European Journal of Medicinal Chemistry 66 (2013) 46–55

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis, self-aggregation and biological properties of alkylphosphocholine and alkylphosphohomocholine derivatives of cetyltrimethylammonium bromide, cetylpyridinium bromide, benzalkonium bromide (C16) and benzethonium chloride

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ARTICLE INFO

Article history:
Received 4 February 2013
Received in revised form
21 May 2013
Accepted 22 May 2013
Available online 3 June 2013

Keywords:
Acanthamoeba
Alkylphosphocholines
Candida albicans
Critical micelle concentration
Cytotoxic activity
Quaternary ammonium compounds

ABSTRACT

A series of alkylphosphocholine and alkylphosphohomocholine derivatives of cetyltrimethylammonium bromide, cetylpyridinium bromide, benzalkonium bromide (C16) and benzethonium chloride have been synthesized. Their physicochemical properties were also investigated. The critical micelle concentration (cmc), the surface tension value at the cmc (γ_{cmc}), and the surface area at the surface saturation per head group (A_{cmc}) were determined by means of surface tension measurements. The prepared compounds exhibit significant cytotoxic, antifungal and antiprotozoal activities. Alkylphosphocholines and alkylphosphohomocholines possess higher antifungal activity against *Candida albicans* in comparison with quaternary ammonium compounds in general. However, quaternary ammonium compounds exhibit significantly higher activity against human tumor cells and pathogenic free-living amoebae *Acanthamoeba lugdunensis* and *Acanthamoeba quina* compared to alkylphosphocholines. The relationship between structure, physicochemical properties and biological activity of the tested compounds is discussed.

1. Introduction

Cetyltrimethylammonium bromide (**CTAB**), cetylpyridinium bromide (**CPB**), benzalkonium bromide/chloride, and benzethonium chloride (**BztCl**) are well known quaternary ammonium compounds (QUATs) (Fig. 1) [1]. They are typical representatives of this group of amphiphilic compounds. All four surfactants have considerable chemotherapeutic properties. They possess antibacterial [2–4], antimycotic [5], antiprotozoal [6] or antiproliferative activities [7,8]. These properties of QUATs are utilized in many products used in industry, healthcare or daily usage: cleaning and disinfecting products, nose decongestant lotions, baby lotions, sun protection creams, acne treatment, eye-wash, pain relief creams, make-up removal products, mouthwashes, tooth paste, etc. [1].

Insertion of the ethyl phosphate group to the molecule of **CTAB** leads to the formation of hexadecylphosphocholine (**HPC**, miltefosine) (Fig. 1), the main representative of alkylphosphocholines (APCs). **HPC** has a wide spectrum of biological activities. It possesses antineoplastic [9], antimycotic [10], antibacterial [11], and antiprotozoal activities [12]. **HPC** has been registered as a drug for the topical treatment of breast cancer (Miltex®) and as the first oral drug for treatment of visceral and cutaneous leishmaniasis (Impavido®) [13].

Another interesting group of APCs are compounds which have three methylene groups in the spacer between phosphate anion and ammonium cation. They contain a moiety which is called homocholine. Therefore, these compounds can be named alkylphosphohomocholines (APHCs). Erucylphosphohomocholine (ErPC3) (Fig. 1),

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http://dx.doi.org/10.1016/j.ejmech.2013.05.033

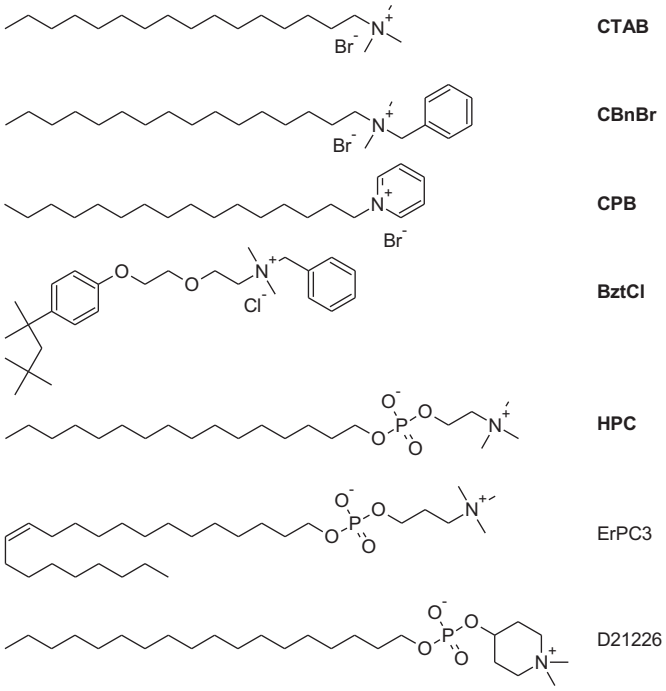


Fig. 1. Structures of well-known QUATs, APC, and APHCs.

which represents APHCs, is the first intravenously applicable APC/APHC studied in potential treatment of human acute myelogenous leukemia [14]. The compound lacks hemolytic toxicity, forms a clear solution in water and is therefore suitable for intravenous administration [15]. Perifosine (D-21226) (Fig. 1) is another antiproliferative active APHC which also contains a homocholine moiety in the molecule enclosed in a piperidine ring. It has been investigated in clinical studies for cancer treatment [16,17].

QUATs, APCs and APHCs are representatives of two groups of amphiphilic compounds, surfactants. QUATs represent cationic surfactants and APCs and APHCs are typical representatives of zwitterionic surfactants. They are not interesting only for their biological activities but their physicochemical properties are also intensively studied. Knowledge about their aggregation [18–20], solubilization [21,22] or biomembrane mimics properties [23,24] help explain biological activities or predict their practical applications.

In our previous studies [25–27], we presented a series of some new APCs and QUATs derived from **HPC** and **CTAB** with potential biological activities which result from their molecular structures, intra- and intermolecular interactions as well as packing ability at the air/water interface. The main goal of this work is the preparation and study of physicochemical and biological properties of a series of some new compounds not synthesized yet and prepared for the first time in our laboratories. We evaluated well-known QUATs, **CTAB**, *N*-benzyl-*N*,*N*-dimethyl-*N*-hexadecylammonium bromide (benzalkonium bromide with hexadecyl alkyl chain, **CBnBr**), **CPB** and **BztCl** (Fig. 1), and APCs and APHCs derived from them. The physicochemical properties were studied by the measurement of the surface tension of the surfactants aqueous solutions. The investigations of biological activities were made on human cancer and non-cancer cell lines, on a strain of pathogenic yeast *Candida albicans*, and on two clinical isolates of amphizoic amoebae *Acanthamoeba lugdunensis* and *Acanthamoeba quina*. The effects of phosphate groups, ammonium cations, and length of the spacers between anions and cations of the prepared compounds on the physicochemical and biological properties were also compared. Our decision for the preparation of APHCs beside APCs was made because the knowledge about this type of compounds is poor and recent literature [14,15,28,29] showed that such compounds in comparison with analogical APCs could have interesting biological activities. Their chemotherapeutic activities in many cases were higher than corresponding APCs, moreover their toxicity was lower.

2. Results and discussion

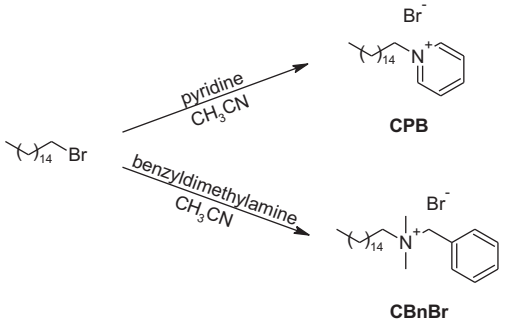
2.1. Chemistry

The synthetic strategies in the preparation of the QUATs are depicted in Scheme 1. **CPB** and **CBnBr** were prepared by an S_N2 reaction of a tertiary amine (pyridine, benzyldimethylamine) with 1-bromohexadecane in a polar solvent using a convenient procedure [30].

