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Synthesis, physicochemical properties and biological activities of novel alkylphosphocholines with foscarnet moiety

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ABSTRACT

A series of alkylphosphocholines with foscarnet moiety was synthesized. The structure of these zwitterionic amphiphiles was modified in both polar and non-polar parts of surfactant molecule. Investigations of physicochemical properties are represented by the determination of critical micelle concentration, the surface tension value at the cmc and the surface area per surfactant head group utilising surface tension measurements. Hydrodynamic diameter of surfactant micelles was determined using the dynamic light scattering technique. Alkylphosphocholines exhibit significant cytotoxic, anticandidal (*Candida albicans*) and antiamoebal (*Acanthamoeba* spp. T4 genotype) activity. The relationship between the structure, physicochemical properties and biological activity of the tested compounds revealed that lipophilicity has a significant influence on biological activity of the investigated surfactants. More lipophilic alkylphosphocholines with octadecyl chains show cytotoxic activity against cancer cells which is higher than that of the compounds with shorter alkyl chains. The opposite situation was observed in case of anticandidal and antiamoebal activity of these surfactants. The most active compounds were found to have pentadecyl chains. The foscarnet analogue of miltefosine **C15-PFA-C** showed the highest anticandidal activity. The minimum value of anticandidal activity of this compound is 1,4 μM thus representing the highest anticandidal activity found within the group of alkylphosphocholines.

1. Introduction

Alkylphosphocholines (APCs) are amphiphilic compounds with a characteristic molecular structure composed of an alkyl chain and a phosphocholine moiety. Hydrophobic and hydrophilic molecular parts can have diverse structures. The alkyl groups are mostly represented by straight hydrocarbon chains. The best known APCs are miltefosine and perifosine with their molecular structure shown in Fig. 1 [1]. The straight alkyl chains can have double bonds in their structure as well. The APCs with unsaturated docos-13-enyl chain are represented by erucylphosphocholine and erufosine (Fig. 1) [1]. The alkyl chains can be also branched [2,3], with fluorocarbon parts [3,4], cycles [5–10] or bonded to bioactive compounds [11,12]. Phosphocholine moiety is mostly composed of choline group bonded to a phosphate group. However, this part of molecule often undergoes structural changes. As the numerous examples indicate, aminium cation can be closed to a heterocycle [1,9,13,14], methyl group of choline can be exchanged for a longer alkyl chain [4,15,16] or phosphate group can be replaced with a phosphonate group [4].

The phosphocholine moiety in APCs can be prepared by several synthetic approaches. Precursors for phosphate groups can be represented by phosphoryl chloride, dimethylchlorophosphite, 2-bromoethyldichlorophosphate, 2-chloro-1,3,2-dioxaphospholane or 2-chloro-2-oxo-1,3,2-dioxaphospholane. Aminium cations in phosphocholine moiety are provided by quaternization of tertiary amines with phosphate precursors (2-bromoethyl phosphate, 2-alkyloxy-2-oxo-1,3,2-dioxolane) or aminium cations are introduced to molecules of APCs as choline salts [17]. If a phosphate group is replaced with a phosphonate group, the bond between carbon and phosphorus atoms is conventionally synthesized by the Michaelis-Arbuzov reaction. Heating

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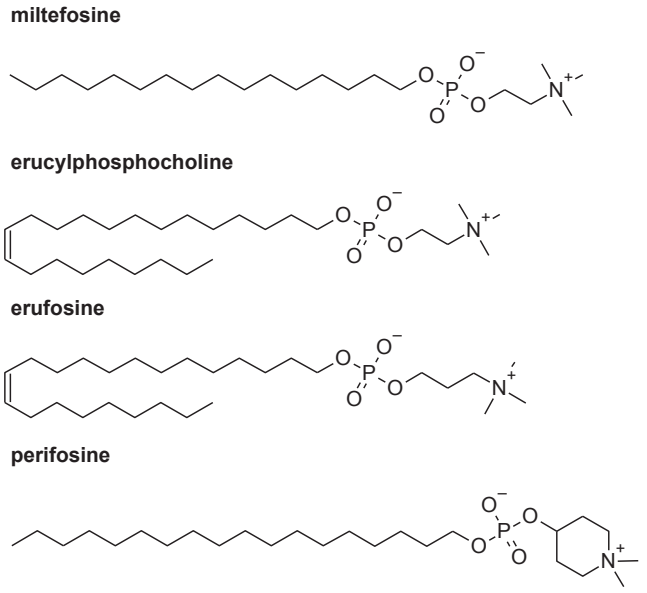


Fig. 1. Structure of well-known APCs.

of trialkyl phosphites with alkyl halogenides results in the formation of dialkyl alkylphosphonates that can be hydrolysed to free phosphonate acids [4]. The most versatile method of the preparation of APCs is depicted in Scheme 1. It provides the synthesis of compounds with the different types of alkyl chains bonded to phosphate/phosphonate groups, with the different length of the spacer between anionic and cationic part of the molecule and with various types of alkyl chains bonded to the cationic part of surfactant molecule.

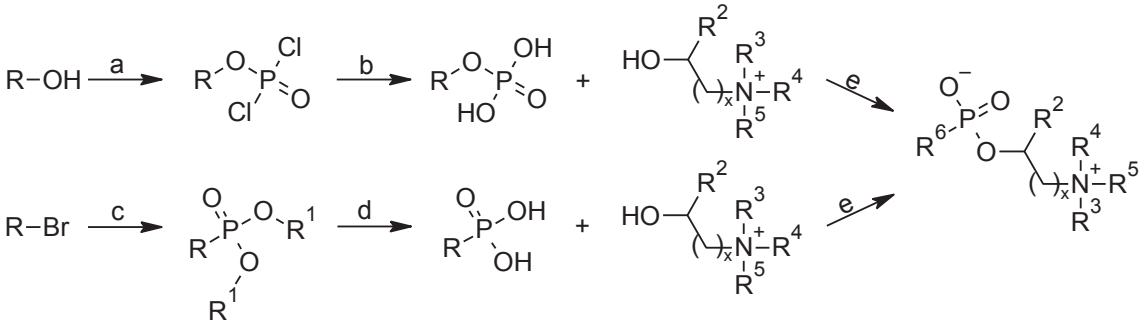
In the past, APCs were investigated as the compounds with cytotoxic activities against cancer cells [18,19]. Edelfosine, miltefosine, erucylphosphocholine and perifosine were the first candidates that were intensively studied. APCs caused cell death by either non-specific or specific action. They caused lysis of plasmatic membrane at the high concentration as indicate haemolytic activity of APCs [13]. The specific mode of action can be explained at several levels. APCs target plasmatic membranes, especially lipid rafts. They alter phospholipase C and D signalling cascades. These cascades modulate the PI3K/Akt/mTOR and RAS/RAF/MEK/ERK pathways. These changes at the subcellular levels lead to the arrest of cell cycle at G2/M-phase and result in the programmed cell death [1,20].

Antimicrobial activities of APCs showed that several bacteria are sensitive to their action, such as *Staphylococcus aureus*, *Enterococcus* sp., *Streptococcus pneumoniae* [21] or *Acinetobacter baumannii* [22]. However, *Escherichia coli* or *Pseudomonas aeruginosa* were resistant to miltefosine [21]. The highest antimicrobial activity was observed against yeast and fungi. Widmer et al. [23] showed that miltefosine has a broad antimycotic spectrum, as well. This APC inhibits growth of several species of the genera *Candida*, *Cryptococcus*, *Aspergillus*, *Fusarium* or *Scedosporium*. The mixture of miltefosine with amphotericin B represent a promising combination of drugs with a potential application in the treatment of multi-drug resistant candidiasis [24].

Antiprotozoal activity belongs to one of the most studied properties of APCs. Miltefosine is a drug which is used in the treatment of severe diseases caused by *Leishmania*. These protozoans are causative agents of cutaneous or visceral leishmaniasis (kala-azar). Leishmaniasis results in 700 000 to 1 million new cases annually worldwide [25]. Miltefosine is the only oral drug approved for the treatment of this disease [26–28]. Its activity is not limited to *Leishmania*, the spectrum of sensitive protozoans is broader. Miltefosine was used in the treatment of disseminated *Acanthamoeba* sp. infection [29], *Acanthamoeba* keratitis [30], and primary amoebic meningoencephalitis caused by *Naegleria fowleri* [31]. Many other genera of protozoans show sensitivity to miltefosine and other APCs. They are represented by *Entamoeba* [32], *Trichomonas* [33–35], *Balamuthia* [36] and Trypanosoma [37,38].

Foscarnet, phosphonoformic acid, is an organic analogue of inorganic pyrophosphate. It inhibits viral DNA-polymerases and reverse transcriptases. Foscarnet trisodium salt is used in the treatment of several viral diseases. This compound is active against herpes viruses, cytomegalovirus, hepatitis B viruses or human immunodeficiency viruses [39]. Biological activity of foscarnet is not limited only to viruses but it is active against cancer cells. Rose [40] documented antineoplastic activity of this drug against human anaplastic thyroid carcinoma cells. The main disadvantage of foscarnet is its low oral bioavailability. It can be increased by the preparation of lipid prodrugs. Foscarnet binding to alkylglycerols or alkyltioglycerols not only increases its bioavailability but also enhances its antiviral activity [41–43].

