.95 ($CH_3\underline{C}H_2CH_2$), 18.48 (CH_{3Pyr}), 22.81 (CH_2), 22.98 ($CH_2\underline{C}H_2CH_2O$), 24.93 ($CH_2$), 29.28 ($CH_2$), 29.42 ($CH_2$), 29.48 ($CH_2$), 29.64 (s ,$CH_2$), 29.77 ($CH_2$), 32.04 ($CH_2$), 34.15 ($CH_2$), 36.33 ($CH_2$), 49.90 ($CH_3N^+$), 50.00 ($CH_3N^+$), 60.53 ($CH_2$), 61.29 ($CH_2$), 62.54 ($CH_2$), 65.65 ($CH_2$), 100.68 ($CH_3CH_2CH_2\underline{C}H$), 117.85 ($C_{Pyr}$), 131.36 ($C_{Pyr}$), 143.89 ($C_{Pyr}$), 148.63 ($C_{Pyr}$), 150.30 ($C_{Pyr}$), 173.83 (C=O). ESI-HRMS m/z: 519.4162 $[M\text{-}Cl]^+$ (calculated for $[C_{31}H_{55}N_2O_4]^+$ - 519.4156).

4.1.3.33. 5-(Methylene(N,N-dimethyl-N-(3-palmitoyloxypropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9l)**

The reaction was carried out following the general procedure with compound **5b** (0.31 g, 1.1 mmol), $NaHCO_3$ (0.09 g, 1.1 mmol) and compound **8f** (0.37 g, 1.1 mmol). Yield 48% (0.30 g); beige solid; mp 130 - 135 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.6 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.98 (t, 3H, $^3J_{HH}$ = 7.3 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.24 - 1.30 (br s, 24H, $12CH_2$), 1.49 - 1.57 (m, 4H, $2CH_2$), 1.78 - 1.83 (m, 2H, CH_2), 2.18 - 2.25 (m, 2H, $CH_2\underline{CH_2}CH_2O$), 2.31 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.42 (s, 3H, CH_{3Pyr}), 3.35 (s, 3H, CH_3N^+), 3.39 (s, 3H, CH_3N^+), 3.72 - 3.77 (m, 2H, CH_2), 4.19 (br s, 2H, CH_2), 4.99, 5.11 (AB, 2H, $^2J_{HH}$ = - 13.2 Hz, CH_2), 5.09 (t, 1H, $^3J_{HH}$ = 5.2 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.10, 5.43 (AB, 2H, $^2J_{HH}$ = - 16.1 Hz, CH_2), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.06 ($\underline{C}H_3CH_2CH_2$), 14.26 (CH_3), 16.98 ($CH_3\underline{C}H_2CH_2$), 18.90 (CH_{3Pyr}), 22.82 (CH_2), 22.99 ($CH_2\underline{C}H_2CH_2O$), 24.94 ($CH_2$), 29.29 (s ,$CH_2$), 29.43 ($CH_2$), 29.49 ($CH_2$), 29.64 ($CH_2$), 29.83 ($CH_2$), 32.05 ($CH_2$), 34.15 ($CH_2$), 36.37 ($CH_2$), 49.90 ($CH_3N^+$), 49.98 ($CH_3N^+$), 60.51 ($CH_2$), 61.20 ($CH_2$), 62.73 ($CH_2$), 65.64 ($CH_2$), 100.58 ($CH_3CH_2CH_2\underline{C}H$), 117.33 ($C_{Pyr}$),130.47 ($C_{Pyr}$), 144.63 ($C_{Pyr}$), 148.39 ($C_{Pyr}$), 150.86 ($C_{Pyr}$), 173.82, (C=O). ESI-HRMS m/z: 547.4475 $[M\text{-}Cl]^+$ (calculated for $[C_{33}H_{59}N_2O_4]^+$ - 547.4469).

4.1.3.34. 5-(Methylene(N,N-dimethyl-N-(3-myristoyloxypropyl)ammonium))-2-pentyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9m)**

The reaction was carried out following the general procedure with compound **5c** (0.21 g, 0.7 mmol), $NaHCO_3$ (0.06 g, 0.7 mmol) and compound **8d** (0.23 g, 0.7 mmol). Yield 70% (0.70 g); beige solid; mp 143-147 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 7.0 Hz, CH_3), 0.89 (t, 3H, $^3J_{HH}$ = 7.4 Hz, CH_3), 1.23-1.27 (m, 20H, $10CH_2$), 1.30-1.33 (m, 4H, $2CH_2$), 1.44-1.55 (m, 4H, $2CH_2$), 1.75-1.83 (m, 2H, CH_2), 2.18-2.25 (m, 2H, $CH_2\underline{CH_2}CH_2$), 2.29 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.40 (s, 3H, CH_{3Pyr}), 3.34 (s, 3H, CH_3N^+), 3.38 (s, 3H, CH_3N^+), 3.63 - 3.79 (m, 2H, CH_2), 4.17 (t, 2H, $^3J_{HH}$ = 6.0 Hz, CH_2), 4,98, 5.07 (AB, $^2J_{HH}$ = 13.6 Hz, 2H, CH_2), 5.07, 5.40 (AB, $^2J_{HH}$ = 16.4 Hz, 2H, CH_2), 5.06 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3(CH_2)_4\underline{CH}$), 8.16 (t, 1H, $^3J_{HH}$ = 5.1 Hz, NH), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.09 (CH_3), 14.23 (CH_3), 18.90 (CH_{3Pyr}), 22.63 (CH_2), 22.79 ($CH_2\underline{C}H_2CH_2O$), 22.93 ($CH_2$), 23.24 ($CH_2$), 24.90 ($CH_2$), 29.26 ($CH_2$), 29.41 ($CH_2$), 29.46 ($CH_2$), 29.62 ($CH_2$), 29.75 ($CH_2$), 29.77 ($CH_2$), 31.63 ($CH_2$), 32.02 ($CH_2$), 34.11 ($CH_2$), 34.29 ($CH_2$), 49.86 ($CH_3N^+$), 49.92 ($CH_3N^+$), 60.52 ($CH_2$), 61.02 ($CH_2$), 62.60 ($CH_2$), 65.57 ($CH_2$), 100.68 ($CH_3(CH_2)_4\underline{C}H$), 117.35 ($C_{Pyr}$), 130.32 ($C_{Pyr}$), 144.72 ($C_{Pyr}$), 148.28 ($C_{Pyr}$), 150.76 ($C_{Pyr}$), 173.79 (C=O). ESI-HRMS m/z: 547.4475 $[M\text{-}Cl]^+$ (calculated for $[C_{33}H_{59}N_2O_4]^+$ - 547.4469).