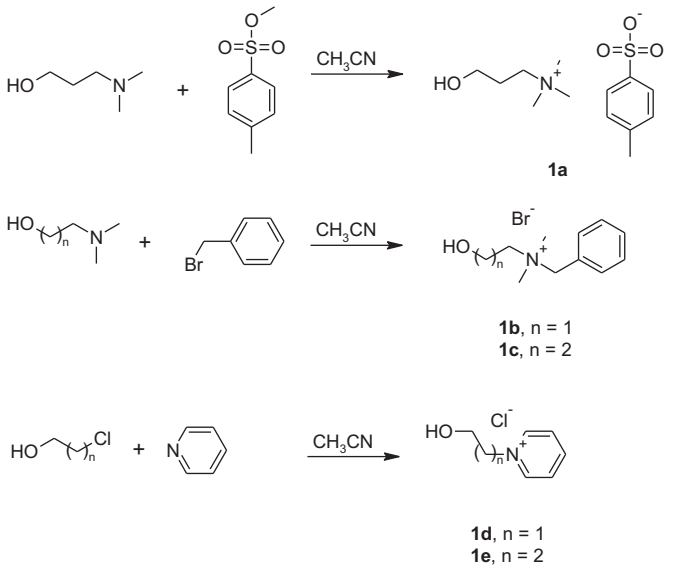
APCs are commonly synthesized by the reaction of alkyl dichloro phosphates [31,32] or alkyl dihydrogen phosphates [33,34] with appropriate choline salts. Other synthetic strategies involve the reaction of cyclic phosphate intermediates with tertiary amines [35,36]. In our study, APCs were obtained from alkyl dihydrogen phosphates (hexadecyl dihydrogen phosphate (**2**) or 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethyl dihydrogen phosphate (**4**)) and choline salts in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TIPS) (Schemes 3 and 4). Choline salts were used as tosylates (**1a**), bromides (**1b**, **1c**) or chlorides (**1d**, **1e**). They were prepared by an S_N reaction of alkylhalogenides or alkyl *p*-toluenesulfonates with tertiary amines (Scheme 2). Hexadecyl dihydrogen phosphate (**2**) was prepared by dissolving hexadecane-1-ol in an excess of $POCl_3$ and subsequently by hydrolysis with water (Scheme 2). 2-[4-(2,4,4-Trimethylpentan-2-yl)phenoxy]ethyl dihydrogen phosphate (**4**) was prepared in two steps (Scheme 4), alkylation of *tert*-octylphenol with 2-chloroethanol and phosphorylation of 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol (**3**). The purities of the compounds were confirmed by ^{31}P NMR spectroscopy; one signal of phosphate group in the spectra was observed.

2.2. Surface-active properties

The surface-active properties of the investigated compounds were studied by measurements of surface tension of surfactants aqueous solutions by the Wilhelmy plate technique. The plots of surface tension *vs.* log concentration curves of the surfactants are shown in Fig. 2 for QUATs, Fig. 3 for APCs, and Fig. 4 for APHCs. Critical micelle concentration (cmc), surface tension at the cmc (γ_{cmc}), and the surface area at the surface saturation per head group (A_{cmc}) of the QUATs, APCs and APHCs are shown in Table 1.



Scheme 1. Preparation of **CPB** and **CBnBr**.



Scheme 2. Preparation of choline derivatives.

The cmc values of **CTAB**, **CBnBr**, **CPB**, **BztCl** and **HPC** corresponded with previously published literature data ($cmc_{(\mathbf{CTAB})}$ = 8.76 × 10^{-4} mol dm^{-3} [37], $cmc_{(\mathbf{CBnBr})}$ = 2.73 × 10^{-4} mol dm^{-3} at 31 °C [38], $cmc_{(\mathbf{CPB})}$ = 6.40 × 10^{-4} mol dm^{-3} [39], $cmc_{(\mathbf{BztCl})}$ = 1.8 × 10^{-3} mol dm^{-3} [40], $cmc_{(\mathbf{HPC})}$ = 1.2 × 10^{-5} mol dm^{-3} [41]). The values of cmcs of QUATs are approximately about 1.5 orders of magnitude higher than the values of the corresponding APCs or APHCs. It is well known that the cmcs of cationic surfactants are higher than the cmcs of zwitterionic surfactants with the same length of alkyl chains [26,42]. APHCs have slightly higher cmcs than APCs. The increasing of cmcs of zwitterionic surfactants with three methylene groups between anion and cation in comparison with two methylenes is caused by the repulsive dipole–dipole interactions between neighboring headgroups [43]. QUATs, APCs and APHCs with hexadecyl alkyl chains and benzyldimethylammonium or pyridinium cations have lower cmcs than the corresponding compounds with trimethylammonium cations. However, **BztCl** and its APCs and APHCs analogs have cmcs higher than **CTAB**, **HPC**, and **HPHC**, respectively.

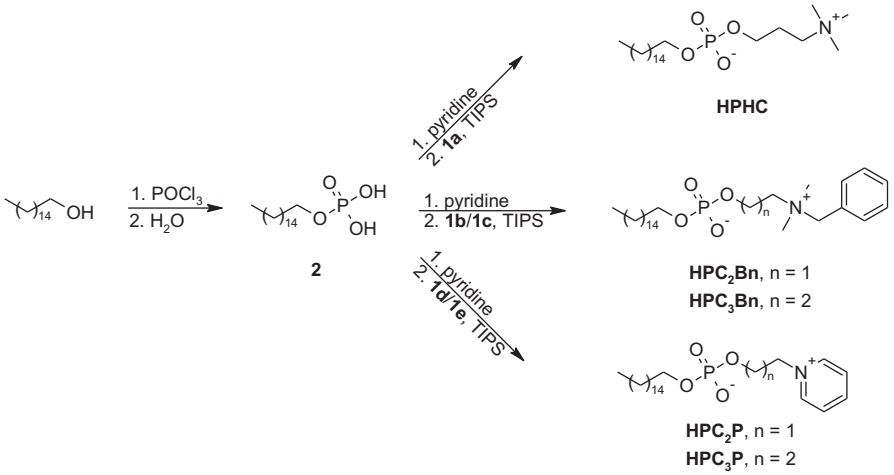
The γ_{cmc} of all compounds are in the range from 35.1 to 39.3 mN m^{-1}, only **PC_2Bzt** and **PC_3Bzt** have lower values. These compounds have two lipophilic parts and the length of the parts is similar. Alkylphosphocholines with two alkyl chains, dialkylphosphocholines (DAPCs), or APCs with branched alkyl chain have lower γ_{cmc} than the APCs with one alkyl chain with the same length of alkyl chain as the sum of two alkyl chains of DAPCs or APCs with branched alkyl chains. **PC_2Bzt** and **PC_3Bzt** are more similar to DAPCs than to APCs. E.g., **HPC** has higher γ_{cmc} (38.3 mN m^{-1}) than octyl 2-[dimethyl(octyl)ammonio]ethyl phosphate (γ_{cmc} = 32 mN m^{-1}) [44] and 2-hexyldecyl 2-(trimethylammonio)ethyl phosphate (γ_{cmc} = 26.1 mN m^{-1}) [45]. Similar influence of the benzyl group on γ_{cmc} is visible in the case of **HPC_2Bn** and **HPC_3Bn**. The benzyl group can be considered as a short lipophilic part. Therefore, **HPC_2Bn** and **HPC_3Bn** could also be considered as dialkylphosphocholines with one long and one short alkyl chain. Influence of short alkyl chain on γ_{cmc} is visible from the values of γ_{cmc} of APCs and APHCs with hexadecyl alkyl chains and trimethylammonium, pyridinium and benzyldimethylammonium cations. APCs and APHCs with benzyldimethylammonium cations have had lower values of γ_{cmc} than APCs and APHCs which do not contain lipophilic part bonded on ammonium cations. Although the length of the alkyl chains of **HPC_2Bn** and **HPC_3Bn** (one long and one short alkyl chain) is different in comparison with **PC_2Bzt** and **PC_3Bzt** (two alkyl chains with not too very different length), the decrease of γ_{cmc} is not as strong as in the case of **PC_2Bzt** and **PC_3Bzt**. Similar influence of different length of the alkyl chains on γ_{cmc} of dialkylphosphocholines with the same empirical formula can be also seen in the results described by Peresypkin and Menger [44]. E.g., compounds with one longer and one shorter alkyl chain namely 2-[dimethyl(dodecyl)ammonio]ethyl octyl phosphate (γ_{cmc} = 26 mN m^{-1}) and dodecyl 2-[dimethyl(octyl)ammonio]ethyl phosphate (γ_{cmc} = 28 mN m^{-1}) have had higher values of γ_{cmc} than compounds with alkyl chains with the same length namely decyl 2-[dimethyl(decyl)ammonio]ethyl phosphate (γ_{cmc} = 24 mN m^{-1}).

A_{cmc}s of QUATs are slightly higher than A_{cmc}s of APCs and APHCs; only **CTAB** with a small polar head group is densely packed. The values of APHCs are slightly higher in comparison with values of APCs in many cases. It is caused by a bigger head group of APHCs in comparison with APCs.