The present study is focused on the preparation, characterisation and investigation of the alkylphosphocholines that contain a foscarnet moiety in their molecular structure. The insertion of a phosphonofromate group between an alkyl chain and a choline results in the formation of a novel structural type of APCs. The main intention of our study is to investigate the relationship between structural changes, physicochemical properties and biological activity of the prepared compounds. The structural changes in the molecule of the model APC (miltefosine) are represented by the alkyl chain extension, the addition of phosphonofromate group and the modification of choline moiety. Physicochemical properties of APCs in aqueous solutions were studied by determining the surface activity and the micelle size utilising surface tension and dynamic light scattering experimental techniques. Biological activity was tested on cancer cells, bacteria, yeast and *Acanthamoeba*. Diseases caused by cancer cells or by the microorganisms mentioned above often lack efficient therapy or they show resistance to conventionally used drugs. Therefore, there is a demand for



Scheme 1. Synthetic approach of preparation of alkylphosphocholines. Abbreviations: R, R^1, R^2, R^3, R^4, R^5 are alkyl chains; R^2 and R^3can be connected to alkane-α,ω-diyls (formed heterocycles); R^6 is alkyloxy or alkyl; x represented different number of methylene groups. Reagents: a) $POCl_3$; b) H_2O; c) trialkyl phosphite; d) HBr or $(CH_3)_3SiBr/CH_3OH$; e) (i) pyridine, (ii) 1-(methylsulfonyl)3-nitro-1*H*-1,2,4-tirazole or 2,4,6-triisopropylbenzenesulfonyl chloride.

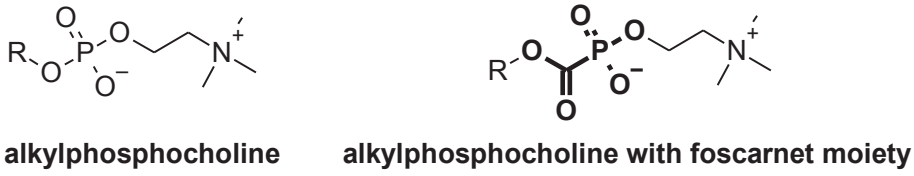


Fig. 2. Structural differences between alkylphosphocholines and alkylphosphocholines with foscarnet moiety.

new bioactive compounds. Some of the presented APCs show very attractive levels of antimicrobial activity against microorganisms causing severe illnesses and can provide a promising solution in the search for novel drugs in the treatment of yeast and protozoal infections.

2. Results and discussion

2.1. Chemistry

Prepared alkylphosphocholines (APCs) represent zwitterionic amphiphilic compounds with the structure similar to miltefosine (**HPC**). All prepared compounds contain foscarnet (phosphonoformic) moiety in the molecular structure. Structural comparison of both types of compounds is depicted in Fig. 2. Phosphonoformate group (the respective molecule part in Fig. 2 is marked bold) was inserted between the alkyl chain and the choline moiety. The compound **C15-PFA-C** ($C_{21}H_{44}NO_5P$; Scheme 1) shows the closest structural resemblance to **HPC** ($C_{21}H_{46}NO_4P$; Fig. 1) within the tested compounds. It contains 16 carbon atoms in the lipophilic part of molecule. However, one carbon atom is isolated from pentadecyl chain by oxygen and this carbon atom is bonded to another oxygen by a double bond. This connection of atoms (R—O—C(=O)—) represents the ester group (Fig. 2). **C16-PFA-C** can be also considered as an analogue to **HPC** because the structures are very similar to each other. They have hexadecyl chains, but **C16-PFA-C** has a carbonyl group inserted between oxygen and phosphorus of the phosphate group. **C16-PFA-C** contains only one more carbonyl group in comparison with **HPC**. We have prepared a perifosine analogue, as well. The relationship between **C18-PFA-4P** and perifosine is the same as the relationship between **C16-PFA-C** and **HPC**. These compounds differ in the presence or absence of carbonyl group in the structures. **C18-PFA-4P** represents an APC with the heterocyclic moiety. The piperidinium skeleton replaced the choline in the polar part of amphiphile. Other types of heterocyclic APCs are represented by compounds where trimethylaminium of the choline group is replaced with *N*-methylpiperidinium, *N*-methylazepanium or *N*-methylmorpholinium groups (Scheme 1).

The synthetic strategy in the preparation of alkylphosphocholines (APCs) is depicted in Scheme 2. The compounds were synthesized in several steps. Alkoxycarbonylphosphonic acids **3** were prepared from the respective fatty alcohols. The alcohols reacted with triphosgene to produce alkoxycarbonylchlorides **1**. The next step was Michaelis-Arbuzov reaction. The carbonylchlorides are more reactive than alkylhalides. Therefore, the formation of phosphonates **2** required lower temperature than generally needed. Phosphonic acids **3** were released from diesters by the reaction with trimethylsilylbromide and methanol. The alkoxycarbonylphosphonic acids were prepared according to the modified methods published by Rosowsky et al. [44]. The last step of synthesis was the reaction of alkoxycarbonylphosphonic acid **3** with choline tosylates in the presence of 2,4,6-triisopropylbenzenesulfonylchloride (TIPS-Cl). Cholines contain trimethylaminium, piperidinium, morpholinium or azepanium cations. The synthesis was performed according to the procedure described for dialkyphosphocholines [4]. The synthesized APCs are illustrated in Scheme 2.

2.2. Physicochemical properties of APCs

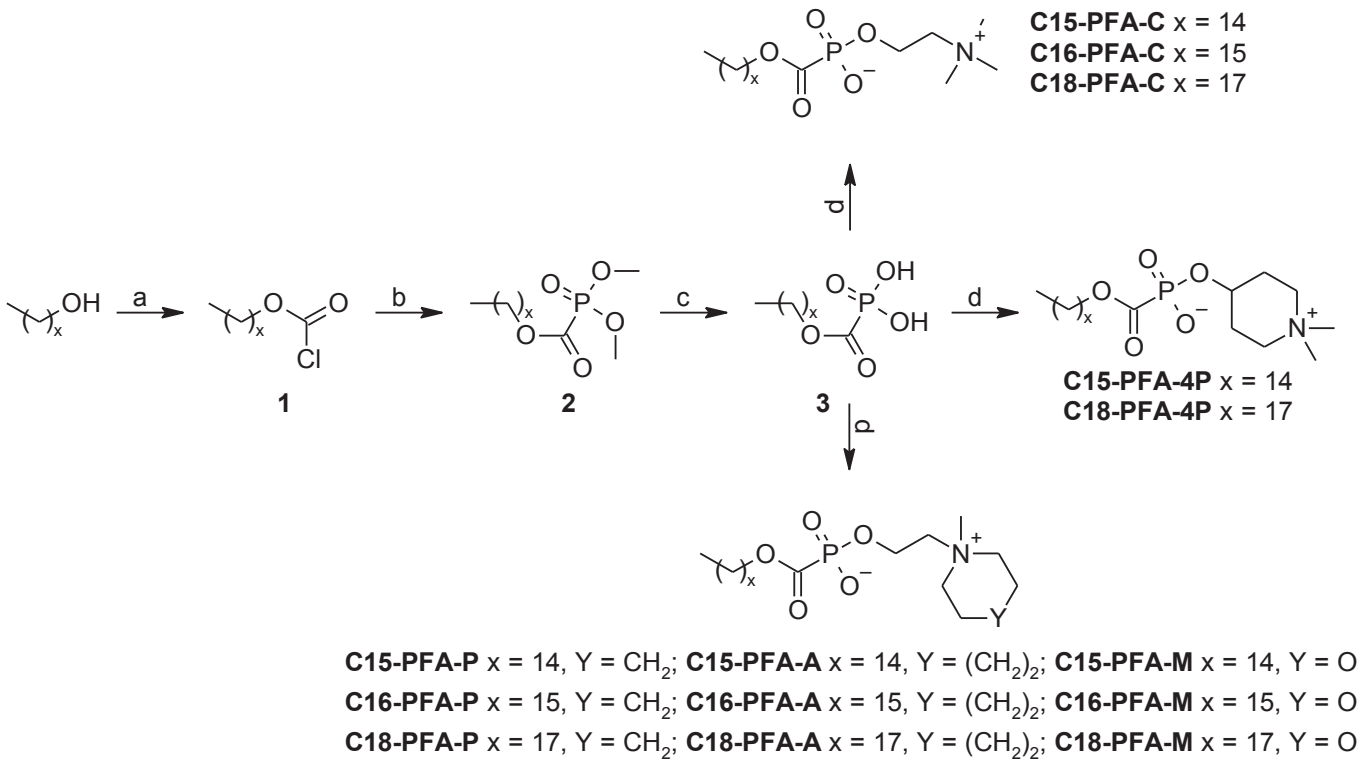
Physicochemical properties of the compounds were investigated by the measurements of surface tension of their aqueous solutions. The plots of surface tension *vs.* log concentration curves of APCs are shown in Fig. 3. The critical micelle concentration (cmc), the surface tension value at the cmc (γ_{cmc}) and, the surface area per head group at the surface saturation (A_{cmc}) were determined. The compounds **C18-PFA-A** and **C18-PFA-4P** have low solubility in water and could not be measured in aqueous solutions. The results are summarized in the Table 1.

The comparison of cmc values of **HPC** and its structural analogues **C15-PFA-C** and **C16-PFA-C** shows that the values are very similar. **C15-PFA-C** is slightly more hydrophilic than **HPC**. Its increased hydrophilicity is caused by the presence of an extra oxygen atom in the structure of **C15-PFA-C** in comparison with **HPC**. **C16-PFA-C** is more hydrophobic than **HPC**. The extension of the alkyl chain of **C16-PFA-C** by single methylene group in comparison with **C15-PFA-C** results in the cmc decrease. The cmc of **C16-PFA-C** is half the value of cmc of **C15-PFA-C**. Another insertion of two methylene groups in the alkyl chain of **C16-PFA-C** results in the cmc decrease, however, not so significantly. The difference between the cmc of **C18-PFA-C** and **C16-PFA-C** is lower than the difference between cmc values **C16-PFA-C** and **C15-PFA-C**. This complies with the published data [46]. The extension of the alkyl chain above 16 carbon atoms shows only marginal influence on the cmc values. The lipophilicity of alkyl chains has the same influence on cmc in the series of APCs with heterocycle moiety in the polar part of molecules.