4.1.3.35. 5-(Methylene(N,N-dimethyl-N-(3-myristoyloxypropyl)ammonium))-2-octyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(9n)**

The reaction was carried out following the general procedure with compound **5d** (0.21 g, 0.6 mmol), $NaHCO_3$ (0.21 g, 0.6 mmol) and compound **8d** (0.20 g, 0.6 mmol). Yield 73% (0.29 g); beige solid; mp 145 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.9 Hz, CH_3), 0.87 (t, 3H, $^3J_{HH}$ = 6.2 Hz, CH_3), 1.23-1.35 (m, 30H, $15CH_2$), 1.43-1.56 (m, 4H, $2CH_2$), 1.74-1.85 (m, 2H, CH_2), 2.20-2.25 (m, 2H, $CH_2\underline{CH_2}CH_2O$), 2.30 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.41 (s, 3H, CH_{3Pyr}), 3.35 (s, 3H, CH_3N^+), 3.39 (s, 3H, CH_3N^+), 3.65-3.81 (m, 2H, CH_2), 4.18 (t, 2H, $^3J_{HH}$ = 5.5 Hz, CH_2), 5.00, 5,09 (AB, $^2J_{HH}$ = - 13.8 Hz, 2H, CH_2), 5.06 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3(CH_2)_7\underline{CH}$), 5.08, 5.41 (AB, $^2J_{HH}$ = - 16.6 Hz, 2H, CH_2), 8.16 (s, 1H, CH_{Pyr}). ^{13}C NMR ($CDCl_3$) δ: 14.24 (CH_3), 18.91 (CH_{3Pyr}), 22.80 (CH_2), 22.95 ($CH_2\underline{C}H_2CH_2O$), 23.59 ($CH_2$), 24.92 ($CH_2$), 29.28 ($CH_2$), 29.36 ($CH_2$), 29.42 ($CH_2$), 29.48 ($CH_2$), 29.52 ($CH_2$) 29.60 ($CH_2$), 29.64 ($CH_2$), 29.77 ($CH_2$), 29.79 ($CH_2$), 31.99 ($CH_2$), 32.03 ($CH_2$), 34.12 ($CH_2$), 34.36 ($CH_2$), 49.87 ($CH_3N^+$), 49.92 ($CH_3N^+$), 60.51 ($CH_2$), 61.06 ($CH_2$), 62.64 ($CH_2$), 65.59 ($CH_2$), 100.72 ($CH_3(CH_2)_7\underline{C}H$), 117.34 ($C_{Pyr}$), 130.36 ($C_{Pyr}$), 144.68 ($C_{Pyr}$), 148.31 ($C_{Pyr}$), 150.78 ($C_{Pyr}$), 173.80 (C=O). ESI-HRMS m/z: 588.4944 $[M-Cl]^+$ (calculated for $[C_{36}H_{65}N_3O_4]^+$ - 588.4939).

4.1.4. 5-Hydroxymethyl-6-chloromethyl-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin **(11)**

Triphenylphosphine (1.21 g, 4.6 mmol) and NCS (0.61 g, 4.6 mmol) were added portionwise to a solution of compound **10** (1.10 g, 4.6 mmol) in 50 ml of dichloromethane, then the reaction mixture was stirred at room temperature for 0.5 h., concentrated under vacuum and purified by column chromatography [eluent petroleum ether/diethyl ether = 1:2]. Yield 54% (0.64 g); white solid; mp 120-121 °C. ^{1}H NMR ($CDCl_3$) δ: 1.54 (s, 6H, $2CH_3$), 2.37 (s, 3H, CH_{3Pyr}), 4.67 (s, 2H, CH_2), 4.73 (s, 2H, CH_2), 4.97 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ: 18.38 (CH_{3Pyr}), 24.85 ($(\underline{C}H_3)_2C$), 45.43 (CH_2Cl), 57.83 (CH_2O), 58.94 (CH_2O), 100.03 ($(CH_3)_2\underline{C}$), 127.41 (C_{Pyr}), 128.29 (C_{Pyr}), 144.51 (C_{Pyr}), 146.63 (C_{Pyr}), 147.50 (C_{Pyr}). ESI-HRMS m/z: 258.0897 $[M+H]^+$ (calculated for $[C_{12}H_{17}ClNO_3]^+$ - 258.0891).