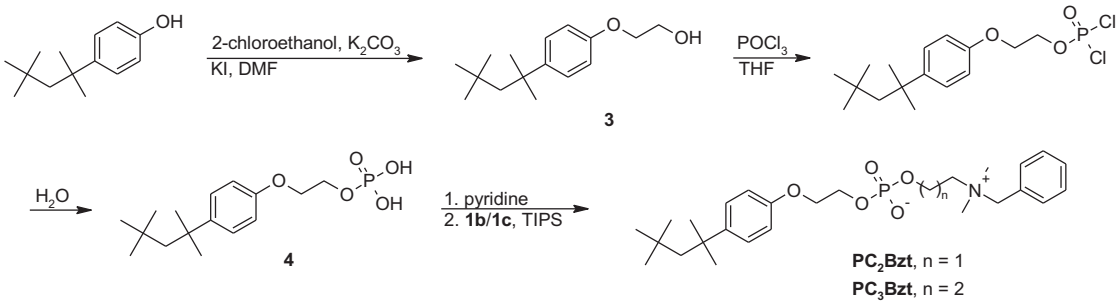
2.3. Biological activities

2.3.1. Antiproliferative activities

The antiproliferative activities of QUATs, APCs and APHCs (Table 2) were determined against acute T-lymphoblastic leukemia cells (CCRF-CEM), human cervical adenocarcinoma cells (HeLa),



Scheme 3. Preparation of APCs and APHCs with a hexadecyl chain.



Scheme 4. Preparation of **PC_2Bzt** and **PC_3Bzt**.

human lung adenocarcinoma cells (A-549), MCF-7 cells (human breast adenocarcinoma, estrogen receptor-positive) and MDA-MB-231 cells (human breast adenocarcinoma, estrogen receptor-negative). The antiproliferative activities of the compounds were compared with their activities against non-cancer cells, human umbilical vein endothelial cells (HUVEC).

CTAB was considered as a standard compound of QUATs, and **HPC** as a standard compound of APCs and APHCs. QUATs had a higher antiproliferative activity in comparison with APCs and APHCs but they were also more toxic against HUVEC than APCs or APHCs. All tested compounds with IC_{50} values shown are more active against cancer cells than non-cancer cells (**PC_2Bzt** and **PC_3Bzt** are excluded from the comparison because their activities against cancer and non-cancer cells are higher than the highest tested concentration of these compounds). It can be expressed by the selectivity index [IC_{50}(HUVEC)/IC_{50}(neoplastic cells)]. In general, the best selectivity indexes were observed in the case of antiproliferative activities of the tested compounds against A549. **HPC**, **CBnBr**, **CPB** and **BztCl** were more than 10-times more active against lung carcinoma than against HUVEC. However, the highest selectivity index can be reported for the activity of **CPB** against HUVEC/MCF-7; its value is 64. The activities of **HPC_2P** against MCF-7 and **BztCl** against CCRF-CEM also showed selectivity index higher than 10.

The standard of QUATs is less antineoplastically active than **CBnBr** and **CPB**. However, the activities of **BztCl** were lower in comparison with **CTAB** apart from its activity against CCRF-CEM. Nevertheless, **BztCl** had the best selectivity indexes of the studied QUATs, if the activity of **CPB** against MCF-7 is not considered. They are in the range of 8–15. These results are in agreement with previous observations, which described **BztCl** as promising antiproliferative compounds [7].

APCs and APHCs with hexadecyl alkyl chains possessed IC_{50} values lower than 85 μM against all tested neoplastic cells. A different situation was observed in the case of **PC_2Bzt** and **PC_3Bzt**. These compounds can be considered antiproliferatively inactive. **PC_2Bzt** and **PC_3Bzt** have cmcs about one order of magnitude higher than other APCs and APHCs which also means that **PC_2Bzt** and **PC_3Bzt** possess lower lipophilicity than other APCs and APHCs. This can be the reason why these compounds did not express antiproliferative activities in tested ranges of concentrations. It is well known that antiproliferative activity increases with increasing lipophilicity of APCs [46].

Comparison of antiproliferative activities of choline and homocholine derivatives showed that APCs are more active than APHCs in many cases. We previously obtained similar results in a comparison of choline and homocholine derivatives of dialkylphosphocholines (DAPCs) [28]. However, Rübel et al. [47] studied the efficacy of ionizing radiation in combination with erucylphosphocholine (ErPC) and ErPC3 in human malignant glioma cell lines *in vitro*. ErPC3 improved efficacy in comparison with ErPC, so they observed that APHC was more active than APC.

2.3.2. *Candida albicans*

Antimycotic activities of the compounds were determined against *C. albicans* (Table 2) which is a representative of pathogenic yeasts.

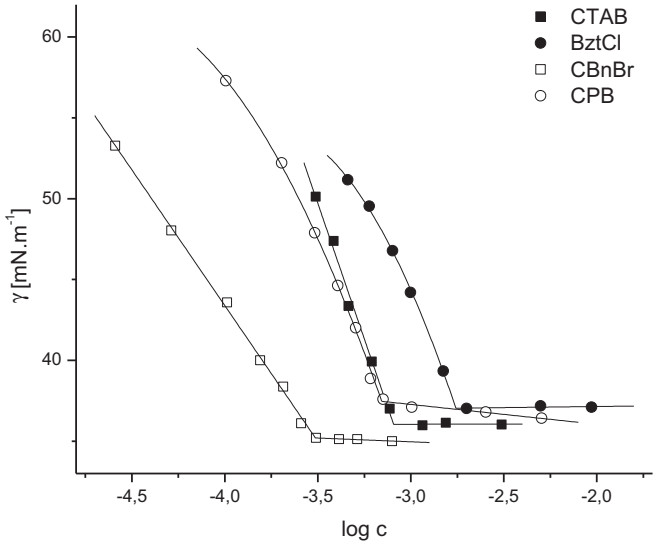


Fig. 2. Plots of surface tension *vs.* log concentration curves of QUATs.

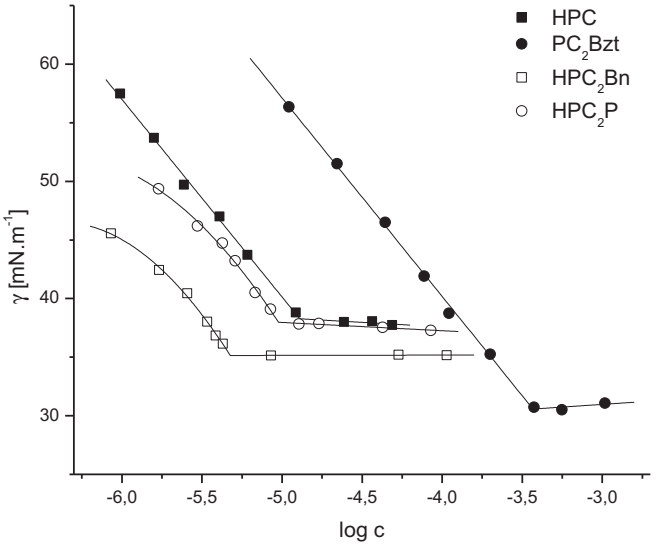


Fig. 3. Plots of surface tension *vs.* log concentration curves of APCs.

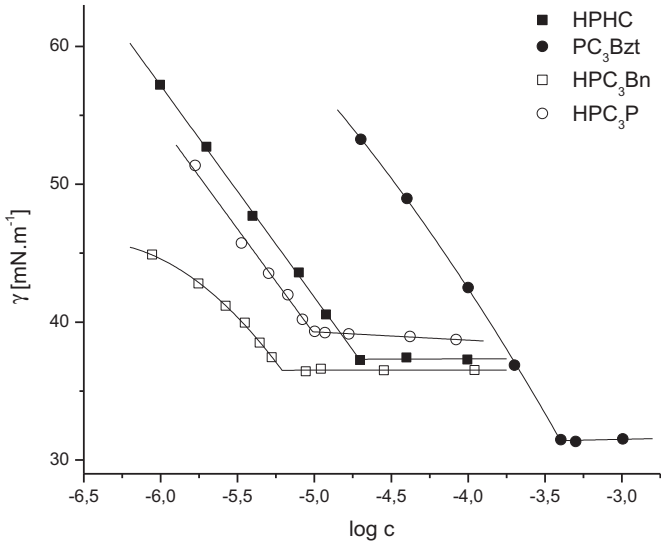


Fig. 4. Plots of surface tension *vs.* log concentration curves of APHCs.