Heterocyclic substitution on aminium nitrogen of APCs with pentadecyl chain results in the cmc decrease in comparison with the choline derivative. The size of rings affects the cmc values. The insertion of single methylene group into a heterocycle results in the cmc decrease. **C15-PFA-A** with azepane has cmc smaller than that of **C15-PFA-P** with piperidine. On the other hand, the insertion of hydrophilic oxygen into the heterocycle causes the cmc increase. The morpholine derivative **C15-PFA-M** shows the cmc larger than that of the piperidine derivative **C15-PFA-P**. We observed the same influence of the heterocycle presence on cmc values in the case of **HPC** derivatives [45]. The comparison of two piperidine derivatives **C15-PFA-P** and **C15-PFA-4P** shows that the removal of ethylene bridge between phosphonate and heterocycle results in the cmc increase. **C15-PFA-4P** is less lipophilic than **C15-PFA-P**. The cmc values of the series of heterocyclic APCs with hexadecyl and octadecyl chains are very low. Therefore, it is difficult to corelate the structural changes with the cmc values. Their values are in the range 4–4.6 $\times$ 10^{-6} mol·dm^{-3} for the derivatives with hexadecyl chains and 2.6–2.7 $\times$ 10^{-6} mol·dm^{-3} for derivatives with octadecyl chains, respectively.

The surface tension value at the cmc (γ_{cmc}) is found to be between 30.2 mN·m^{-1} and 36.0 mN·m^{-1}. The compounds with octadecyl chains have γ_{cmc} slightly higher than APCs with shorter alkyl chains. The insertion of an oxygen atom into heterocyclic ring also causes a slight increase in γ_{cmc}. The same influence was observed in the case of heterocyclic derivatives of **HPC** [45].

The surface area values per head group at the surface saturation (A_{cmc}) of choline derivatives **C15-PFA-C** and **C16-PFA-C** correlate well with the previously published data of **HPC** [45]. However, **C18-PFA-C** is densely packed at the water/air interface which results in its value of A_{cmc} being only 0.37 nm^2. The same results were observed in the case of heterocyclic APCs with the longest alkyl chains. The lipophilicity increase may result in the increased intensity of Van der Waals interactions between molecules of amphiphiles. These interactions lead to the decrease in A_{cmc}. Piperidine and azepane derivatives of **C15-PFA-C** and



Scheme 2. Synthesis of alkylphosphocholines derived from foscarnet. Reagents: (a) triphosgene; (b) trimethyl phosphite, 80 °C; (c) i: trimethylsilylbromide, ii: methanol; (d) TIPS-Cl, pyridine, choline tosylates.

C16-PFA-C have A_{cmc} larger than that of choline derivatives. Heterocyclic cations are bulkier than trimethylaminium cations and require more space at the water/air interface. On the other hand, morpholine derivatives are packed very tightly. Their A_{cmc} values are smaller than the values of **C15-PFA-C** and **C16-PFA-C**. We assume that the interaction of oxygen atom of the morpholine ring with water molecules through hydrogen bonds is responsible for the dense arrangement of APC molecules as compared with other heterocyclic derivatives.

Hydrodynamic diameter (d_H) of APCs was determined at the concentrations that were 200-times higher than cmc. The data are summarized in the Table 2. The compounds with pentadecyl and hexadecyl chains formed small micelles. Their diameter values are in the range from 5.5 nm to 7.6 nm. The values correspond with the data published for **HPC** (d_H = 5.8 ± 1.0 nm) and similar nitrogen heterocyclic APCs published previously [45]. A different situation is observed in the case of APCs with octadecyl chains. Two compounds (**C18-PFA-A**, **C18-PFA-4P**) were poorly soluble in water. Therefore, their hydrodynamic diameter could not be measured. **C18-PFA-P** shows a bimodal particle size spectrum with one peak assigned to small micelles (d_H = 7.5 nm) and the second assigned to large aggregates (d_H = 299 nm). Large aggregates, bigger than 240 nm in size, are also formed by **C18-PFA-C** and **C18-PFA-M**. The aggregates are > 50 times larger than the aggregates of structurally similar compounds with shorter alkyl chains. Higher lipophilicity of these compounds may result in the aggregation of micelles into large intermicellar clusters or in the formation of vesicles. We observed a similar behaviour in case of APCs with branched alkyl chains [2]. The increased molecular lipophilicity may be responsible for the formation of large aggregates along with the presence of small micelles in aqueous solution of the studied compounds.

2.3. Biological activity

2.3.1. Cytotoxic activity

Cytotoxic activity of APCs was determined against cancer cells. They are represented by human cervical adenocarcinoma cells (HeLa) and human breast adenocarcinoma cells, estrogen receptor positive (MCF-7). Cytotoxic activity of the compounds is compared with the activity against non-cancer cells fibroblasts 3T3. The values of the half maximal inhibitory concentration (IC_{50}) are summarized in Table 3.

The results show that the APCs with pentadecyl chains are practically inactive against the tested cells. Cytotoxic activity is only observed in the case of **C15-PFA-C** and **C15-PFA-A**. The activity of **C15-PFA-C** is practically identical with that of the standard **HPC**. Both compounds show cytotoxic activity against HeLa. MCF-7 was resistant to the action of the compounds. A different situation is observed in the case of **C15-PFA-A**. The compound is inactive against HeLa, but it shows cytotoxic activity against MCF-7. However, the activity is very low.

The compounds with hexadecyl and octadecyl chains have higher cytotoxic activity against cancer cells than APCs with a shorter alkyl chain. The activity increases with the increasing alkyl chain length. Generally, APCs with octadecyl chains are more active than the compounds with hexadecyl chains. We have made a similar observation in the case of dialkylphosphocholines. The compounds with the longest alkyl chain (eicosanyl) showed the highest cytotoxicity [15]. The cytotoxicity increase with the increasing lipophilicity of APCs was also observed in other series of compounds [47,48]. Lipophilicity is one of the most important factors that affects cytotoxic activity of APCs. The cmc is a parameter that has a strong relationship to lipophilicity. Higher cytotoxic activity of compounds is observed for APCs with smaller cmc. Another reason of high cytotoxic activity of these compounds is that they can form different types of aggregates. Large intermicellar clusters or vesicles can be expected as showed the measurement of the hydrodynamic diameters of APCs with octadecyl chain. They may interact more easily with plasmatic membrane of cancer cells than micelles. An example of the compound with high lipophilicity is erufosine that has been widely tested as potential chemotherapeutic [49–53].

The insertion of the foscarnet moiety into the structure of APCs has no effect on cytotoxic activity. **C15-PFA-C**, **C16-PFA-C** and **HPC** have approximately the same activity. Their cytotoxicity also correlates with their cmc values. All three compounds have similar lipophilicity and

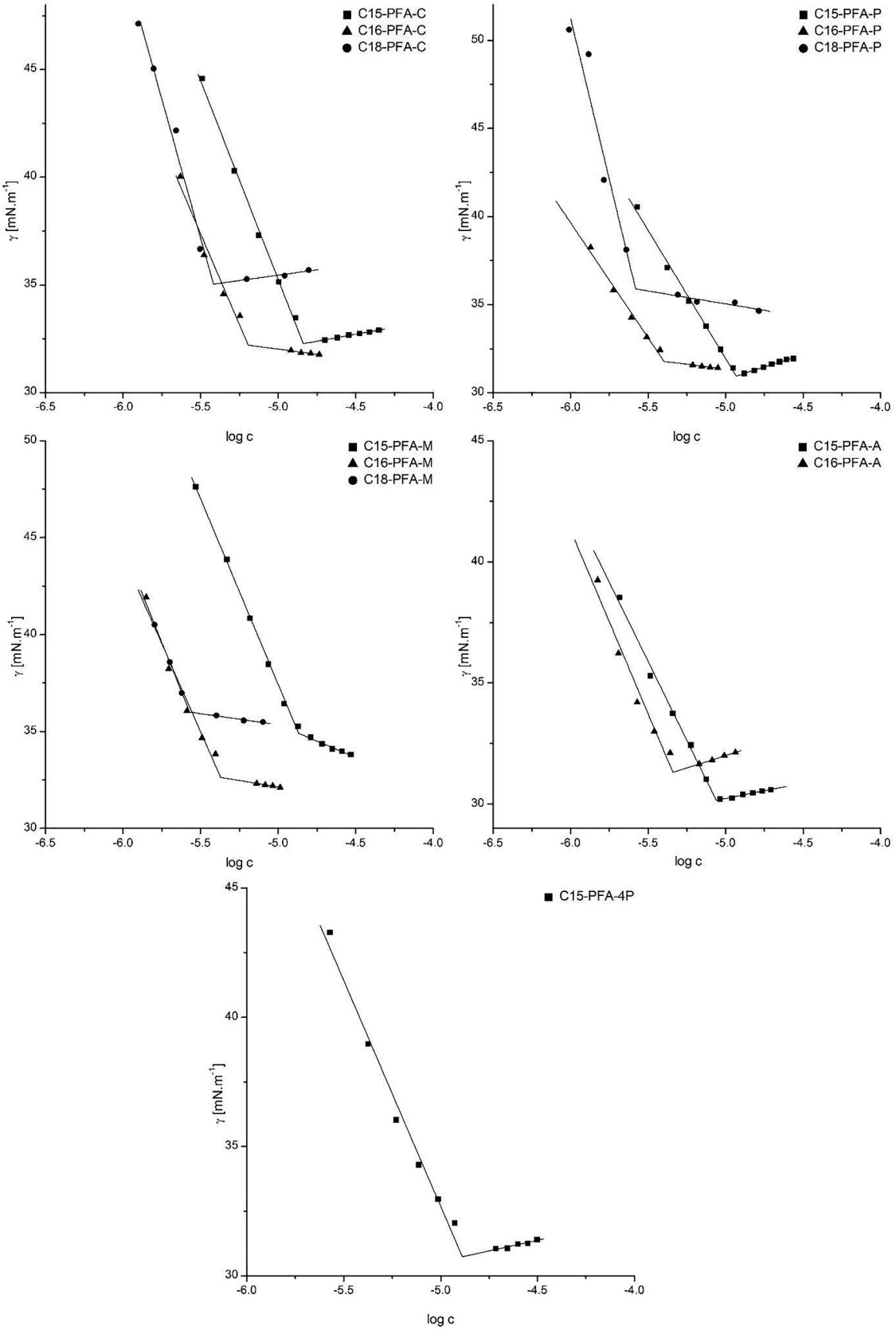


Fig. 3. Plots of surface tension *vs.* log concentration of APCs.

their cytotoxic activity has been changed minimally, as well.