4.1.5. 5-Hydroxymethyl-6-chloromethyl-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin **(14)**

Triphenylphosphine (2.58 g, 9.9 mmol) and NCS (1.31 g, 9.9 mmol) were added portionwise to a solution of compound **14** (2.50 g, 9.9 mmol) in 50 ml of dichloromethane, then the reaction mixture was stirred at room temperature for 0.5 h., concentrated under vacuum and purified by column chromatography [eluent petroleum ether/diethyl ether = 1:2]. Yield 44% (1.18 g); white solid; mp 115-116 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 1.01 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2$), 1.52-1.62 (m, 2H, $CH_3\underline{CH_2}CH_2$), 1.82-1.90 (m, 2H, $CH_3CH_2\underline{CH_2}$), 2.43 (s, 3H, CH_{3Pyr}), 4.63, 4.71 (AB, 2H, $^3J_{HH}$ = -12.8 Hz, CH_2), 4.77, 4.80 (AB, 2H, $^2J_{HH}$ = 11.6 Hz, CH_2), 5.02 (t, 1H, $^3J_{HH}$ = 5.2 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.04, 5.09 (AB, 2H, $^2J_{HH}$ = 16.2 Hz, CH_2). ^{13}C NMR ($CDCl_3$) δ: 14.03 ($\underline{C}H_3CH_2CH_2$), 17.06 ($CH_3\underline{C}H_2CH_2$), 18.11 (CH_{3Pyr}), 36.31 ($CH_3CH_2\underline{C}H_2$), 45.08 (CH_2Cl), 57.98 (CH_2O), 64.62 (CH_2O), 100.08 ($CH_3CH_2CH_2\underline{C}H$), 128.84 ($C_{Pyr}$), 129.69 ($C_{Pyr}$), 144.91 ($C_{Pyr}$), 147.00 ($C_{Pyr}$), 148.53 ($C_{Pyr}$). ESI-HRMS m/z: 272.1053 $[M+H]^+$ (calculated for $[C_{13}H_{19}ClNO_3]^+$ - 272.1048).

4.1.6. *General procedure for preparation of quaternary ammonium salts* ***6a-u***, ***9a-n***, ***12a-d***, ***15a-d***.

Compounds **3b**,**f, 8c,d** (1 equiv) were added to a solution of compound **11** or **14** (1 equiv) in 20 ml of ethanol. The reaction mixture was heated at 70 °C for 8 h. and the solvent was evaporated under reduced pressure. The product was recrystallized from acetone (compounds **12c, 12d, 15c, 15d**) or purified by column chromatography (compound **12a, 12b, 15a, 15b**) on reversed-fase column (eluent isopropanol/water = from 0:100 to 100:0).

4.1.6.1. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(2-myristoylaminoethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(12a)**

The reaction was carried out following the general procedure with compound **11** (0.20 g, 0.8 mmol) and compound **3b** (0.23 g, 0.8 mmol). Yield 42% (0.18 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^{3}J_{HH}$ = 7.0 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.20- 1.29 (m, 20H, $10CH_2$), 1.52 (s, 6H, $(CH_3)_2C$), 1.53-1.57 (m, 2H, CH_2), 2.20 (t, 2H, $^{3}J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.33 (s, 3H, CH_{3Pyr}), 3.32 (s, 6H, $(CH_3)_2N^+$), 3.78-3.82 (m, 2H, CH_2), 3.84-3.88 (m, 2H, CH_2), 4.63 (s, 2H, CH_2), 4.86 (s, 2H, CH_2), 4.95 (s, 2H, CH_2), 8.40 (t, 1H, $^{3}J_{HH}$ = 5.0 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 14.20 (CH_3), 18.68 (CH_{3Pyr}), 22.77 (CH_2), 24.85 (($\underline{C}H_3)_2C$), 25.63 (CH_2), 29.44 (CH_2), 29.49 (CH_2), 29.55 (CH_2), 29.65 (CH_2), 29.75 (CH_2), 29.77 (CH_2), 32.00 (CH_2), 34.28 (CH_2), 36.38 (CH_2NH), 52.12 (CH_3N^+), 55.83 (CH_2), 59.08 (CH_2), 64.49 (CH_2), 100.25 (($CH_3)_2\underline{C}$), 127.12 (C_{Pyr}), 132.12 (C_{Pyr}), 136.92 (C_{Pyr}), 146.99 (C_{Pyr}), 147.47 (C_{Pyr}), 174.84 (C=O). ESI-HRMS m/z: 520.4109 $[M-Cl]^+$ (calculated for $[C_{30}H_{54}N_3O_4]^+$ - 520.4114). HPLC analysis: retention time 6.3 min; purity 98.2%.