The most active evaluated compounds were **HPC** and **HPC_3P**. Other APCs and APHCs had two or more times higher values of MIC than these two compounds but they also had very significant activity too. There are only two substances, **PC_2Bzt**, **PC_3Bzt**, with no anticandidal activity detected in the tests. It is interesting that the extension of the spacer between the phosphate group and the ammonium cation by one methylene group in the case of **HPC** led to a substantial decrease in activity. However, the same structural change in a pyridinium analog of **HPC** led to a slight increase of activity and the activity of the pair **HPC_2Bn**-**HPC_3Bn** was practically not affected. The cmcs of **HPC** and **HPC_3P** are very similar and it can be one of the reasons of the similarity of these two compounds' anticandidal action. The second reason in similarity of anticandidal activity of these two compounds can be caused by their interaction with enzymes or subcellular compartments of yeast. Widmer et al. [10] have shown that one mode of action of **HPC** can be dedicated to inhibition of fungal phospholipase B1. One can expect that **HPC** and **HPC_3P** have the optimal design of polar head group which caused the highest inhibition of activity of the enzyme.

The most active QUAT was **CBnBr**. The MIC of **CPB** was approximately two times higher than the MIC of **CBnBr** and other two QUATs were less active than the pyridinium salt or **CBnBr**.

2.3.3. *Acanthamoeba spp.*

Antiprotozoal activities of the compounds were determined against two clinical isolates of *A. lugdunensis* and *A. quina* (Table 2), causative agents of amoebic keratitis.

Table 1
Physicochemical properties of QUATs, APCs, and APHCs.

Compound	cmc [mol dm^{-3}]	γ_{cmc} [mN m^{-1}]	A_{cmc} [Å^2]
CTAB [18]	$(8.5 \pm 0.1) \times 10^{-4}$	36.0	58 ± 3
CBnBr	$(3.1 \pm 0.1) \times 10^{-4}$	35.2	113 ± 2
CPB	$(7.3 \pm 0.1) \times 10^{-4}$	37.2	69 ± 2
BztCl	$(2.0 \pm 0.1) \times 10^{-3}$	37.0	77 ± 3
HPC [18]	$(1.3 \pm 0.1) \times 10^{-5}$	38.3	57 ± 3
HPHC	$(2.0 \pm 0.1) \times 10^{-5}$	37.2	62 ± 2
HPC_2Bn	$(5.0 \pm 0.2) \times 10^{-6}$	35.1	58 ± 4
HPC_3Bn	$(6.2 \pm 0.1) \times 10^{-6}$	36.4	66 ± 2
HPC_2P	$(9.5 \pm 0.2) \times 10^{-6}$	38.0	50 ± 3
HPC_3P	$(9.8 \pm 0.3) \times 10^{-6}$	39.3	62 ± 3
PC_2Bzt	$(3.8 \pm 0.1) \times 10^{-4}$	30.4	56 ± 1
PC_3Bzt	$(4.3 \pm 0.1) \times 10^{-4}$	31.3	56 ± 2

QUATs expressed very good amebicidal activities. **CTAB**, **CBnBr** and **CPB** possessed values of trophocidal activities against both strains equal to 15.6 μM. Only **BztCl** was less active. The activities of the investigated QUATs are comparable with activities of dialkylamino and nitrogen heterocyclic derivatives of **CTAB**, which were published previously [25] (Table 2).

APCs and APHCs were less active than QUATs. Only the activity of **HPC_3P** against *A. quina* reached the activities observed in the case of **CTAB**, **CBnBr** or **CPB**. However, surprisingly, the activity of the compound against *A. lugdunensis* was relatively weak. It was comparable to the activity of the standard, **HPC**. The second pyridinium compound, **HPC_2P**, was also markedly active. The MTCs of the compound against both strains were 62.5 μM. The value in the case of *A. lugdunensis* was the lowest of all investigated APCs and APHCs. Comparison of the activities of APCs and APHCs showed that the activities of APHCs are generally better than the activities of APCs with the same hydrophobic part and architecture of the ammonium cation. Only **HPC_2P** was more active than **HPC_3P** against *A. lugdunensis* and the activities of **PC_2Bzt** and **PC_3Bzt** against the same strain were identical.

2.3.4. *Structure–action relationship of QUATs, APCs, APHCs*

From the results presented we can say that the antineoplastic and antiprotozoal activities of QUATs studied in this paper are higher than that of respective APCs and APHCs. However, the activities of QUATs against the yeast *Candida* are in general lower than the activities of APCs and APHCs. In their study Šeršeň et al. [48] used model membranes for comparison of perturbation effects of cationic and zwitterionic tensides with the same lipophilic parts on such models. They found that QUAT possessed somewhat higher perturbation effect than similar zwitterionic tenside. It is very likely that the antineoplastic and antiprotozoal activities of tensides could be mainly caused by a non-specific mode of action. Such a phenomenon, namely the biophysical destruction of the cell membrane as a basic reason for chemotherapeutic effect by QUATs, APCs and APHCs was described also in the literature [12,49,50]. The amphiphilic compounds incorporated into biological membranes do interfere with the formation and behavior of the membrane's microdomains, lipid-based signal transduction pathways or phospholipid turnover [12,49–51].

The higher antimycotic activities of APCs and APHCs against *C. albicans* compared with QUATs could be ascribed to specific mode of action of APCs and APHCs against yeasts. Widmer et al. [10] did show that the **HPC** acts at the subcellular level, e.g. inhibiting the phospholipase B1. However, this mode of action could hardly be the only one which could explain the antimycotic efficacy of APCs and APHCs. As an example, the paper of Zhou at al. [52] could be recommended. The authors studied the apoptosis-like cell death caused by **HPC**. The model organisms were the cells of *Saccharomyces cerevisiae* yeasts. The results show that the **HPC** is responsible for the death of the cells by their specific interaction with the substructure of Cox9p of the mitochondrial Cytochrome C Oxidase (COX). **HPC** disintegrates the electron transport chain by a partial destruction of COX which in turn leads to an apoptosis-like cell death. This mode of action could play a significant role with other yeasts e.g. *Candida* too.

If we compare the cationic parts of the molecules and their biological activities it is obvious that the pyridinium salts are in this study the most antimicrobially active compounds. Moreover, from all in this work investigated compounds in most of the cases the pyridinium QUAT tested against cancer cells possesses the lowest value of the IC_{50}. The substitution of the methyl group by a benzyl moiety in the cationic parts of the molecules was generally less advantageous from the biological activity point of view.

Table 2
Cytotoxic, antiprotozoal, and anticandidal activities of QUATs, APCs, and APHCs.

Co.	IC_{50} (μM)						MIC (μM)	MTC (μM)	
	CEM	HeLa	A549	MCF	MDA	HUVEC	C.a.	A.l.	A.q.
CTAB	4.0	4.5	1.3	1.3	3.1	6.1	22.0	**15.6**	**15.6**
CBnBr	0.90	2.8	0.63	1.0	3.5	7.5	4.5	**15.6**	**15.6**
CPB	0.67	4.3	0.42	**0.07**	2.1	4.5	9.9	**15.6**	**15.6**
BztCl	3.8	5.7	3.2	5.0	4.8	47	35.7	250	500
HPC	20.7	74.4	8.6	18.9	34.8	>100	**2.2**	250	125
HPHC	6.2	84.4	41.5	45.2	61.7	>100	18.2	125	62.5
HPC_2Bn	13.8	55.7	73.2	76.0	44.3	>100	8.1	>500	>500
HPC_3Bn	62.2	62.1	57.6	57.6	43.7	>100	7.9	125	250
HPC_2P	22.9	52.1	9.9	8.1	45.8	94	4.0	62.5	62.5
HPC_3P	34.6	74.7	61.6	50.4	59.9	>100	**2.1**	250	**15.6**
PC_2Bzt	>100	>100	>100	>100	>100	>100	>100	250	500
PC_3Bzt	>100	>100	>100	>100	>100	>100	>100	250	125

Co. – compound, C.a. – *Candida albicans*, A.l. – *Acanthamoeba lugdunensis*, A.q. – *Acanthamoeba quina*. Values in bold character correspond to the most active compounds.

If we compare the biological activities of APCs with the APHCs which are bearing the same hexadecyl alkyl chain we can see that the APCs possess in general higher antineoplastic activities. However, interestingly, the reversed trend was shown by the antiprotozoal activity. In general, compared to APCs, the bigger head-groups of APHCs caused an easier destruction of the cytoplasmic membrane of protozoae. Similar results were also observed with heterocyclic derivatives of **HPC**. The most effective APCs where those with the biggest heterocyclic rings in the cationic part of the molecules namely derivatives of azepane and azocane. Nevertheless, the opposite trend was shown with short alkyl chains in the cationic part of the molecule. Some of those molecules could be considered as "open" heterocycles (e.g. the diethyl derivative could be considered as an "open" pyrrolidine, the dipropyl derivative as an "open" azepane, etc.). So when two methyl groups were substituted by two ethyl groups (an open pyrrolidine) the antiprotozoal activity increased. Further increase of the chains length (two propyl groups instead of two methyl groups; "open" azepane) decreased the activity. The biological activity is also dependent on the size of the cationic head-group. Molecular modeling studies showed that two flexible propyl groups occupy sterically more space than the rigid cyclic azepane ring [27]. Therefore, when the heterocyclic ring was "opened" and the size of the head-group exceeded the optimal limit, the antiprotozoal activity started to decrease.