The investigations of the activity of APCs against cancer and non-cancer cells revealed that the APCs with hexadecyl chains and **C18-PFA-C** are more active against HeLa and MCF-7 than against 3T3. In contrast, heterocyclic APCs with the longest alkyl chain are more toxic to non-cancer cells in comparison with cancer cells. The compound

Table 1
Surface tension data of APCs.

Compound	cmc [mol.dm^{-3}]	A [Å^2]	γ_{cmc} [mN.m^{-1}]
C15-PFA-C	(1.45 ± 0.03) × 10^{-5}	51 ± 2	32.3 ± 0.2
C15-PFA-P	(1.17 ± 0.02) × 10^{-5}	65 ± 2	31.0 ± 0.1
C15-PFA-A	(8.71 ± 0.27) × 10^{-6}	73 ± 4	30.2 ± 0.2
C15-PFA-M	(1.36 ± 0.01) × 10^{-5}	49 ± 1	34.9 ± 0.1
C15-PFA-4P	(1.29 ± 0.04) × 10^{-5}	54 ± 3	30.7 ± 0.3
C16-PFA-C	(6.41 ± 0.45) × 10^{-6}	56 ± 8	32.2 ± 0.5
C16-PFA-P	(4.01 ± 0.14) × 10^{-6}	72 ± 5	31.8 ± 0.1
C16-PFA-A	(4.58 ± 0.24) × 10^{-6}	62 ± 6	31.3 ± 0.4
C16-PFA-M	(4.26 ± 0.23) × 10^{-6}	52 ± 5	32.6 ± 0.4
C18-PFA-C	(3.83 ± 0.23) × 10^{-6}	37 ± 4	35.0 ± 0.6
C18-PFA-P	(2.63 ± 0.28) × 10^{-6}	26 ± 5	35.9 ± 1.6
C18-PFA-M	(2.69 ± 0.02) × 10^{-6}	47 ± 0	36.0 ± 0.1
HPC [45]	(1.25 ± 0.05) × 10^{-5}	57 ± 3	38.3 ± 0.1

Table 2
Micelle sizes of APCs.

Compound	Micelle size (d_H) [nm]	Compound	Micelle size (d_H) [nm]
C15-PFA-C	5.5 ± 0.4	**C16-PFA-A**	5.7 ± 0.3
C15-PFA-P	5.5 ± 0.0	**C16-PFA-M**	7.6 ± 0.7
C15-PFA-A	5.5 ± 0.3	**C18-PFA-C**	286.6 ± 7.2
C15-PFA-M	5.7 ± 0.3	**C18-PFA-P**	299.3 ± 9.8 7.5 ± 0.6
C15-PFA-4P	6.3 ± 0.2	**C18-PFA-A**	–
C16-PFA-C	6.7 ± 0.7	**C18-PFA-M**	245.1 ± 1.7
C16-PFA-P	6.2 ± 0.6	**C18-PFA-4P**	–

C18-PFA-C shows the highest selectivity. This compound has the lowest values of IC_{50} against HeLa and MCF-7 among all tested APCs and it is approximately two times less active against non-cancer cells 3T3.

2.3.2. Antimicrobial activity

Antimicrobial activity was determined against Gram-positive bacteria *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli* and yeast *Candida albicans*. Minimal inhibition concentration (MIC) values are summarized in Table 3.

The results show that all prepared compounds do not demonstrate significant antimicrobial activity against *S. aureus* and *E. coli*. The standard (**HPC**) is antimicrobially active only against Gram-positive bacteria. In our previous study, we have made a similar observation in case of dialkyl derivatives of **HPC** [16]. Obando et al. [21] obtained the same results in the case of *E. coli*. The modification of the lipophilic part of APC molecule had no effect on antimicrobial activity. However, **HPC** and APCs with higher lipophilicity than **HPC** were active against *S. aureus* and its methicilline-resistant strain.

On the other hand, the antifungal activity values of APCs were found to be significant. The MIC values of all compounds were lower than 40 μM. The activity of compounds with pentadecyl and hexadecyl chains was comparable with the **HPC** standard. These compounds have similar lipophilicity. The carbonyl group of foscarnet moiety affects physico-chemical property only marginally. The structural changes in polar head groups have only small influence on the activity. The highest activity was determined for **C15-PFA-C**. It is twice as active as the **HPC** standard and other APCs tested. The MIC value is 1.4 μM. The same value was obtained by Obando [21] for octadecylphosphocholine. To the best of our knowledge, this is the highest anticandidal activity attained within the group of APCs. The extension of the alkyl chain of the investigated APCs to the length of 18 carbon atoms results in the decrease of anticandidal activity. The compounds with heterocyclic groups in the structure are twelve times less active than their analogues with shorter alkyl chains. Only **C18-PFA-C** shows the activity that is comparable with other APCs of pentadecyl or hexadecyl chain length. These findings are in accordance with similar observations published previously [2,15,21]. The compounds with the highest lipophilicity (i.e. with the lower cmc values) show decreased activity against *Candida albicans*. The relationship between anticandidal activity and the alkyl chain length was studied in case of APCs with branched alkyl chains [2] where the cut-off effect was observed. The cut-off effect is typical for biological activity of a series of amphiphilic compounds with different alkyl chain length [54]. In other words, the highest activity is observed for a compound with specific lipophilicity. The compounds with very short or very long alkyl chains were less active.

2.3.3. Acanthamoeba spp.

Antiprotozoal activity of the APCs with foscarnet moiety was determined against two clinical isolates of *A. lugdunensis* and *A. quina* of T4 genotype, which were causative agents of *Acanthamoeba* keratitis. The values of minimal trophocidal concentrations (MTC) and half maximal effective concentration (EC_{50}) are summarized in Table 3.

The MTC values of APCs with foscarnet moiety show that only two compounds with pentadecyl chains have demonstrable trophocidal activity. **C15-PFA-A** shows the activity against both *A. lugdunensis* and *A. quina*. **C15-PFA-P** is active only against *A. lugdunensis* isolate. These two compounds reach or exceed the level of trophocidal activity of the standard **HPC**. On the basis of MTC values, the other tested compounds can be classified as trophocidally inactive. However, this does not mean

Table 3
Cytotoxic, antimicrobial and antiprotozoal activity of APCs.

Compound	IC_{50} (μM)			MIC (μM)			MTC (μM)		EC_{50} (μM)	
	HeLa	MCF-7	3T3	*S.a.*	*E.c.*	*C.a.*	*A.l.*	*A.q.*	*A.l.*	*A.q.*
C15-PFA-C	72.5	> 100	> 100	> 1000	> 1000	**1.39**	> 500	> 500	96	95
C15-PFA-P	> 100	> 100	> 100	> 1000	> 1000	2.59	500	> 500	34	104
C15-PFA-A	> 100	94.3	> 100	> 1000	> 1000	5.05	**500**	**500**	**25**	**9.6**
C15-PFA-M	> 100	> 100	> 100	> 1000	> 1000	2.56	> 500	> 500	75	59
C15-PFA-4P	> 100	> 100	> 100	> 1000	> 1000	2.65	> 500	> 500	84	61
C16-PFA-C	86.4	> 100	> 100	> 1000	> 1000	2.71	> 500	> 500	371	273
C16-PFA-P	85.0	> 100	> 100	> 1000	> 1000	2.49	> 500	> 500	> 500	419
C16-PFA-A	64.7	88.2	> 100	> 1000	> 1000	2.44	> 500	> 500	> 500	> 500
C16-PFA-M	77.5	> 100	> 100	> 1000	> 1000	2.49	> 500	> 500	> 500	356
C18-PFA-C	**46.9**	**50.6**	**91.4**	> 1000	> 1000	5.05	> 500	> 500	> 500	> 500
C18-PFA-P	57.3	55.0	52.6	> 1000	> 1000	38.1	> 500	> 500	> 500	> 500
C18-PFA-A	44.8	50.9	40.8	> 1000	> 1000	36.8	> 500	> 500	> 500	> 500
C18-PFA-M	88.5	72.7	57.5	> 1000	> 1000	n.d.	> 500	> 500	> 500	> 500
C18-PFA-4P	50.4	58.4	40.7	> 1000	> 1000	n.d.	> 500	> 500	> 500	469
HPC	77.8	> 100	> 100	11.5	> 1000	2.87	> 500	500	42	45

A.l. – Acanthamoeba lugdunensis, A. q. – Acanthamoeba quina, C.a. – Candida albicans, E.c. – Escherichia coli, n.d. – not determined, S.a. – Staphylococcus aureus.

that they are inactive against acanthamoebae. All investigated compounds inhibit the growth of *Acanthamoeba* trophozoites. The half maximal effective concentration shows that the compounds with pentadecyl chains and several compounds with longer alkyl chains are strongly trophostatic. The concentration of these APCs lower than 500 μM is capable of reducing the cell populations by 50%. The values of EC_{50} show that lipophilicity is crucial for a good activity. All APCs with alkyl chains with 15 carbon atoms have EC_{50} lower than 100 μM. Only **C15-PFA-P** has EC_{50} slightly higher in the case of *A. quina*. This value was 104 μM. APCs with the alkyl chain length above 15 carbon atoms were less active. The values of EC_{50} lower than 500 μM were observed in the case of **C16-PFA-C, C16-PFA-P, C16-PFA-M** and **C18-PFA-4P**. The first compound is active against both strains. The other compounds show activity only against *A. quina*. However, their values of EC_{50} are > 200 μM.