4.1.6.2. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(2-myristoyloxyethyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(12b)**

The reaction was carried out following the general procedure with compound **11** (0.20 g, 0.8 mmol) and compound **8c** (0.23 g, 0.8 mmol). Yield 40% (0.17 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^{3}J_{HH}$ = 6.9 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.22-1.28 (m, 20H, $10CH_2$), 1.52 (s, 6H, $(CH_3)_2C$), 1.54-1.58 (m, 2H, CH_2), 2.30 (t, 2H, $^{3}J_{HH}$ = 7.7 Hz, $CH_2C(O)$), 2.32 (s, 3H, CH_{3Pyr}), 3.41 (s, 6H, $(CH_3)_2N^+$), 4.09-4.12 (m, 2H, CH_2), 4.63-4.65 (m, 4H, $2CH_2$), 4.63 (s, 2H, CH_2), 4.94 (s, 2H, CH_2), 4.97 (s, 2H, CH_2), 5.82 (br s, 1H, $CH_2\underline{OH}$). ^{13}C NMR ($CDCl_3$) δ: 14.20 (CH_3), 18.67 (CH_{3Pyr}), 22.76 (CH_2), 24.76 (($\underline{C}H_3)_2C$), 24.86 (CH_2), 29.19 (CH_2), 29.33 (CH_2), 29.42 (CH_2), 29.53 (CH_2), 29.68 (CH_2), 29.72 (CH_2), 29.75 (CH_2), 31.99 (CH_2), 34.14 (CH_2), 52.47 (CH_3N^+), 55.56 (CH_2), 58.03 (CH_2), 59.15 (CH_2), 63.52 (CH_2), 64.45 (CH_2), 100.21 (($CH_3)_2\underline{C}$), 127.21 (C_{Pyr}), 132.66 (C_{Pyr}), 137.08 (C_{Pyr}), 146.95 (C_{Pyr}), 147.22 (C_{Pyr}), 173.00 (C=O). ESI-HRMS m/z: 521.3954 $[M-Cl]^+$ (calculated for $[C_{30}H_{53}N_2O_5]^+$ - 521.3949).

4.1.6.3. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(3-myristoylaminopropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(12c)**

The reaction was carried out following the general procedure with compound **11** (0.20 g, 0.8 mmol) and compound **3f** (0.24 g, 0.8 mmol). Yield 56% (0.24 g); white solid; mp 135 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.83 (t, 3H, $^{3}J_{HH}$ = 7.1 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.19-1.26 (s, 20H, $10CH_2$), 1.51 (s, 6H, $(CH_3)_2C$), 1.53-1.58 (m, 2H, CH_2), 2.09-2.16 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.21 (t, 2H, $^{3}J_{HH}$ = 7.0 Hz, CH_2CO), 2.33 (s, 3H, CH_{3Pyr}), 3.24 (s, 6H, $(CH_3)_2N^+$), 3.29-3.34 (m, 2H, CH_2), 3.78-3.82 (m, 2H, CH_2), 4.60 (s, 2H, CH_2), 4.74 (s, 2H, CH_2), 4.95 (s, 2H, CH_2), 7.96 (t, 1H, $^{3}J_{HH}$ = 5.5 Hz, NH), 5.84 (br s, 1H, $CH_2\underline{OH}$). ^{13}C NMR ($CDCl_3$) δ: 14.16 (CH_3), 18.62 (CH_{3Pyr}), 22.72 (CH_2), 23.07 ($CH_2\underline{CH_2}CH_2NH$), 24.82 (($\underline{C}H_3)_2C$), 25.89 ($CH_2$), 29.40 ($CH_2$), 29.50 ($CH_2$), 29.59 ($CH_2$), 29.66 ($CH_2$), 29.70 ($CH_2$), 29.74 ($CH_2$), 31.96 ($CH_2$), 36.24 ($CH_2$), 36.41 ($CH_2$), 51.46 ($CH_3N^+$), 55.64 ($CH_2$), 59.08 ($CH_2$), 64.09 ($CH_2$), 64.38 ($CH_2$), 100.25 (($CH_3)_2\underline{C}$), 127.05 ($C_{Pyr}$), 132.40 ($C_{Pyr}$), 136.93 ($C_{Pyr}$), 147.01 ($C_{Pyr}$), 147.40 ($C_{Pyr}$), 174.65 (C=O). ESI-HRMS m/z: 534.4271 $[M-Cl]^+$ (calculated for $[C_{31}H_{56}N_3O_4]^+$ - 534.4265).

4.1.6.4. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(3-myristoyloxypropyl)ammonium))-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(12d)**

The reaction was carried out following the general procedure with compound **11** (0.25 g, 1.0 mmol) and compound **8d** (0.30 g, 1.0 mmol). Yield 41% (0.23 g); white solid; mp 117-120 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.83 (t, 3H, $^{3}J_{HH}$ = 6.9 Hz, $\underline{CH_3}C_{12}H_{24}$), 1.21-1.26 (m, 20H, $10CH_2$), 1.51 (s, 6H, $(CH_3)_2C$), 1.52-1.56 (m, 2H, CH_2), 2.25-2.30 (m, 4H, $2CH_2$), 2.32 (s, 3H, CH_{3Pyr}), 3.36 (s, 6H, $(CH_3)_2N^+$), 3.75 (br s, 2H, CH_2), 4.17 (br s, 2H, CH_2), 4.63 (br s, 2H, CH_2), 4.86 (br s, 2H, CH_2), 4.96 (s, 2H, CH_2), 5.90 (br s, 1H, $CH_2\underline{OH}$). ^{13}C NMR ($CDCl_3$) δ: 14.15 (CH_3), 18.64 (CH_{3Pyr}), 22.71 (CH_2), 22.87 ($CH_2\underline{C}H_2CH_2O$), 24.82 (($\underline{C}H_3)_2C$), 24.88 ($CH_2$), 29.20 ($CH_2$), 29.33 ($CH_2$), 29.38 ($CH_2$), 29.52 ($CH_2$), 29.68 ($CH_2$), 29.71 ($CH_2$), 31.94 ($CH_2$), 34.12

(CH_2), 51.91 (CH_3N^+), 55.44 (CH_2), 59.14 (CH_2), 60.85 (CH_2), 62.55 (CH_2), 62.96 (CH_2), 100.16 (($CH_3)_2\underline{C}$), 127.11 (C_{Pyr}), 132.72 (C_{Pyr}), 137.16 (C_{Pyr}), 146.89 (C_{Pyr}), 147.06 (C_{Pyr}), 173.64 (C=O). ESI-HRMS m/z: 535.4105 $[M\text{-}Cl]^+$ (calculated for $[C_{30}H_{53}N_2O_5]^+$ - 535.4111).