It is obvious that the action of APCs and APHCs against yeasts is significantly sensitive to the size of the cationic polar head-group. From the results the conclusion could be taken that the **HPC** and **HPC_3P** possess the optimal design and size of the polar head-groups concerning the biological activity against yeasts. Generally, antifungal activities of APCs are very sensitive to modifications of the chemical structure of the compounds as we discussed previously [45].

3. Conclusion

Twelve compounds were studied, four QUATs, four APCs and four APHCs. We investigated the effect of a polar head group of the QUATs, APCs and APHCs on physicochemical properties and cytotoxic, antifungal and amebicidal activities. In this study, we focused on modifying the ammonium cation of **HPC** and **CTAB**, and the length of the spacer between the ammonium cation and the phosphate group of alkylphosphocholines which led to the preparation of some new APCs and APHCs derived from well-known QUATs.

The physicochemical properties of the compounds were studied by the measurement of surface activity of their aqueous solutions. The most important parameter obtained from the measurement was the critical micelle concentration. The cmc could be taken as the measure of lipophilicity [53]. APCs and APHCs have about 1.5 orders of magnitudes lower values than QUATs. APHCs have slightly higher cmcs than APCs. It is probably caused by the repulsive dipole–dipole interactions between neighboring head-groups.

The biological activities of QUATs, APCs and APHCs were examined on five cancer cell lines (CCRF-CEM, HeLa, MDA-MB-231, MCF-7 and A-549), non-cancer cells (HUVEC), *C. albicans* and two protists *A. lugdunensis* and *A. quina*. The comparison of cytotoxic activities of QUATs, APCs and APHCs showed that the QUATs exhibited higher cytotoxic activities against the APCs or APHCs. The highest activity was exhibited by **CPB** against MCF-7. Generally, better fungicidal activities against *C. albicans* were determined for the APCs and APHCs in comparison with QUATs. The most active compounds were **HPC** and **HPC_3P**. A different situation was observed in the case of antiprotozoal action of QUATs, APCs and APHCs against *A. lugdunensis* and *A. quina* amoebae. The QUATs were found to have higher trophocidal activity. **CTAB**, **CBnBr** and **CPB** were the most active, and the same activities were observed against both investigated strains. Only one APHC possessed comparable activity with QUATs. The MTC of **HPC_3P** against *A. quina* showed a value similar to **CTAB**, **CBnBr**, and **CPB**. We can conclude that the antineoplastic and antiprotozoal activities of the studied QUATs are generally higher than those of APCs or APHCs. However, the anticandidal activities of QUATs are generally lower than the activities of APCs and APHCs.

4. Experimental part

4.1. Chemistry

4.1.1. Materials

All chemicals used for the synthesis were purchased from commercial suppliers. **CTAB** and **BztCl** were purchased from Aldrich. **HPC** was prepared previously [25]. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian Mercury plus spectrometer operating at 300, 75.5, and 121.5 MHz, respectively, with ^{13}C and ^{31}P spectra being recorded with proton-decoupling. The spectra were measured in $CDCl_3$ or DMSO-d_6 relative to the internal standard TMS for ^{1}H and ^{13}C NMR spectra and to the external standard 85% H_3PO_4 for ^{31}P NMR spectra. Infrared spectra were recorded on an FT-IR Impact 400 D spectrophotometer as potassium bromide discs. Molecular masses of final compounds were measured by high resolution spectrometer ESI-LTQ Orbitrap XL Thermo Scientific.

4.1.2. Preparation of quaternary ammonium compounds

1-Bromohexadecane (0.01 mol) and a tertiary amine, *N*-benzyl-*N*,*N*-dimethylamine or pyridine, respectively (0.011 mol) were added to 25 ml of dry acetonitrile and heated at reflux for 24 h. Solvent was evaporated, the residue was co-evaporated with benzene and crystallized from acetone.

*4.1.2.1. Hexadecylpyridinium bromide (**CPB**).* Yield 75%; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, J = 6.7 Hz, 3H, CH_2CH_3), 1.25 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.98–2.12 (m, 2H, $OCH_2CH_2(CH_2)_{13}CH_3$), 4.98 (t, J = 7.5, 2H, $OCH_2CH_2(CH_2)_{13}CH_3$), 8.19 (t, J = 7.2 Hz, 2H, H_{meta}), 8.56 (t, J = 7.8 Hz, 1H, H_{para}), 9.47 (d, J = 5.6 Hz, 2H, H_{ortho}); ^{13}C NMR ($CDCl_3$) δ: 14.2, 22.7, 26.1, 29.1, 29.4, 29.5, 29.6, 29.7, 32.0, 32.1, 62.2, 128.6, 145.2, 145.3; IR υ_{max}/cm^{-1} 3381, 2914, 2850, 1636, 1486, 1473, 1176, 777, 715, 681; HRMS calcd. for $C_{21}H_{38}N$ = 304.2999; found m/z: 304.2996 $[M - Br]^+$.

*4.1.2.2. N-Benzyl-N,N-dimethylhexadecane-1-ammonium bromide (**CBnBr**).* Yield 84%; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, J = 6.7 Hz, 3H, CH_2CH_3), 1.26 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.73–1.82 (m, 2H, $OCH_2CH_2(CH_2)_{13}CH_3$), 3.31 (s, 6H, $N^+(CH_3)_2$), 3.50–3.62 (m, 2H, $OCH_2CH_2(CH_2)_{13}CH_3$), 5.11 (s, 2H, CH_2Ph), 7.38–7.51 (m, 3H, H_{meta} and H_{para}), 7.68 (dd, J = 7.6 Hz, J = 1.8 Hz, 2H, H_{ortho}); ^{13}C NMR ($CDCl_3$) δ: 14.2, 22.8, 23.0, 26.4, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 32.0, 49.7, 63.8, 67.4, 127.4, 129.3, 130.8, 133.3; IR υ_{max}/cm^{-1} 2916, 2849, 1473, 1464, 883, 758, 726, 705; HRMS calcd. for $C_{25}H_{46}N$ = 360.3625; found m/z: 360.3620 $[M - Br]^+$.

4.1.3. General procedure for the preparation of choline salts

A tertiary amine (20 mmol) and a halogen alkane or an alkyl tosylate (22 mmol) were dissolved in 25 ml of acetonitrile and refluxed for 4 h (in the case of pyridinium salts the time was extended to 8 h). After cooling down, the acetonitrile was evaporated in vacuum. The resulting mixture was crystallized from acetone or from a mixture of acetone/methanol. The quaternary salts were obtained as white, hygroscopic solids.

3-Hydroxy-*N,N,N*-trimethylpropane-1-ammonium *p*-toluenesulfonate was prepared by quaternization of 3-(dimethylamino) propan-1-ol and methyl *p*-toluenesulfonate; yield: 76%; ^{1}H NMR (DMSO-d_6) δ: 1.77–1.89 (m, 2H, $N^+CH_2CH_2CH_2O$), 2.29 (s, 3H, $PhCH_3$), 3.04 (s, 9H, $N^+(CH_3)_3$), 3.30–3.52 (m, 4H, $N^+CH_2CH_2CH_2O$ and $N^+CH_2CH_2CH_2O$), 4.76–4.88 (m, 1H, OH), 7.12 (d, J = 7.9 Hz, 2H, H_{meta}), 7.48 (d, J = 7.9 Hz, 2H, H_{ortho}); ^{13}C NMR (DMSO-d_6) δ: 20.8, 25.7, 52.1, 57.7, 63.7, 125.5, 128.1, 137.7, 145.7.

1-(2-Hydroxyethyl)pyridinium chloride was prepared by quaternization of pyridine and 2-chloroethanol; yield: 73%; ^{1}H NMR (DMSO-d_6) δ: 3.80–3.93 (m, 2H, $N^+CH_2CH_2OH$), 4.79 (t, J = 5.0 Hz, 2H, $N^+CH_2CH_2OH$), 5.66–5.80 (m, 1H, OH), 8.20 (t, J = 7.2 Hz, 2H, H_{meta}), 8.66 (t, J = 7.9 Hz, 1H, H_{para}), 9.19 (d, J = 5.6 Hz, 2H, H_{ortho}); ^{13}C NMR (DMSO-d_6) δ: 60.1, 62.8, 127.7, 145.3, 145.5.