Among all compounds investigated, the compound **C15-PFA-A** shows the highest activity. It has the lowest MTC and EC_{50} values as well. These values are approximately two times (for *A. lugdunensis*) and five times (for *A. quina*) lower than those of the **HPC** standard. It can be assumed that the heterocycle presence may have the influence on biological antiprotozoal activity. We obtained the same results for heterocyclic APCs which were published previously [13]. Azepane and azocane analogues of **HPC** were the most active compounds against *A. lugdunensis*. However, heterocyclic analogues of APCs with a branched alkyl chain do not support this assumption [14]. The antiprotozoal activity of APCs is non-specific and therefore, it depends on the ability to penetrate the plasmatic membrane of trophozoites. Lipophilicity of molecules is more important for antiprotozoal activity than the presence of a heterocycle in the molecule polar part. The changes in the activity are more pronounced as a result of the alkyl chain extension than the structural modification of polar head group [2,14]. The presence or absence of carbonyl group in the molecules of APCs affects antiprotozoal activity only minimally. The most active compounds have cmc approximately 1×10^{-5} mol·dm^{-3}.

3. Conclusions

Within the framework of this study, fourteen novel APCs were synthesized and investigated. The novel compounds represent zwitterionic amphiphiles with phosphocholine moiety where the phosphate group is replaced with phosphonometanoate group. The synthesized compounds represent a new type of APCs that link the structure of miltefosine and its analogues to the antiviral drug foscarnet. We investigated the effect of polar head group and carbonyl group bonded to phosphonate on physicochemical properties, cytotoxic, antimicrobial and anti-*Acanthamoeba* activity. The physicochemical properties of the novel compounds were studied by determining the surface activity and the micelle size utilising surface tension and dynamic light scattering experimental techniques. The most important parameter obtained from the measurements was the critical micelle concentration. The cmc can be regarded as the measure of lipophilicity [17]. The insertion of carbonyl group between oxygen and phosphorus atoms of the phosphate group of **HPC** resulted in the cmc decrease. However, the compound **C15-PFA-C** that has the number of carbon atoms identical with **HPC** and the only structural difference is the presence of two hydrogen atoms and one oxygen atom in its molecular structure, has cmc larger than that of **HPC**. The presence of hydrophilic oxygen atom decreases the lipophilicity of APC with foscarnet moiety. The size of the aggregates formed in aqueous solution indicates that APCs with pentadecyl and hexadecyl chain formed small micelles. However, complex aggregates large in size are typical for the most lipophilic APCs among the investigated compounds.

Biological activity of APCs was examined on two cancer cell lines (HeLa, MCF-7), non-cancer cells (3T3), two bacteria (*S. aureus*, *E. coli*), one yeast (*C. albicans*) and two protozoan parasites (*A. lugdunensis* and *A. quina*). The highest cytotoxic activity against cancer cells was observed for **C18-PFA-C**. Its value was approximately twice the value of **HPC**. APCs with foscarnet moiety were antibacterially inactive. However, anticandidal activity was determined in case of all tested compounds. The highest activity was found for the compound **C15-PFA-C** with the MIC = 1.4 μM. It is the highest anticandidal activity ever determined within the group of APCs. The highest antiprotozoal activity was found in the case of **C15-PFA-A**. This compound is more active against both pathogenic strains of *Acanthamoeba* (T4 genotype) than **HPC**. One can conclude that more lipophilic APCs with octadecyl chain have higher cytotoxic activity against cancer cells than the compounds with shorter alkyl chains. The opposite situation was observed for anticandidal and antiprotozoal activity. The most active compounds are those with pentadecyl chains.

Increasing resistance of *Candida* spp to antimycotics and inadequate treatment of acanthamoebiasis raise demand for new antimicrobial compounds. As the results obtained within our study indicate, two of our novel compounds show promising potential. The compound **C15-PFA-C** might be efficient in the treatment of candidiasis whereas the compound **C15-PFA-A** represents a potential high efficient APC in the treatment of *Acanthamoeba* infections.

4. Experimental part

Chemicals for synthesis were obtained from the following commercial suppliers: CentralChem, Slovakia – acetone, acetonitrile, diethyl ether, chloroform, propan-2-ol, pyridine, tetrahydrofurane, triethylamine; Fluka, Germany – phosphorus oxychloride, methyl 4-methylbenzenesulphonate, *N*,*N*-dimethyl-2-aminoethanol; Merck, Germany – pentadecane-1-ol, hexadecane-1-ol, octadecane-1-ol, *N*-methylpiperidine-4-ol, Amberlite MB-3; Chemicals were used as supplied. Solvents for synthesis were purified and dried before use according to Perrin and Armarego [55]. 2-(piperidine-1-yl)ethanol, 2-(azepane-1-yl)ethanol, 2-(morpholine-1-yl)ethanol were prepared by hydroxyethylation [56]. Choline salts were dried at 61 °C at diminished pressure prior to use.

^{1}H, ^{13}C and ^{31}P NMR spectra were measured on a Varian MERCURY plus spectrometer working at frequency 300, 75 and 121.5 MHz respectively. ^{13}C and ^{31}P NMR spectra were decoupled against protons. Spectra were measured in $CDCl_3$ or DMSO-d_6. TMS was used as the internal standard for ^{1}H and ^{13}C NMR spectra and 85% H_3PO_4 was used as the external standard for ^{31}P NMR spectra. Mass spectra were recorded on an LTQ Orbitrap XL hybrid FTMS spectrometer (Thermo Fisher Scientific) using electrospray Ion Max-ESI in positive mode. Elemental analysis was carried out using FLESCH 2000 (Thermo Fischer Scientific).

A. lugdunensis and *A. quina* were clinical isolates of free-living amoebae isolated from corneas of patients with *Acanthamoeba* keratitis [57].

4.1. Synthesis

4.1.1. General procedure for preparation of choline salts

A solution of methyl 4-methylbenzenesulfonate (50 mmol) was added to a solution of tertiary amine (2-(*N*,*N*-dimethylamino)ethanol, 2-(piperidine-1-yl)ethanol, 2-(azepane-1-yl)ethanol, 2-(morpholine-1-yl)ethanol or *N*-methylpiperidine-4-ol; 50 mmol) in acetonitrile (30 mL). The reaction mixture was refluxed for 4 h. The solvent was evaporated in vacuum and the crude solid was left for crystallisation from the mixture of acetone and methanol yielding a white, hygroscopic solid as the product. The product was kept in vacuum desiccator over P_4O_{10}.

4.1.1.1. 2-hydroxy-(N,N,N-trimethyl)ethane-1-aminium 4-methylbenzenesulfonate. Yield: 93.8%; ^{1}H NMR (DMSO-d_6) δ 2.29 (s, 3H); 3.10 (s, 9H); 3.37–3.41 (m, 2H); 3.81–3.84 (m, 2H); 5.28 (t, 1H, J = 5.3 Hz); 7.12 (d, 2H, J = 8.4 Hz); 7.48 (d, 2H, J = 6.6 Hz); ^{13}C NMR (DMSO-d_6)

δ 20.8; 53.1; 53.2; 55.1; 66.9; 125.5; 128.1; 137.6; 145.7; HRMS calcd. for $C_5H_{14}ON^+$ = 104.1070; found *m/z*: $[M]^+$ 104.1069.

4.1.1.2. N-(2-hydroxyethyl)-N-methylpiperidinium 4-methylbenzenesulfonate. Yield: 86.0%; ^{1}H NMR (DMSO-d_6) δ 1.49–1.56 (m, 2H); 1.75–1.79 (m, 4H); 2.29 (s, 3H); 3.06 (s, 3H); 3.30–3.44 (m, 6H); 3.83–3.84 (m, 2H); 5.27 (t, 1H, J = 4.8 Hz); 7.12 (d, 2H, J = 7.8 Hz); 7.48 (d, 2H, J = 7.8 Hz); ^{13}C NMR (DMSO-d_6) δ 19.3; 20.6; 20.8; 48.2; 54.6; 60.8; 63.7; 125.5; 128.1; 137.6; 145.7; HRMS calcd. for $C_8H_{18}ON^+$ = 144.1383; found *m/z*: $[M]^+$ 144.1381.

4.1.1.3. N-(2-hydroxyethyl)-N-methylazepanium 4-methylbenzenesulfonate. Yield: 89.0%; ^{1}H NMR (DMSO-d_6) δ 1.56–1.60 (m, 4H); 1.81 (m, 4H); 2.29 (s, 3H); 3.05 (s, 3H); 3.32–3.40 (m, 4H); 3.51–3.59 (m, 2H); 3.84 (m, 2H); 5.29 (t, 1H, J = 4.8 Hz); 7.11 (d, 2H, J = 7.9 Hz); 7.47 (d, 2H, J = 7.9 Hz); ^{13}C NMR (DMSO-d_6) δ 20.7; 20.7; 27.1; 50.8; 55.0; 63.9; 65.4; 125.4; 127.9; 137.5; 145.7; HRMS calcd. for $C_9H_{20}ON^+$ = 158.1539; found *m/z*: $[M]^+$ 158.1536.

4.1.1.4. N-(2-hydroxyethyl)-N-methylmorpholinium 4-methylbenzenesulfonate. Yield: 94.4%; ^{1}H NMR (DMSO-d_6) δ 2.29 (s, 3H); 3.20 (s, 3H); 3.38–3.58 (m, 6H); 3.86–3.91 (m, 6H); 5.33 (t, 1H, J = 4.8 Hz); 7.12 (d, 2H, J = 8.2 Hz); 7.48 (d, 2H, J = 7.9 Hz); ^{13}C NMR (DMSO-d_6) δ 21.3; 48.1; 54.9; 55.0; 60.2; 60.3; 65.2; 126.0; 128.5; 138.1; 146.2; HRMS calcd. for $C_7H_{16}O_2N^+$ = 146.1176; found *m/z*: $[M]^+$ 146.1174.