4.1.6.5. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(2-myristoylaminoethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(15a)**

The reaction was carried out following the general procedure with compound **13** (0.15 g, 0.6 mmol) and compound **3b** (0.16 g, 0.6 mmol). Yield 44% (0.14 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 3H, $^3J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.99 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.21-1.29 (m, 20H, $10CH_2$), 1.49-1.59 (m, 4H, $2CH_2$), 1.77-1.90 (m, 2H, CH_2), 2.21 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.35 (s, 3H, CH_{3Pyr}), 3.33 (s, 6H, $(CH_3)_2N^+$), 3.78-3.81 (m, 2H, CH_2), 3.85-3.88 (m, 2H, CH_2N^+), 4.57, 4.64 (AB, $^2J_{HH}$ = -13.2 Hz, 2H, CH_2), 4.86, 4.91 (AB, $^2J_{HH}$ = -14.0 Hz, 2H, CH_2), 4.98 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.02 (s, 2H, CH_2), 8.45 (t, 1H, $^3J_{HH}$ = 4.8 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 14.00 ($\underline{C}H_3CH_2CH_2$), 14.24 (CH_3), 17.00 ($CH_3\underline{C}H_2CH_2NH$), 18.65 ($CH_{3Pyr}$), 22.80 ($CH_2$), 25.64 ($CH_2$), 29.48 ($CH_2$), 29.51 ($CH_2$), 29.57 ($CH_2$), 29.67 ($CH_2$), 29.78 ($CH_2$), 29.80 ($CH_2$), 32.02 ($CH_2$), 34.34 ($CH_2$), 36.24 ($CH_3CH_2\underline{C}H_2$), 36.41 ($CH_2$), 52.22 ($CH_3N^+$), 52.33 ($CH_3N^+$), 55.72 ($CH_2$), 64.29 ($CH_2$), 64.54 ($CH_2$), 64.73 ($CH_2$), 99.98 ($CH_3CH_2CH_2\underline{C}H$), 128.79 ($C_{Pyr}$), 132.40 ($C_{Pyr}$), 137.66 ($C_{Pyr}$), 147.14 ($C_{Pyr}$), 148.64 ($C_{Pyr}$), 174.86 (C=O). ESI-HRMS m/z: 534.4271 $[M\text{-}Cl]^+$ (calculated for $[C_{31}H_{56}N_3O_4]^+$ - 534.4265).

4.1.6.6. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(2-myristoyloxyethyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(15b)**

The reaction was carried out following the general procedure with compound **13** (0.19 g, 0.6 mmol) and compound **8c** (0.21 g, 0.6 mmol). Yield 48% (0.19 g); colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^3J_{HH}$ = 6.7 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.98 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.19-1.28 (m, 20H, $10CH_2$), 1.49-1.58 (m, 4H, $2CH_2$), 1.78-1.87 (m, 2H, CH_2), 2.30 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.34 (s, 3H, CH_{3Pyr}), 3.41 (s, 6H, $2CH_3N^+$), 4.10 (br s, 2H, CH_2), 4.59, 4.68 (AB, 2H, $^2J_{HH}$ = -13.2 Hz, CH_2), 4.65 (br s, 2H, CH_2), 4.95 (s, 2H, CH_2), 4.99 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.04 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ: 13.99 ($\underline{C}H_3CH_2CH_2$), 14.23 (CH_3), 16.99 ($CH_3\underline{C}H_2CH_2$), 18.62 (CH_{3Pyr}), 22.79 (CH_2), 24.76 (CH_2), 29.21 (CH_2), 29.35 (CH_2), 29.45 (CH_2), 29.55 (CH_2), 29.70 (CH_2), 29.74 (CH_2), 29.77 (CH_2), 32.01 (CH_2), 34.14 (CH_2), 36.23 ($CH_3CH_2\underline{C}H_2$), 52.47 (CH_3N^+), 55.50 (CH_2), 58.00 (CH_2), 63.53 (CH_2), 64.34 (CH_2), 64.80 (CH_2), 99.95 ($CH_3CH_2CH_2\underline{C}H$), 128.97 ($C_{Pyr}$), 133.03 ($C_{Pyr}$), 137.69 ($C_{Pyr}$), 146.94 ($C_{Pyr}$), 148.67 ($C_{Pyr}$), 173.01 (C=O). ESI-HRMS m/z: 535.4111 $[M\text{-}Cl]^+$ (calculated for $[C_{31}H_{55}N_2O_4]^+$ - 535.4105).