1-(3-Hydroxypropyl)pyridinium chloride was prepared by quaternization of pyridine and 3-chloropropanol; yield: 68%; ^{1}H NMR (DMSO-d_6) δ: 2.03–2.18 (m, 2H, $N^+CH_2CH_2CH_2O$), 3.38–3.52 (m, 2H, $N^+CH_2CH_2CH_2OH$), 4.73 (t, J = 7.0 Hz, 2H, $N^+CH_2CH_2CH_2OH$), 4.98 (t, J = 4.7 Hz, 1H, OH), 8.16 (t, J = 7.2 Hz, 2H, H_{meta}), 8.60 (t, J = 7.8 Hz, 1H, H_{para}), 9.18 (d, J = 5.3 Hz, 2H, H_{ortho}); ^{13}C NMR (DMSO-d_6) δ: 33.4, 57.1, 58.6, 128.0, 145.1, 145.5.

N-benzyl-2-hydroxy-*N,N*-dimethylethanammonium bromide was prepared by quaternization of 2-(dimethylamino)ethanol and benzyl bromide; yield: 85%; ^{1}H NMR (DMSO-d_6) δ: 3.46 (s, 6H, $N^+(CH_3)_2$), 3.42–3.52 (m, 2H, $N^+CH_2CH_2OH$), 3.90–4.00 (m, 2H, $N^+CH_2CH_2OH$), 4.74 (s, 2H, CH_2Ph), 5.39 (t, J = 5.0 Hz, 1H, OH), 7.47–7.58 (m, 3H, H_{meta} and H_{para}), 7.60–7.69 (m, 2H, H_{ortho}); ^{13}C NMR (DMSO-d_6) δ: 49.8, 54.9, 64.8, 67.2, 128.2, 128.9, 130.3, 133.2.

N-benzyl-3-hydroxy-*N,N*-dimethylpropanammonium bromide was prepared by quaternization of 3-(dimethylamino) propan-1-ol and benzyl bromide; yield: 83%; ^{1}H NMR (DMSO-d_6) δ: 2.13–2.26 (m, 2H, $N^+CH_2CH_2CH_2O$), 3.21 (s, 6H, $N^+(CH_3)_2$), 3.73–3.91 (m, 2H, $N^+CH_2CH_2CH_2OH$), 3.83–3.92 (m, 2H, $N^+CH_2CH_2CH_2OH$), 4.35–4.44 (m, 1H, OH), 4.88 (s, 2H, CH_2Ph), 7.41–7.51 (m, 3H, H_{meta} and H_{para}), 7.65 (d, J = 7.4 Hz, 2H, H_{ortho}); ^{13}C NMR (DMSO-d_6) δ: 25.4, 49.1, 57.6, 61.3, 65.8, 128.1, 128.7, 130.1, 132.9.

4.1.4. Synthesis of 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy] ethanol

4-(2,4,4-Trimethylpentan-2-yl)phenol (0.05 mol) and 2-chloroethanol (0.1 mol) were dissolved in DMF. K_2CO_3 (0.2 mol) and KI (1 g) were subsequently suspended in the solution. The mixture was refluxed for 8 h. After cooling down, the suspension was filtered and the solution was evaporated. The product was distilled at reduced pressure, b.p. = 192–193 °C 2.4 kPa; yield: 77%; ^{1}H NMR ($CDCl_3$) δ: 0.71 (s, 9H, $(CH_3)_3CCH_2(CH_3)_2C$), 1.34 (s, 6H, $(CH_3)_3CCH_2(CH_3)_2C$), 1.70 (s, 2H, $(CH_3)_3CCH_2(CH_3)_2C$), 2.12 (t, J = 6.2 Hz, 1H, OH), 3.91–3.97 (m, 2H, $PhOCH_2CH_2OH$), 4.04–4.09 (m, 2H, $PhOCH_2CH_2OH$), 6.83 (d, J = 8.9 Hz, 2H, $H_{meta(Ph)}$), 7.27 (d, J = 8.9 Hz, 2H, $H_{ortho(Ph)}$); ^{13}C NMR ($CDCl_3$) δ: 31.7, 31.8, 32.3, 38.0, 57.0, 61.6, 69.1, 113.7, 127.1, 142.7, 156.2.

*4.1.5. Synthesis of hexadecyl dihydrogen phosphate (**2**)*

Hexadecan-1-ol (25 mmol) was dissolved in 25 ml of $POCl_3$ and the solution was stirred at ambient temperature for 48 h. After 2 days, hexadecyl dichloro phosphate and unreacted $POCl_3$ were decomposed with water. The water solution was extracted with $CHCl_3$ (3 × 50 ml). The organic phase was dried with anhydrous Na_2SO_4 and the solvent was evaporated. The product was crystallized from hexane. Compound **2** was obtained as a white solid; yield = 53%; ^{1}H NMR ($CDCl_3$:DMSO-d_6 5:1) δ: 0.88 (t, J = 6.7 Hz, 3H, CH_2CH_3), 1.25 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.59–1.72 (m, 2H, OCH_2CH_2), 3.89–4.02 (m, 2H, OCH_2CH_2), 6.48 (br s, 2H, $PO(OH)_2$); ^{13}C NMR ($CDCl_3$:DMSO-d_6 5:1) δ: 14.1, 22.6, 25.5, 29.2, 29.5, 29.6, 30.3, 30.4, 31.8, 66.5.

4.1.6. General procedure for the synthesis of APCs and APHCs

APCs and APHCs were synthesized from 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol or hexadecyl dihydrogen phosphate. The alcohol was phosphorylated first. The alcohol (1 mmol) was treated with phosphorous oxychloride (1.1 mmol) and triethylamine (3 mmol) in anhydrous THF. The solution was hydrolyzed with 1.5 ml H_2O for 1 h and evaporated *in vacuo*. Pyridinium salts of alkyl dihydrogen phosphates were prepared by treatment with pyridine (8 ml) for 2 h at 50 °C. The excess pyridine was evaporated and the rest was dissolved in 5 ml of anhydrous pyridine. Appropriate choline (1.5 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (2 mmol) in pyridine (15 ml) were added to the solution of alkyl dihydrogen phosphate. The solution was stirred at 40 °C overnight. After cooling, the mixture was hydrolyzed by addition of H_2O (1.5 ml) and stirred for 1 h at r.t. The solvents were evaporated in vacuum, and the resulting crude solid was dissolved in a mixture of tetrahydrofuran and water (5:1, V/V). To the stirred solution, exchange resin Amberlite MB3 was added sequentially until the color of the resin ceased to change. Then the resin was filtered off and the solvents were evaporated in vacuum. The remaining mixture was several times coevaporated with propan-2-ol. The resulting crude solid (**PC_2Bzt** and **PC_3Bzt**) was purified by flash chromatography over silica gel using $CHCl_3$/MeOH/H_2O (65/25/4, V/V/V) as a liquid phase. After chromatography the solvents were evaporated *in vacuo*. The residue was dissolved in chloroform and precipitated with acetone or petroleum ether. APC was filtered off and dried in vacuum dessicator over P_4O_{10}. The other APCs and APHCs were purified by crystallization from a mixture of chloroform and acetone.

*4.1.6.1. Hexadecyl 3-(trimethylammonio)propyl phosphate (**HPHC**).* Yield 63%; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, J = 6.3 Hz, 3H, CH_2CH_3), 1.25 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.46–1.60 (m, 2H, OCH_2CH_2),

2.05–2.17 (m, 2H, $N^+CH_2CH_2CH_2O$), 3.30 (s, 9H, $N^+(CH_3)_3$), 3.60–3.84 (m, 4H, $N^+CH_2CH_2CH_2O$ and OCH_2CH_2), 3.90–4.01 (m, 2H, $N^+CH_2CH_2CH_2O$); ^{13}C NMR ($CDCl_3$) δ: 14.1, 22.7, 24.8, 26.0, 29.4, 29.6, 29.7, 29.8, 31.1, 31.9, 53.3, 61.6, 64.1, 65.3; ^{31}P NMR ($CDCl_3$) δ: −0.33; IR υ_{max}/cm^{-1} 3400, 2917, 2850, 1660, 1493, 1470, 1241, 1053, 943, 837, 719; HRMS calcd. for $C_{22}H_{48}O_4NPNa$ = 444.3213; found *m/z*: 444.3209 $[M + Na]^+$.