4.1.1.5. 4-hydroxy-N,N-dimethylpiperidinium 4-methylbenzenesulfonate. Yield: 86.5%; ^{1}H NMR (DMSO-d_6) δ 1.71–1.72 (m, 2H); 1.92–2.00 (m, 2H); 2.29 (s, 3H); 3.04 (s, 3H); 3.08 (s, 3H); 3.24–3.31 (m, 2H); 3.37–3.46 (m, 2H); 3.74–3.78 (m, 1H); 5.08 (d, 1H, J = 3.8 Hz); 7.11 (d, 2H, J = 8.5 Hz); 7.46 (d, 2H, J = 7.9 Hz); ^{13}C NMR (DMSO-d_6) δ 20.7; 27.6; 49.7; 51.3; 58.1; 60.8; 125.4; 127.9; 137.5; 145.7; HRMS calcd. for $C_7H_{16}ON^+$ = 130.1226; found *m/z*: $[M]^+$ 130.1225.

4.1.2. General procedure for preparation of dimethyl alkyloxycarbonylphosphonates

Primary alcohol (pentadecane-1-ol, hexadecane-1-ol, octadecane-1-ol; 10 mmol) was dissolved in 30 mL of tetrachloromethane and pyridine was added (11 mmol). The solution was cooled to −20 °C and triphosgene (5 mmol) was added at vigorous stirring. The mixture was stirred another 20 min at −20 °C. Then, the solution was warmed to room temperature and stirred overnight. The precipitate was filtered and the solution was evaporated. The obtained alkyloxycarbonylchloride was used in the next reaction step without further purification.

Alkyloxycarbonylchloride (10 mmol) was mixed with trimethyl phosphite (10 mL) and heated at 80 °C overnight. After cooling, the excess of trimethyl phopshite was evaporated and the raw product was crystalized from hexane. The esters were obtained as white crystalline compounds.

4.1.2.1. Dimethyl pentadecyloxycarbonylphosphonate. Yield: 51.4%; m.p. = 26–28 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.6 Hz); 1.26–1.36 (m, 24H); 1.72 (qi, 2H, J = 6.9 Hz); 3.93 (d, 6H, J = 11.1 Hz); 4.27 (t, 2H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.8; 28.4; 29.1; 29.4; 29.5; 29.6; 29.7; 31.9; 54.5; 54.6; 66.3; 164.6; 168.2; ^{31}P NMR ($CDCl_3$) δ –2.26.

4.1.2.2. Dimethyl hexadecyloxycarbonylphosphonate. Yield: 57.5%; m.p. = 32–33 °C; ^{1}H NMR ($CDCl_3$, TMS) 0.88 (t, 3H, J = 6.7 Hz); 1.26–1.36 (m, 26H); 1.72 (qi, 2H, J = 6.7 Hz); 3.93 (d, 6H, J = 11.1 Hz); 4.27 (t, 2H, J = 6.7 Hz); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1; 22.7; 28.4; 29.1; 29.4; 29.5; 29.6; 29.7; 31.9; 53.7; 63.1; 164.4; 168.0; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ –2.24.

4.1.2.3. Dimethyl octadecyloxycarbonylphosphonate. Yield: 88.7%; m.p. = 41–42 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.26–1.33 (m, 30H); 1.72 (qi, 2H, J = 6.8 Hz); 3.93 (d, 6H, J = 11.1 Hz); 4.27 (t, 3H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.8; 28.4; 29.1; 29.4; 29.5; 29.6; 29.7; 31.9; 54.5; 54.6; 66.3; 164.6; 168.1; ^{31}P NMR ($CDCl_3$) δ –2.27.

4.1.3. General procedure for preparation of alkyloxycarbonylphosphonic acids

Dimethyl alkyloxycarbonylphosphonate (10 mmol) was dissolved in 20 mL tetrachloromethane and bromotrimethylsilane (30 mmol) was added. The reaction mixture was stirred at r.t. for 20 h. After that, the volatile compounds were evaporated and methanol was added. The mixture was heated up to 65 °C for 3 h and methyl trimethylsilyl ether was distilled off during the reaction. The unreacted methanol was evaporated, and the crude acid was crystalized from hexane. The compounds were obtained as white amorphous powders.

4.1.3.1. Pentadecyloxycarbonylphosphonic acid. Yield: 62.0%; m.p. = 31–32 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.26 (m, 24H); 1.67–1.70 (m, 2H); 3.71 (t, 2H, J = 6.6 Hz); 9.53 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.6; 28.3; 29.3; 29.4; 29.6; 29.7; 31.9; 63.4.

4.1.3.2. Hexadecyloxycarbonylphosphonic acid. Yield: 64.0%; m.p. = 42–44 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.26–1.30 (m, 26H); 1.52–1.61 (m, 2H); 3.64 (t, 2H, J = 6.6 Hz); 9.45 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.7; 29.4; 29.6; 29.7; 32.8; 63.1.

4.1.3.3. Octadecyloxycarbonylphosphonic acid. Yield: 78.3%; m.p. = 81–82 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.25–1.30 (m, 30H); 1.67–1.72 (m, 2H); 3.61 (t, 3H, J = 6.6 Hz); 9.52 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.8; 29.4; 29.5; 29.7; 32.0; 32.6; 62.8.

4.1.4. General procedure for preparation of alkyloxycarbonylphosphonatocholines

Alkyloxycarbonylphosphonic acid (5 mmol) was dissolved in pyridine (15 mL) and the solution was stirred at 50 °C for 2 h. After that, pyridine was evaporated, and another 20 mL of pyridine was added. The appropriate choline derivative (7.5 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (10 mmol) in pyridine (20 mL) were added to the solution of pyridinium salt of alkyloxycarbonylphosphonic acid. The solution was stirred at 50 °C overnight. After cooling, the mixture was hydrolysed by the addition of H_2O (1.5 mL) and stirred at r.t. for 1 h. Solvents were evaporated at vacuum and the crude solid was diluted in tetrahydrofuran and water (5:1). Exchange resin Amberlite MB3 was added to the stirred solution until the resin stopped changing colour. The resin was filtered off and solvents were evaporated in vacuum. The product was purified by chromatography using silica gel with the liquid phase containing $CHCl_3$:CH_3OH:H_2O (65:25:1 → 65:25:3). After purification, solvents were evaporated in vacuum, the product was dried by azeotropic distillation using propan-2-ol, dissolved in chloroform and precipitated with acetone. The product was filtered off as white hygroscopic solid and was stored in desiccator over phosphorus pentoxide.

*4.1.4.1. 2-(trimethylaminio)ethyl pentadecyloxycarbonylphosphonate (**C15-PFA-C**)*. Yield: 18.8%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.25–1.30 (m, 24H); 1.68 (qi, 2H, J = 7.0); 3.33 (s, 9H); 3.79–3.82 (m, 2H); 4.12 (t, 2H, J = 7.0 Hz); 4.44 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.2; 22.7; 25.9; 28.6; 29.3; 29.4; 29.6; 29.7; 32.0; 54.6; 59.8; 64.5; 66.6; 173.5 (d, J = 240 Hz); ^{31}P NMR ($CDCl_3$) δ –5.39; HRMS calcd. for $C_{21}H_{45}O_5NP$ = 422.3030; found *m/z*: $[M + H]^+$ 422.3037; Anal. calcd. for $C_{21}H_{44}O_5NP \times 0.9\ H_2O$: C: 57.62; H: 10.55;

N: 3.20; found: C: 57.72; H: 10.22; N: 2.98.

*4.1.4.2. 2-(N-methylpiperidinio-1-yl)ethyl pentadecyloxycarbonylphosphonate (**C15-PFA-P**)*. Yield: 23.6%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.25–1.33 (m, 26H); 1.63–1.75 (m, 2H); 1.89–1.91 (m, 4H); 3.36 (s, 3H); 3.51–3.56 (m, 2H); 3.69–3.77 (m, 2H); 3.92 (m, 2H); 4.10 (t, 2H, J = 6.9 Hz); 4.48 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 20.2; 21.0; 22.7; 26.0; 28.6; 29.4; 29.6; 29.7; 31.9; 48.6; 59.1; 62.2; 64.2; 69.6; 174.2 (d, J = 238 Hz); ^{31}P NMR ($CDCl_3$) δ –4.84; HRMS calcd. for $C_{24}H_{49}O_5NP$ = 462.3342; found m/z: $[M + H]^+$ 462.3342; Anal. calcd. for $C_{24}H_{48}O_5NP \times 0.5\ H_2O$: C: 61.25; H: 10.50; N: 2.98; found: C: 61.16; H: 10.23; N: 2.73.

*4.1.4.3. N,N-dimethylpiperidinio-4-yl Pentadecyloxycarbonylphosphonate (**C15-PFA-4P**)*. Yield: 14.9%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.25 (m, 24H); 2.20 (m, 4H); 3.22 (s, 3H); 3.29 (s, 3H); 3.48–3.52 (m, 2H); 3.66–3.70 (m, 2H); 4.09 (t, 2H, J = 7.0); 4.62–4.65 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.9; 27.2; 28.7; 29.4; 29.6; 29.7; 31.9; 48.9; 55.2; 58.8; 64.1; 64.8; 174.1 (d, J = 235 Hz); ^{31}P NMR ($CDCl_3$) δ –5.73; HRMS calcd. for $C_{23}H_{47}O_5NP$ = 448.3186; found m/z: $[M + H]^+$ 448.3189; Anal calcd. for $C_{23}H_{46}O_5NP \times 0.75\ H_2O$: C: 59.91; H: 10.38; N: 3.04; found: C: 60.00; H: 10.17; N: 2.80.