4.1.6.7. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(3-myristoylaminopropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(15c)**

The reaction was carried out following the general procedure with compound **13** (0.15 g, 0.6 mmol) and compound **3f** (0.17 g, 0.6 mmol). Yield 83% (0.27 g); white solid; mp 150 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, 3H, $^3J_{HH}$ = 7.0 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.97 (t, 3H, $^3J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.19-1.28 (m, 20H, $10CH_2$), 1.48-1.57 (m, 4H, $2CH_2$), 1.79-1.85 (m, 2H, CH_2), 2.10-2.17 (m, 2H, $CH_2\underline{CH_2}CH_2NH$), 2.22 (t, 2H, $^3J_{HH}$ = 7.6 Hz, $CH_2C(O)$), 2.36 (s, 3H, CH_{3Pyr}), 3.23 (s, 3H, CH_3N^+), 3.25 (s, 3H, CH_3N^+), 3.31 - 3.35 (m, 2H, CH_2), 3.78 - 3.82 (m, 2H, CH_2), 4.56, 4.62 (AB, 2H, $^2J_{HH}$ = - 13.2 Hz, CH_2), 4.74, 4.77 (AB, 2H, $^2J_{HH}$ = -13.6 Hz, CH_2), 4.98 (t, 1H, $^3J_{HH}$ = 5.1 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.02 (s,2H, CH_2), 7.93 (t, 1H, $^3J_{HH}$ = 5.6 Hz, NH). ^{13}C NMR ($CDCl_3$) δ: 13.95 ($\underline{C}H_3CH_2CH_2$), 14.18 (CH_3), 16.96 ($CH_3\underline{C}H_2CH_2NH$), 18.50 ($CH_{3Pyr}$), 22.75 ($CH_2$), 23.10 ($CH_2\underline{C}H_2CH_2$), 25.91 ($CH_2$), 29.43 ($CH_2$), 29.53 ($CH_2$), 29.62 ($CH_2$), 29.65 ($CH_2$), 29.69 ($CH_2$), 29.74 ($CH_2$), 29.78 ($CH_2$), 31.99 ($CH_2$), 36.21 ($CH_2$), 36.44 ($CH_2$), 51.47 ($CH_3N^+$), 55.70 ($CH_2$), 64.08 ($CH_2$), 64.43 ($CH_2$), 64.75 ($CH_2$), 100.03 ($CH_3CH_2CH_2\underline{C}H$), 129.00 ($C_{Pyr}$), 132.80 ($C_{Pyr}$), 137.46 ($C_{Pyr}$), 147.11 ($C_{Pyr}$), 148.77 ($C_{Pyr}$), 174.69 (C=O). ESI-HRMS m/z: 548.4427 $[M\text{-}Cl]^+$ (calculated for $[C_{32}H_{58}N_3O_4]^+$ - 548.4422).

4.1.6.8. 5-Hydroxymethyl-6-(methylene(N,N-dimethyl-N-(3-myristoyloxypropyl)ammonium))-2-propyl-8-methyl-4H-[1,3]dioxino[4,5-c]pyridin chloride **(15d)**

The reaction was carried out following the general procedure with compound **13** (0.19 g, 0.7 mmol) and compound **8d** (0.22 g, 0.7 mmol). Yield 66% (0.27 g); white solid; mp 142-145 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, $^{3}J_{HH}$ = 6.8 Hz, $\underline{CH_3}C_{14}H_{28}$), 0.98 (t, 3H, $^{3}J_{HH}$ = 7.4 Hz, $\underline{CH_3}CH_2CH_2CH$), 1.23-1.29 (m, 20H, $10CH_2$), 1.49-1.59 (m, 4H, $2CH_2$), 1.77-1.86 (m, 2H, CH_2), 2.25-2.23 (m, 4H, $2CH_2$), 2.35 (s, 3H, CH_{3Pyr}), 3.37 (s, 6H, $2CH_3N^+$), 3.73-3.77 (m, 2H, CH_2), 4.20 (t, 2H, $^{3}J_{HH}$ = 6.0 Hz, CH_2), 4.60, 4.68 (AB, 2H, $^{2}J_{HH}$ = - 13.2 Hz, CH_2), 4.88 (s, 2H, CH_2), 4.99 (t, 1H, $^{3}J_{HH}$ = 5.2 Hz, $CH_3CH_2CH_2\underline{CH}$), 5.04 (s, 2H, CH_2), 5.93 (br s, 2H, CH_2OH). ^{13}C NMR ($CDCl_3$) δ: 13.96 ($\underline{C}H_3CH_2CH_2$), 14.19 (CH_3), 16.97 ($CH_3\underline{C}H_2CH_2$), 18.46 (CH_{3Pyr}), 22.75 (CH_2), 22.91 ($CH_2\underline{C}H_2CH_2$), 24.92 (CH_2), 29.24 (CH_2), 29.37 (CH_2), 29.42 (CH_2), 29.57 (CH_2), 29.72 (CH_2), 31.98 (CH_2), 34.15 (CH_2), 36.22 ($CH_2CH_2\underline{C}H_2$), 51.92 (CH_3N^+), 55.47 (CH_2), 60.83 (CH_2), 62.69 (CH_2), 62.84 (CH_2), 64.83 (CH_2), 100.10 ($CH_3CH_2CH_2\underline{C}H$), 129.08 ($C_{Pyr}$), 133.22 ($C_{Pyr}$), 137.64 ($C_{Pyr}$), 146.75 ($C_{Pyr}$), 148.72 ($C_{Pyr}$), 173.68 (C=O). ESI-HRMS m/z: 549.4267 $[M-Cl]^+$ (calculated for $[C_{32}H_{57}N_2O_5]^+$ - 549.4262).