*4.1.6.2. 2-[Benzyl(dimethyl)ammonio]ethyl hexadecyl phosphate (**HPC$_2$Bn**).* Yield 58%; ^1H NMR ($CDCl_3$) δ: 0.88 (t, J = 6.6 Hz, 3H, CH_2CH_3), 1.25 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.50–1.61 (m, 2H, OCH_2CH_2), 3.30 (s, 6H, $N^+(CH_3)_2$), 3.79–3.98 (m, 4H, $N^+CH_2CH_2O$ and OCH_2CH_2), 4.34–4.43 (m, 2H, $N^+CH_2CH_2O$), 4.89 (s, 2H, CH_2Ph), 7.36–7.46 (m, 3H, H_{meta} and H_{para}), 7.68 (d, J = 6.6 Hz, 2H, H_{ortho}); ^{13}C NMR ($CDCl_3$) δ: 14.2, 22.8, 26.0, 29.5, 29.6, 29.7, 29.8, 31.1, 31.3, 32.0, 50.5, 59.2, 64.1, 65.7, 69.0, 127.8, 129.1, 130.6, 133.6; ^{31}P NMR ($CDCl_3$) δ: 0.31; IR υ_{max}/cm^{-1} 3443, 2920, 2851, 1632, 1469, 1241, 1068, 988, 928, 762, 719; HRMS calcd. for $C_{27}H_{50}O_4NPNa$ = 506.3370; found *m/z*: 506.3360 $[M + Na]^+$.

*4.1.6.3. 2-[Benzyl(dimethyl)ammonio]propyl hexadecyl phosphate (**HPC$_3$Bn**).* Yield: 70%; ^1H NMR ($CDCl_3$) δ: 0.88 (t, J = 6.7 Hz, 3H, CH_2CH_3), 1.25 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.43–1.62 (m, 2H, OCH_2CH_2), 2.18–2.38 (m, 2H, $N^+CH_2CH_2CH_2O$), 3.14 (s, 6H, $N^+(CH_3)_2$), 3.62–3.82 (m, 4H, $N^+CH_2CH_2CH_2O$ and OCH_2CH_2), 3.85–4.11 (m, 2H, $N^+CH_2CH_2CH_2O$), 4.80 (s, 2H, CH_2Ph), 7.28–7.43 (m, 3H, H_{meta} and H_{para}), 7.45–7.67 (m, 2H, H_{ortho}); ^{13}C NMR ($CDCl_3$) δ: 14.2, 22.8, 24.7, 26.1, 29.4, 29.6, 29.8, 31.2, 31.3, 32.0, 49.5, 61.7, 62.5, 65.4, 67.5, 128.0, 129.1, 130.4, 133.5; ^{31}P NMR ($CDCl_3$) δ: −0.53; IR υ_{max}/cm^{-1} 3414, 2920, 2851, 1632, 1487, 1468, 1240, 1094, 1068, 961, 834, 777, 732, 704; HRMS calcd. for $C_{28}H_{52}O_4NPNa$ =
520.3526; found *m/z*: 520.3522 $[M + Na]^+$.

*4.1.6.4. Hexadecyl 2-pyridinium-1-ylethyl phosphate (**HPC$_2$P**).* Yield 42%; ^1H NMR ($CDCl_3$) δ: 0.88 (t, J = 6.7 Hz, 3H, CH_2CH_3), 1.25 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.43–1.54 (m, 2H, OCH_2CH_2), 3.62–3,73 (m, 2H, OCH_2CH_2), 4.22–4.38 (m, 2H, $N^+CH_2CH_2O$), 4.91–5.08 (m, 2H, $N^+CH_2CH_2O$), 8.03 (t, J = 6.7 Hz, 2H, H_{meta}), 8.42 (t, J = 7.9 Hz, 1H, H_{para}), 9.25 (d, J = 5.6 Hz, 2H, H_{ortho}); ^{13}C NMR ($CDCl_3$) δ: 14.1, 22.7, 25.9, 29.4, 29.5, 29.6, 29.7, 29.8, 31.0, 31.1, 32.0, 62.2, 63.8, 65.6, 128.1, 145.2, 146.1; ^{31}P NMR ($CDCl_3$) δ: 0.55; IR υ_{max}/cm^{-1} 3382, 2917, 2850, 1690, 1637, 1493, 1471, 1239, 1218, 1079, 1048, 1028, 918, 790, 782, 746, 719, 685; HRMS calcd. for $C_{23}H_{42}O_4NPNa$ = 450.2744; found *m/z*: 450.2734 $[M + Na]^+$.

*4.1.6.5. Hexadecyl 3-pyridinium-1-ylpropyl phosphate (**HPC$_3$P**).* Yield 35%; ^1H NMR ($CDCl_3$) δ: 0.88 (t, J = 6.7 Hz, 3H, CH_2CH_3), 1.25 (br s, 26H, $OCH_2CH_2(CH_2)_{13}CH_3$), 1.50–1.63 (m, 2H, OCH_2CH_2), 2.23–2.36 (m, 2H, $N^+CH_2CH_2CH_2O$), 3.75–3.81 (m, 2H, OCH_2CH_2), 3.81–3.92 (m, 2H, $N^+CH_2CH_2CH_2O$), 5.03 (t, J = 6.2 Hz, 2H, $N^+CH_2CH_2CH_2O$), 8.07 (t, J = 7.0 Hz, 2H, H_{meta}), 8.39 (t, J = 7.2 Hz, 1H, H_{para}), 9.52 (d, J = 5.9 Hz, 2H, H_{ortho}); ^{13}C NMR ($CDCl_3$) δ: 14.1, 22.7, 26.0, 29.4, 29.5, 29.6, 29.7, 29.9, 31.0, 31.9, 33.6, 58.9, 60.5, 65.5, 128.3, 144.6, 146.1; ^{31}P NMR ($CDCl_3$) δ: 0.80; IR υ_{max}/cm^{-1} 3298, 2915, 2849, 1636, 1488, 1471, 1255, 1233, 1119, 1093, 1045, 1025, 1009, 975, 831, 814, 717, 675; HRMS calcd. for $C_{24}H_{44}O_4NPNa$ = 464.2900; found *m/z*: 464.2892 $[M + Na]^+$.

*4.1.6.6. 2-[Benzyl(dimethyl)ammonio]ethyl 2-[(2,2,4,4-tetramethylpentane-2-yl)oxy]ethyl phosphate (**PC$_2$Bzt**).* Yield 22%; ^1H NMR ($CDCl_3$) δ: 0.68 (s, 9H, $(CH_3)_3CCH_2(CH_3)_2C$), 1.29 (s, 6H, $(CH_3)_3CCH_2(CH_3)_2C$), 1.66 (s, 2H, $(CH_3)_3CCH_2(CH_3)_2C$), 3.19 (s, 6H, $N^+(CH_3)_2$), 3.81–3.88 (m, 2H, $N^+CH_2CH_2O$), 4.06–4.14 (m, 2H, $PhOCH_2CH_2O$), 4.18–4.26 (m, 2H, $PhOCH_2CH_2O$), 4.42–4.52 (m, 2H, $N^+CH_2CH_2O$), 4.75 (s, 2H, CH_2Ph), 6.72 (d, J = 8.8 Hz, 2H, $H_{meta(Ph)}$), 7.19 (d, J = 8.8 Hz, 2H, $H_{ortho(Ph)}$), 7.29–7.39 (m, 3H, $H_{meta(Bn)}$ and $H_{para(Bn)}$), 7.50 (d, J = 7.7 Hz, 2H, $H_{ortho(Bn)}$); ^{13}C NMR ($CDCl_3$) δ: 31.6, 31.7, 32.3, 37.9, 50.4, 56.8, 59.2, 63.7, 64.2, 68.0, 69.0, 113.7, 127.1, 127.7, 129.1, 130.4, 133.3, 142.4, 156.3; ^{31}P NMR ($CDCl_3$) δ: −0.71; IR υ_{max}/cm^{-1} 3348, 2954, 2892, 1632, 1611, 1512, 1484, 1251, 1080, 1059, 985, 795, 763, 710, 541; HRMS calcd. for $C_{27}H_{42}O_5NPNa$ = 514.2693; found *m/z*: 514.2685 $[M + Na]^+$.