*4.1.4.4. 2-(N-methylazepanio-1-yl)ethyl pentadecyloxycarbonylphosphonate (**C15-PFA-A**)*. Yield: 39.1%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.21–1.33 (m, 24H); 1.65–1.71 (m, 6H); 1.91 (m, 4H); 3.35 (s, 3H); 3.48–3.55 (m, 2H); 3.77–3.89 (m, 4H); 4.09 (t, 2H, J = 7.1 Hz); 4.48 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.8; 22.7; 26.0; 27.9; 28.7; 29.3; 29.6; 29.7; 31.9; 51.6; 59.7; 64.1; 65.5; 174.2 (d, J = 234 Hz); ^{31}P NMR ($CDCl_3$) δ –5.64; HRMS calcd. for $C_{25}H_{51}O_5NP$ = 476.3499; found m/z: $[M + H]^+$ 476.3501; Anal. calcd. for $C_{25}H_{50}O_5NP \times 0.4\ H_2O$: C: 62.19; H: 10.60; N: 2.90; found: C: 62.10; H: 10.56; N: 2.80.

*4.1.4.5. 2-(N-methylmorpholinio-1-yl)ethyl pentadecyloxycarbonylphosphonate (**C15-PFA-M**)*. Yield: 27.9%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 7.1 Hz); 1.21–1.25 (m, 24H); 1.63–1.68 (m, 2H); 3.50 (s, 3H); 3.66–3.81 (m, 4H); 4.02–4.11 (m, 8H); 4.47 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.9; 28.6; 29.4; 29.6; 29.7; 31.9; 48.1; 59.2; 60.7; 60.8; 64.3; 64.7; 173.9 (d, J = 236 Hz); ^{31}P NMR ($CDCl_3$) δ –5.84; HRMS calcd. for $C_{23}H_{47}O_6NP$ = 464.3136; found m/z: $[M + H]^+$ 464.3135; Anal calcd. for $C_{23}H_{46}O_6NP \times 0.75\ H_2O$: C: 57.90; H: 10.04; N: 2.94; found: C: 57.97; H: 9.87; N: 2.81.

*4.1.4.6. 2-(trimethylaminio)ethyl hexadecyloxycarbonylphosphonate (**C16-PFA-C**)*. Yield: 26.9%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.25–1.30 (m, 26H); 1.68 (qi, 2H, J = 7.0 Hz); 3.32 (s, 9H); 3.78 (m, 2H); 4.12 (t, 2H, J = 7.0 Hz); 4.44 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.2; 22.7; 25.9; 28.6; 29.3; 29.4; 29.6; 29.7; 54.6; 59.7; 64.5; 66.6; 173.4 (d, J = 238 Hz); ^{31}P NMR ($CDCl_3$) δ –5.32; HRMS calcd. for $C_{22}H_{47}O_5NP$ = 436.3186; found m/z: $[M + H]^+$ 436.3192; Anal. calcd. for $C_{22}H_{46}O_5NP \times 0.8\ H_2O$: C: 58.72; H: 10.66; N: 3.11; found: C: 58.70; H: 10.35; N: 2.93.

*4.1.4.7. 2-(N-methylpiperidinio-1-yl)ethyl hexadecyloxycarbonylphosphonate (**C16-PFA-P**)*. Yield: 21.2%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.25–1.33 (m, 26H); 1.65–1.75 (m, 4H); 1.89–1.91 (m, 4H); 3.36 (s, 3H); 3.50–3.57 (m, 2H); 3.69–3.77 (m, 2H); 3.90–3.93 (m, 2H); 4.09 (t, 2H, J = 7.0 Hz); 4.48 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 20.2; 21.0; 22.7; 26.0; 28.6; 29.4; 29.6; 29.7; 31.9; 48.6; 59.1; 62.1; 64.2; 174.1 (d, J = 235 Hz); ^{31}P NMR ($CDCl_3$) δ –4.37; HRMS calcd. for $C_{25}H_{51}O_5NP$ = 476.3499; found m/z: $[M + H]^+$ 476.3503; Anal calcd. for $C_{25}H_{50}O_5NP \times 0.75\ H_2O$: C: 61.39; H: 10.61; N: 2.86; found: C: 61.51; H: 10.27; N: 2.66.

*4.1.4.8. 2-(N-methylazepanio-1-yl)ethyl hexadecyloxycarbonylphosphonato (**C16-PFA-A**)*. Yield: 16.8%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.25 (m, 26H); 1.65–1.72 (m, 6H); 1.91 (m, 4H); 3.35 (s, 3H); 3.49–3.54 (m, 2H); 3.76–3.87 (m, 4H); 4.10 (t, 2H, J = 6.9 Hz); 4.49 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.8; 22.1; 26.0; 27.8; 28.7; 29.4; 29.6; 29.7; 51.8; 59.6; 64.2; 65.5; 174.4 (d, J = 233 Hz); ^{31}P NMR ($CDCl_3$) δ –5.76; HRMS calcd. for $C_{26}H_{53}O_5NP$ = 490.3656; found m/z: $[M + H]^+$ 490.3660; Anal. calcd. for $C_{26}H_{52}O_5NP \times 0.6\ H_2O$: C: 62.40; H: 10.71; N: 2.80; found: C: 62.39; H: 10.56; N: 2.71.

*4.1.4.9. 2-(N-methylmorpholinio-1-yl)ethyl hexadecyloxycarbonylphosphonate (**C16-PFA-M**)*. Yield: 25.5%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.19–1.25 (m, 26H); 1.63–1.68 (m, 2H); 3.48 (s, 3H); 3.65–3.79 (m, 4H); 4.02–4.11 (m, 8H); 4.47 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.9; 28.7; 29.4; 29.6; 29.7; 31.9; 48.2; 59.2; 60.7; 60.8; 64.4; 173.7 (d, J = 237 Hz); ^{31}P NMR ($CDCl_3$) δ –5.99; HRMS calcd. for $C_{24}H_{49}O_6NP$ = 478.3292; found m/z: $[M + H]^+$ 478.3293; Anal. calcd. for $C_{24}H_{48}O_6NP \times 0.7\ H_2O$: C: 58.80; H: 10.16; N: 2.86; found: C: 58.76; H: 10.05; N: 2.70.

*4.1.4.10. 2-(trimethylaminio)ethyl octadecyloxycarbonylphosphonate (**C18-PFA-C**)*. Yield: 21.2%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.6 Hz); 1.25 (m, 30H); 1.65–1.70 (m, 2H); 3.32 (s, 9H); 3.77–3.80 (m, 2H); 4.12 (t, 2H, J = 7.0 Hz); 4.44 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.2; 22.7; 25.9; 29.4; 29.6; 29.7; 54.6; 59.8; 64.6; 66.6; 173.3 (d, J = 241 Hz); ^{31}P NMR ($CDCl_3$) δ –5.49; HRMS calcd. for $C_{24}H_{51}O_5NP$ = 464.3499; found m/z: $[M + H]^+$ 464.3499; Anal calcd. for $C_{24}H_{50}O_5NP \times 1.1\ H_2O$: C: 59.63; H: 10.88; N: 2.90; found: C: 59.56; H: 10.64; N: 2.66.

*4.1.4.11. 2-(N-methylpiperidinio-1-yl)ethyl octadecyloxycarbonylphosphonate (**C18-PFA-P**)*. Yield: 15.6%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.25 (m, 36H); 1.65–1.73 (m, 4H); 3.29 (s, 3H); 3.45–3.50 (m, 2H); 3.60–3.64 (m, 2H); 3.82 (m, 2H); 4.11 (t, 2H, J = 7.0 Hz); 4.46 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 20.1; 21.0; 22.7; 25.9; 26.8; 28.6; 29.4; 29.6; 29.7; 31.9; 48.6; 59.1; 62.3; 64.4; 174.3 (d, J = 236 Hz); ^{31}P NMR ($CDCl_3$) δ –5.40; HRMS calcd. for $C_{27}H_{55}O_5NP$ = 504.3812; found m/z: $[M + H]^+$ 504.3814; Anal. calcd. for $C_{27}H_{54}O_5NP \times 0.5\ H_2O$: C: 63.25; H: 10.81; N: 2.73; found: C: 63.19; H: 10.55; N: 2.50.

*4.1.4.12. N,N-dimethylpiperidinio-4-yl octadecyloxycarbonylphosphonate (**C18-PFA-4P**)*. Yield: 25.5%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.6 Hz); 1.26–1.31 (m, 30H); 1.63–1.70 (m, 2H); 2.05–2.25 (m, 4H); 3.09 (s, 3H); 3.19 (s, 3H); 3.25–3.29 (m, 2H); 3.70–3.73 (m, 2H); 4.13 (t, 2H, J = 6.9 Hz); 4.63 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2; 22.8; 26.0; 26.9; 27.0; 28.7; 29.4; 29.5; 29.7; 29.8; 32.0; 48.1; 55.9; 58.6; 64.6; 173.2 (d, J = 244 Hz); ^{31}P NMR ($CDCl_3$) δ –6.19; HRMS calcd. for $C_{26}H_{53}O_5NP$ = 490.3656 found m/z: $[M + H]^+$ 490.3647; Anal. calcd. for $C_{26}H_{52}O_5NP \times 0.75\ H_2O$: C: 62.06; H: 10.72; N: 2.78; found: C: 62.07; H: 10.61; N: 2.67.

*4.1.4.13. 2-(N-methylazepanio-1-yl)ethyl octadecyloxycarbonylphosphonate (**C18-PFA-A**)*. Yield: 7.2%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.26 (m, 30H); 1.65–1.72 (m, 6H); 1.91 (m, 4H); 3.36 (s, 3H); 3.50–3.55 (m, 2H); 3.77–3.88 (m, 4H); 4.10 (t, 2H, J = 7.1 Hz); 4.49 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.8; 22.7; 26.0; 27.9; 28.7; 29.4; 29.6; 29.7; 31.9; 51.7; 59.6; 64.2; 65.5; 174.1 (d, J = 235 Hz); ^{31}P NMR ($CDCl_3$) δ –5.54; HRMS calcd. for $C_{28}H_{57}O_5NP$ = 518.3969; found m/z: $[M + H]^+$ 518.3968; Anal. calcd. for $C_{28}H_{56}O_5NP \times 0.7\ H_2O$: C: 63.41; H: 10.91; N: 2.64; found: C: 63.45; H: 10.71; N: 2.55.