4.2. Antibacterial activity

The antibacterial activity of all obtained compounds was carried out againts the following Gram-positive bacteria: methicillin sensitive *Staphylococcus aureus* ATCC®29213 (MSSA), *Micrococcus luteus* (clinical isolate), *Bacillus subtilis 168* and Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC), *Escherichia coli* (MG1655)d. Additionally antimicrobial susceptibility of compounds **6b, 6f, 6i, 6j, 9b, 9h, 9j, 12a, 12b** were tested on a number of various clinical isolates of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus intermedius, Enterococcus faecalis, Acinetobacter species, Pseudomonas species, Klebsiella species, Proteus species, E. coli., Serratia marcescens and Serratia ureilytica,* which were obtained from bacteriology laboratory of Rebublic Clinical Hospital (Kazan, Russia)

The bacterial strains were stored in 10% (V/V) glycerol stocks at -80 °C and freshly streaked on full Muller-Hinton (MH) agar plates (Sigma aldrich) and grown overnight at 37 °C before use. Fresh colony was grown overnight in MH-broth and then used to adjust an optical density of 0.5 McFarland (equivalent to 10^8 cells/mL) in 0.9% NaCl solution that was used as a working suspension. *Salmonella typhimurium* TA1535/pSK1002 was used for SOS-chromotest. *S. typhimurium* TA1535, TA1537, TA98, TA100, TA102 were used in Ames test. Bacteria were maintained and grown on the LB medium containing ampicillin at final concentration of 100 µg/mL. For the biofilm assay the previously developed BM broth (glucose 5 g, peptone 7g, $MgSO_4$× $7H_2O$ 2.0 g and $CaCl_2$× $2H_2O$ 0.05 g in 1.0 liter tap water) where all strains formed rigid biofilms in 2 days was used. For differential CFUs count of *E.coli, P.aeruginos* and *S.aureus*, the Endo-agar (Sigma aldrich), Cetrimide agar (Sigma aldrich) and Salt-mannitol agar (peptones 10g, meat extract 1 g, NaCl 75 g, D-mannitol 10 g, agar-agar 12 g in 1.0 liter tap water) were used, respectively.

The MIC of compounds was determined by the broth microdilution method in 96-well microtiter plates (Eppendorf) according to the EUCAST rules for antimicrobial susceptibility testing [30]. The bacterial culture adjusted to $3\text{-}9\times10^5$ cells/mL in the MH broth was seeded into 96-well polystyrol culture plates (Eppendorf). The concentrations of substances to be tested ranged from 0.25 to 64 µg/ml for. ATCC and 0.03 to 64 µg/ml for clinical isolate. The minimal inhibitory concentration was determined as the lowest concentration of compound for which no visible bacterial growth could be observed after 24 h of incubation.

To determine a minimum bactericidal concentration, a culture liquid from wells without visible growth was diluted a thousand-fold into new 96-well microtiter plates in fresh MH-broth and incubated for 24 h growth at 35 °C. MBC was assumed at concentrations, where no viable planktonic cells were observed.

4.3 Cytotoxicity

The cytotoxicity of compounds **6b, 6f, 6i, 6j, 9b, 9h, 9j, 12a, 12b** was evaluated on HEK 293 (human embryonic kidney), MSK (human mesenchymal stem cells) and HSF (primary human skin fibroblasts) by MTT assay. Cells were cultured in a-MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin. Cells were seeded in 96-well plates at the density of 20 000 cells per well and grown overnight at 37 °C and 5% CO_2 in humidified atmosphere. Then the medium was changed to the fresh one containing compounds to be tested in concentration of 0. 2-100 μg/mL. After 72 h of cultivation the cultural fluid was discarded and MTT solution (in Dulbecco's phosphate-buffered saline) was added to the fresh media until final concentration of 0.5 mg/mL. In 2 h. the liquid was replaced by dimethyl sulfoxide (Sigma-Aldrich, St.Louis, MO) to dissolve formazan crystals, and absorption was measured on Tecan Infinite 200Pro at 557 nm with reference 700 nm. Based on data obtained, the CC50 values (concentrations decreasing the proliferative activity by 2-fold) were calculated.

4.4. Genotoxicity

A SOS-chromotest was performed by using the *Salmonella typhimurium TA1535/pSK1002* as described by [31]. The β-galactosidase activity (OD_{405}) was measured as described in [32] with modifications [33] and normalized by the growth factor (OD_{600}). The induction ratio (IR) of SOS-response was calculated as the ratio of activity in presence of compounds and the solvent control. Mitomycin C (1 μg/ml) was used as positive control.

The Ames test was carried out using *S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537 strains was performed as described in [34]. Due to compounds toxicity to *S. typhimurium*, the spot test modification has been applied. For that, 5 μl of sample (10 mg/mL solution in water) was dropped onto 5-mm filter disk placed on the top agar surface (see Fig S). The amount of revertants was then calculated by using the in-house developed software [35]. The tested compound was considered to be mutagenic if the count of revertants in the experiment was more than 2 times higher than that in the negative control (DMSO) and increased at higher concentrations of compound [35].

4.5. In vivo toxicity

Male and female F1 C57BL/6*CBA mice (weighing 18–22 g) for acute oral toxicity study were purchased from the Federal Research Center Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences. CD-1 female mice, weighing about 34.30 ± 3.20 g between 2 and 3 months of age obtained from Nursery for laboratory animals "Pushchino", Russia were usedy for acute cutaneous toxicity study. All animals acclimatized for 2 weeks before the experiment. Animals were kept in polypropylene cages (6 animals per cage), maintained under standard conditions (12 h light and 12 h dark cycle; 22 ±3 °C and 30–70% relative humidity). They were given standard pellet diet and water *ad libitum* throughout the course of the study. Randomly, they were assigned into groups comprising 6 animals per group. Prior to treatment, animals were weighed, marked. Animals were observed two times once daily. All surviving animals were euthanized with CO_2 inhalation at the end of the study on day 14, and their vital organs were individually observed for overt pathology by necropsy. All experimental procedures were performed in accordance with the Ethical Principles in Animal Research and were approved by the Local Ethics Committee of the Kazan Federal University.