*4.1.6.7. 3-[Benzyl(dimethyl)ammonio]propyl 2-[(2,2,4,4-tetramethylpentane-2-yl)oxy]ethyl phosphate (**PC$_3$Bzt**).* Yield 24%; ^1H NMR ($CDCl_3$) δ: 0.68 (s, 9H, $(CH_3)_3CCH_2(CH_3)_2C$), 1.30 (s, 6H, $(CH_3)_3CCH_2(CH_3)_2C$), 1.67 (s, 2H, $(CH_3)_3CCH_2(CH_3)_2C$), 2.18–2.31 (m, 2H, $N^+CH_2CH_2CH_2O$), 3.01 (s, 6H, $N^+(CH_3)_2$), 3.75–3.82 (m, 2H, $N^+CH_2CH_2CH_2O$), 4.01–4.16 (m, 4H, $PhOCH_2CH_2O$ and $N^+CH_2CH_2CH_2O$), 4.18–4.22 (m, 2H, $PhOCH_2CH_2O$), 4.67 (s, 2H, CH_2Ph), 6.74 (d, J = 8.7 Hz, 2H, $H_{meta(Ph)}$), 7.19 (d, J = 8.7 Hz, 2H, $H_{ortho(Ph)}$), 7.31–7.42 (m, 3H, $H_{meta(Bn)}$ and $H_{para(Bn)}$), 7.51 (d, J = 7.7 Hz, 2H, $H_{ortho(Bn)}$); ^{13}C NMR ($CDCl_3$) δ: 24.6, 31.7, 31.8, 32.3, 37.9, 49.3, 56.9, 61.8, 63.0,63.8, 67.7, 68.1, 113.6, 127.1, 127.7, 129.1, 130.4, 133.3, 142.3, 156.4; ^{31}P NMR ($CDCl_3$) δ: −1.08; IR υ_{max}/cm^{-1} 3427, 2956, 2886, 1632, 1610, 1511, 1480, 1248, 1096, 1067, 961, 832, 768, 735, 706, 534; HRMS calcd. for $C_{28}H_{44}O_5NPNa$ = 528.2850; found *m/z*: 528.2844 $[M + Na]^+$.

4.2. Equilibrium surface tension

The critical micelle concentration values of the surfactants were determined from the surface tension isotherm. The solvent surface tension values were measured by the Wilhelmy plate technique using a Kruss 100 MK2 tensiometer. Deionized water was used in the preparation of all samples. The temperature of the measurements was kept at 25 ± 0.1 °C. Measurements of equilibrium surface tension were taken repeatedly (every 6 min) until the change in surface tension was less than 0.05 mN m^{-1}. (The measurement of one solution with premicellar concentrations of the compounds took 1–2 h, whereas the measurement of the equilibrium surface tension of premicellar solutions of **HPC$_2$Bn** and **HPC$_3$Bn** took 1.5–3 h.) The critical micelle concentrations (cmc) and surface tensions at the cmc (γ_{cmc}) were determined from the intersection of two lines – one premicellar and second one postmicellar – of the surface tension *vs.* log *c* curve. From the surface tension data, the adsorbed amount of surfactant Γ_{cmc} is calculated utilizing the Gibbs adsorption isotherm

$$\Gamma_{cmc} = -[d\gamma/d\log c]_T/(2.303iRT) \quad (1)$$

where γ is the surface tension (mN m^{-1}), c is the surfactant concentration, i is the prefactor (QUATs, i = 2; APCs, i = 1), T is the absolute temperature and R the gas constant. Surface excess may be determined from the slope below the cmc in the surface tension *vs.* log *c* plots. Surface area at the surface saturation per head group (A_{cmc}) is obtained from the equation

$$A_{cmc} = 10^{20}/N_A\Gamma_{cmc} \quad (2)$$

were N_A is the Avogadro constant.

4.3. Biological activities

4.3.1. Cytotoxicity assay

4.3.1.1. Cell culture. The following human cancer cell lines were used for this study: CEM (acute T-lymphoblastic leukemia), HeLa (cervical carcinoma cells), A-549 (human lung adenocarcinoma), MDA-MB-231 and MCF-7 (breast cancer cells). CEM and HeLa cells were maintained in RPMI 1640 medium (PAA Laboratories, Pasching, Austria). MDA-MB-231, MCF-7 and A-549 were maintained in growth

medium consisting of high glucose Dulbecco's Modified Eagle Medium. Both media were supplemented with Glutamax, 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) (all from Invitrogen, Carlsbad, CA, USA), in the atmosphere of 5% CO_2 in humidified air at 37 °C. Cell viability, estimated by trypan blue exclusion, was greater than 95% before each experiment.

4.3.1.2. Primary endothelial cells isolation and culture. Human umbilical vein endothelial cells (HUVEC) were isolated, cultured, and characterized as previously described [54]. Cells were cultured on gelatin-coated dishes in cM199 (=M199 medium supplemented with 10% heat-inactivated human serum, 10% heat-inactivated NBCS, 3.75 μg/ml crude endothelial cell growth factor (ECGF), 5 U/ml heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin) at 37 °C under 5% CO_2/95% air atmosphere.

Twenty-four hours before the experiments were started, ECGF and heparin were withdrawn from the endothelial cell cultures.

4.3.1.3. Assessment of cytotoxicity by MTT assay. The cytotoxic effect of the tested compounds was studied by using colorimetric microculture assay with the MTT end-point. The amount of MTT reduced to formazan was proportional to the number of viable cells [55]. Briefly, 5×10^3 cells were plated per well in 96-well polystyrene microplates (SARSTEDT, Nümbrecht, Germany) in the culture medium containing the tested chemicals at final concentrations 10^{-4} to 10^{-9} mol/L. After 72 h, 10 μl of MTT (5 mg/ml) were added in each well. After additional 4 h, during which insoluble formazan was produced, 100 μL of 10% sodium dodecylsulphate were added in each well and another 12 h were allowed for the formazan to be dissolved. The absorbance was measured at 540 nm using the automated uQuant™ Universal Microplate Spectrophotometer (Biotek). Absorbance of control wells was taken as 100%, and the results were expressed as a percent of control. The cytotoxic activities were expressed as IC_{50}.

4.3.2. Antifungal assay

The antifungal activity of the synthesized compounds was tested using the modified M-27 microdilution assay of NCCLS (National Committee for Clinical Laboratory Standards, USA). The final density of *C. albicans* (ATCC 60193) was adjusted spectrophotometrically ($\lambda = 570$ nm) to 2.5×10^3 CFU/ml. Tested substances were dissolved in Sabouraud medium, sterile filtrated and tested at the range of 64–0.125 μg/ml. Minimum inhibitory concentrations (MICs) were recorded macroscopically and calculated as the percentage of absorbance in control wells after 48 h of cultivation. *C. albicans* cultured in medium without amphiphilic compounds was used as control.

4.3.3. In vitro amebicidal activity assay

The cytotoxic activity of four QUATs, four APCs, four APHCs was tested on two clinical isolates of free-living amoebae, i.e., *A. lugdunensis* AcaVNAK02 and *A. quina* AcaVNAK03, isolated from the corneas of two patients with amoebic keratitis [56]. Both isolates are representatives of group II according to the classification of Pussard and Pons [57]. The species identification was performed according to the identification key of Page [58] based mainly on cyst morphology and temperature tolerance [56]. The molecular classification into the genotype T4 for both strains was revealed [59].

The experiment was performed using modifications of previously described methods [60]. Briefly, from the 2-day monoxenic cultures on agar plates, the trophozoites were axenized by inoculation into the Bacto-Casitone/Serum medium (BCS) with penicillin and ampicillin. After 72 h, the active trophozoites were transferred into peptone–yeast extract–glucose medium (PYG) with penicillin and ampicillin. After 5 passages, the trophozoites were transferred into a PYG medium without antibiotics and subsequently cultivated in this medium. Cytotoxicity measurements were carried out in sterile 96-well microtiter plates. Each well was seeded with 100 μl (2×10^5 cells ml^{-1}) of a trophozoite suspension. Then, 100 μl of a freshly prepared medium containing a tested compound at 10 concentrations was added to all wells except untreated control wells that received 100 μl of pure medium. Each compound was tested at final concentrations of 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, and 0.98 μM. The reduction of trophozoites was recorded after 48 h by counting the surviving cells in a Bürker-Türk hemocytometer. Viability of trophozoites was determined by trypan blue exclusion; 100% eradication was confirmed by transferring 50 μl of the suspension to a PYG medium, then recording the amoeba growth for 14 days. The lowest concentration of tested compounds leading to 100% eradication of the trophozoites was defined as the minimal trophocidal concentration (MTC). The experiments were repeated 8 times for each concentration. The cultivations and the cytotoxicity measurements were carried out at 37 °C.

Acknowledgments

We thank the NMR laboratory, Faculty of Pharmacy, Comenius University in Bratislava for allowing us to conduct the NMR experiments. Further we thank the management of HPL (Ltd.) for laboratory space for testing the amebicidal activities. The contribution of authors to the paper is the following: M.L., J.K., H.H. and F.D. realized the synthesis of the compounds and investigated their physicochemical properties. M.M., M.G. and F.O. tested the amebicidal activities. The cytotoxic activities were tested by G.M., L.V. and J.M., while M.S. studied the activities of the compounds against *Candida albicans*. This work was supported by Grants VEGA 1/0796/12, APVV-0325-07, FaF UK/28/2013, UK/547/2013, UK/360/2012, ESF grant no. NFP 26240120065 and by the Agency of the Slovak Ministry of Education for the Structural Funds of the EU, under project SEPO-II (ITMS code: 26220120039).

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