*4.1.4.14. 2-(N-methylmorpholinio-1-yl)ethyl octadecyloxycarbonylphosphonate (**C18-PFA-M**)*. Yield: 20,9%; ^{1}H NMR

($CDCl_3$) δ 0.88 (t, 3H, J = 6.6 Hz); 1.25–1.30 (m, 30H); 1.65–1.68 (m, 2H); 3.41 (s, 3H); 3.61–3.71 (m, 4H); 3.94–4.01 (m, 6H); 4.12 (t, 2H, J = 6.9 Hz); 4.46 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 25.9; 28.6; 29.3; 29.4; 29.6; 29.7; 32.0; 48.3; 59.0; 60.7; 61.0; 64.6; 65.0; 172.8 (d, J = 241 Hz); ^{31}P NMR ($CDCl_3$) δ –5.81; HRMS calcd. for $C_{26}H_{53}O_6NP$ = 506.3605; found m/z: $[M + H]^+$ 506.3599; Anal. calcd. for $C_{26}H_{52}O_6NP \times 0.9\ H_2O$: C: 59.84; H: 10.39; N: 2.68; zistená: C: 59.90; H: 10.44; N: 2.61.

4.2. Measurements of physicochemical properties

4.2.1. Equilibrium surface tension measurements

The equilibrium surface tension measurements were carried out according to the procedure described previously [58]. The Wilhelmy plate technique was used for the determination of the solvent surface tension values. Measurements were performed with a Krüss 100 MK2 tensiometer. All samples were prepared by dissolving amphiphilic compounds in deionized water. The stock solutions were prepared in volumetric flask. The measurements were performed at 25 ± 0.1 °C. The measurements of equilibrium surface tension were taken repeatedly. The values were recorded every 360 s. The measurement was stopped if the difference between the values of two successive measurements was less than 5×10^{-5}N·m^{-1}. The break point of the linear parts of the surface tension *vs.* log c curve was used for the determination of the critical micelle concentration (cmc) and surface tension at the cmc (γ_{cmc}). The adsorbed amount of surfactant Γ_{cmc} was also determined from surface tension data. The value was calculated using the Gibbs adsorption isotherm:

$$\Gamma_{cmc} = -[d\gamma/dlogc]_T/(2,\ 303iRT)$$

where γ represents the surface tension (mN/m), c is the concentration of surfactant, i represents the prefactor (APCs have i = 1), R is the gas constant, and T represents the absolute temperature. The slope below the cmc in the surface tension *vs.* log c plots was used for the determination of the surface excess. The values of area per head group at the water/air interface (A_{cmc}) was obtained from the following equation:

$$A_{cmc} = 10^{18}/N_A\Gamma_{cmc}$$

where N_A represents the Avogadrós constant.

4.2.2. Dynamic light scattering

The hydrodynamic diameter of APCs was determined according the modified procedure described previously [59] using a dynamic light scattering equipment (Brookhaven BI 9000 digital correlator, Brookhaven SM 200 goniometer, Lexel argon laser). The argon laser at 514.5 nm wavelength was used as the incident light source. The intensity time fluctuations of the scattered light were detected at a scattering angle of 90° and a temperature 25 °C and autocorrelated in the BI 9000 correlator card where the time correlation function was built. The translation diffusion coefficient was calculated from the time correlation function using the method of cumulants. The method of cumulants was used for the calculation of the mean particle diameter from the expansion of logarithm of time correlation function into a series up to the second quadratic term. The diffusion coefficient was determined from the correlation function decay rate and the hydrodynamic diameter d_H was calculated from the diffusion coefficient D using the Stokes–Einstein formula d_H = kT / (3πηD). η is solvent viscosity, k is the Boltzmann constant, and T is absolute temperature. Five independent measurements and calculations of time correlation function were carried out for each APC. The mean value and the standard deviation of d_H for each sample were calculated. The concentration of APCs in deionized water was 200-times higher than cmc. To determine particle size distributions and hence, the population of small micelles and large aggregates of APCs, a numerical algorithm based on the inverse Laplace transformation was applied to the time correlation function. Size of the aggregates was evaluated from the partition size distributions utilizing the CONTIN algorithm [60] with the rejection probability parameter set to 0.5. Due to the large sets of processed data, a custom application software written in Visual Basic was used for automated data format conversion from the measurement files.

4.3. Determination of biological activity

4.3.1. Cytotoxicity assay

Hela cells (human cervical carcinoma) were cultured in RPMI 1640 medium (Biosera, Kansas City, MO, United States). MCF-7 (human breast adenocarcinoma) and 3T3 (murine fibroblasts) cell lines were maintained in a growth medium consisting of high glucose Dulbecco's Modified Eagle Medium with sodium pyruvate (GE Healthcare, Piscataway, NJ, United States). The growth medium was supplemented with a 10% fetal bovine serum, 1X HyClone™ Antibiotic/ Antimycotic Solution (GE Healthcare, Little Chalfont, UK). Cells were cultured in an atmosphere containing 5% CO2 in humidified air at 37 °C. Cell viability, estimated by trypan exclusion, was > 95% before each experiment.

The cytotoxic effect of novel alkylphosphocholines was determined by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5×10^3cells/well in 96-well polystyrene microplates (SARSTEDT, Nümbrecht, Germany). 24 h after cell seeding, tested compounds in various concentrations (1×10^{-4} to 1×10^{-8} mol·l^{-1}) were added. After 72 h, cells in each well were incubated with 10 μL of MTT (5 mg·mL^{-1}, Sigma-Aldrich Chemie, Steinheim, Germany) at 37 °C. After an additional 4 h, during which insoluble formazan was produced, 100 μL of a 10% sodium dodecyl sulphate (SDS) was added in each well and another 12 h were allowed for the formazan to dissolve. The absorbance was measured at 540 nm using the automated Cytation™ 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT, United States). Three independent experiments were performed for each test. Results obtained from MTT assay were used to determine a half maximal inhibitory concentration (IC_{50}) of each tested compound.

4.3.2. Antimicrobial assay

The antimicrobial activity was measured against Gram-positive bacteria *S. aureus* ATCC 29/58, Gram-negative bacteria *E. coli* ATCC 377/79 and yeast *C. albicans* ATCC 8186. The testing was performed by a method used previously by Lukáč et al. [61]. Solutions of studied compounds were prepared in water. The stock solution concentration was 1000 μg/mL. The cultures of bacteria and yeast grew 24 h in blood agar (*S. aureus*), in Endo agar (*E. coli*) and in the Sabouraud agar (*C. albicans*). From these cultures suspensions, of microorganisms were prepared with the concentration of 5×10^7 CFU/mL of bacteria and 5×10^5 CFU/mL of *Candida albicans*. The suspensions were prepared in physiological solution (pH 7.2). The concentration of microorganisms was determined spectrophotometrically and the suspensions were adjusted to the absorbance value 0.35 at 540 nm. The microorganisms suspension (5 mL) was added to the solutions containing the tested compound (100 mL), to double concentrated peptone broth medium (8%) for bacteria and to the Sabouraud medium (12%) for *Candida albicans* (100 mL). For a testing assay the solutions were serially diluted by half (concentrations of the solutions were: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 μg/mL). The tests were performed in 96-well microtiter plates. The microorganisms were incubated for 24 h at 37 °C. From each well, 5 mL of tested suspension was taken and cultured on blood agar (*S. aureus*), on Endo agar (*E. coli*) or on Sabouraud agar (yeast). The Petri dishes were incubated for 24 h at 37 °C. The lowest concentration of APC that prevented colony formation was defined as the minimum inhibitory concentration (MIC).

4.3.3. In vitro amoebicidal activity assay

The stock solutions of the investigated compounds were prepared by dissolving APCs in sterile distilled water. The trophocidal activity

against acanthamoebae was tested according to the previously described procedure [14]. *Acanthamoeba lugdunensis* (strain AcaVNAK02, T4 genotype) and *A. quina* (strain AcaVNAK03, T4 genotype) represented clinical isolates of free-living amoebae, which were isolated from the corneas of two patients who suffered from *Acanthamoeba* keratitis. In brief, from the 2-day monoxenic cultures on agar, the amoebae were axenized by inoculation into the Bacto-Casitone/Serum medium (BCS) with ampicillin and penicillin. Incubation was performed for 72 h. The active trophozoites were then inoculated into peptone-yeast extract-glucose medium (PYG) which contained ampicillin and penicillin. The cultures were passaged five times. PYG medium without antibiotics was then used for axenic cultivation of trophozoites. The measurements of cytotoxicity were performed in 96-well microtiter plates. The cultivations were carried out under sterile conditions and at 37 °C. Trophozoite suspension of the volume of 100 μL (2×10^5 cells·mL^{-1}) was added to each well. Afterwards, 100 μL of the solutions with the tested compounds in PYG medium was added to all wells, except for the untreated control. In this case, the wells were filled with 100 μL of pure medium. Each compound was tested at six concentrations. The final concentrations were 500, 250, 125, 62.5, 31.25 and 15.6 μM. The reduction of trophozoites was investigated after 24 h. The surviving cells were counted in a Bürker-Türk hemocytometer. A method of the trypan blue exclusion was used for the detection of viability of trophozoites. 100% trophocidal activity of the APCs was confirmed by transferring 50 μL of the suspension to a PYG medium. The amoeba growth was investigated afterwards for 14 days. The absence of amoeba in cultures indicated 100% eradication. The linear regression analysis using Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was used for the calculation of the EC_{50} (effective concentration of the tested compound that reduces the survival of amoebae by 50%). All experiments were performed four times for each concentration.

Declaration of Competing Interest

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.

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