A solutions of compounds **6i** and **12a** were prepared by dissolving 1.2 g of substances by water in 10 ml volumetric flask. A solution of compounds at a concentration of 120 mg/ml made it possible to inject a dose of 2000 mg/kg in a volume of 0.5 ml per mouse weighing 30 g.

For acute oral toxicity study prior to treatment, animals were fasted for 3 h with free access to water. Compounds **6i** and **12a** were dissolved in distilled water and administered to the mice by oral gavage at dose 2000 mg/kg body weight.

For acute toxicity evaluation of compounds **6i** and **12a** after a single cutaneous administration dorsal sites of the animals were shaved (not less than 10% of the body surface) 24 hours before the study. Since the permeability of damaged and intact skin is different, skin damage is not allowed. Mice (6 per group) received 2500 mg/kg of compounds **6i** and **12a** as a suspension in distilled water. Compounds were applied in selected area with porous gauze dressing and fixed with tape for 24 h. Animals were caged individually. An equal number of mice)served as a control group, and the control animals received an equal volume of distilled water. Dosage precision was regulated by the variable volume of the drug solution administered (normalized to the animal's body weight) at a constant concentration of the solution. At the end of the exposure period, residual test substances were removed by water.

Clinical signs related with a drug-toxicity and the changes in the general behaviors of the animals were monitored and recorded every 1 h for the first 6 h after treatment, and then once daily over 14 days. Attention was given to changes in skin, fur, mucous membrane, eyes, respiration, behavior patterns, body weight, food and water consumption. Individual body weights were checked immediately before drug treatment and then at day 1, 3, 7, and 14 thereafter.

4.6. Influence of pyridoxine-based QACs on biofilm-embedded cells

To determine the minimal biofilm eradicating concentrations (MBECs) of compounds **6i** and **12a**, bacterial cells were grown in 24-well polystyrol culture plates (Eppendorf) in BM broth for 48 h until a mature biofilm had been formed. Then the broth was exchanged with the fresh one. Antimicrobials were added into wells in concentrations from 8 to 512 μg/mL. After 24 h incubation at 37 °C the number of CFUs was counted by using the drop plate assay. $MBEC_{99.9}$ was defined as the reduction of viable cells by 3 orders of magnitude.

4.7 Testing of the resistance development

The development of bacterial resistance was tested by using serial passages approach as described in [36] with modifications [37]. Briefly, sterile 96-well plates were seeded with bacterial cells at different concentrations of a test compound in a liquid medium (similar to the MIC definition as described above). Plates were incubated for 24 hours at 37 °C, then microorganisms from the last well with a visible growth (i.e. with sub-lethal concentration of a compound) were resuspended in fresh broth and used as inoculum for next seeding into liquid medium with range concentrations of antimicrobial. The procedure was repeated to obtain 14 cycles of passages and MICs of compounds were determined after each passage. Then a series of 7 passages from the last well with a visible growth on antimicrobial-free agar was done and MICs were again determined.

4.8. Membrane potential evaluation

Membrane potential was evaluated by detection of 3,3′-diethyloxacarbocyanine iodide ($DioC_2(3)$) fluorescence as previously described [38, 39]. Briefly, bacteria were grown for 24 h, harvested and washed with PBS. Cells were resuspended until final density of $1\text{-}9\times10^5$ CFU/ml in PBS supplemented with $DioC_2(3)$ until final concentration of 10 μM. After 30 min preincubation at 25 °C, compounds to be tested were added at concentrations corresponding to their respective 1×MIC and 1-4×MBCs and the fluorescence was measured for 35 min with 5-minute interval by using carboxyfluorescein (FAM) filter-set detection (The excitation and

emission wavelengths are 497 and 520 nm, respectively). The fluorescence in samples with untreated cells was considered as a baseline.

4.9. Statistics and data analysis

All experiments were performed in biological triplicates (i.e. newly prepared cultures and medium) with three repeats in each run. The data were analyzed and graphically visualized using GraphPad Prism version 6.00 for Windows [40] (GraphPad Software, USA, www.graphpad.com). Comparison against negative control using the non-parametric Kruskal–Wallis one-way analysis of variance test has been performed in each experiment. Significant differences against respective controls were considered at $p < 0.05$ and specified in the corresponding figure captions.

Declaration of competing interest

The authors declare that they have no known competingfinancial interests or personal relationships that could haveappeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at:

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Highlights

- Design and synthesis of novel "soft antimicrobials" based on quaternary ammonium pyridoxine derivatives.
- The compounds exhibited in vitro promising antibacterial activity against Gram-positive and Gram-negative bacterial strains.
- Lead compounds **6i** and **12a** exhibited promising antibacterial activity on clinical isolates, mono- and dual species bacterial biofilms.
- Lead compounds **6i** and **12a** were non mutagenic neither in SOS-chromotest nor in Ames test and non-toxic in vivo at acute oral (LD_{50} > 2000 mg/kg) and cutaneous administration (LD_{50} >2500 mg/kg) on mice

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Declarations of interest: none