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Synthesis, Aggregation Properties, and Antiprotozoal Activity of Heterocyclic Heterogemini Surfactants

M. Lukáč,[1,2] L. Timko,[1] M. Mrva,[3] F. Ondriska,[4] J. Karlovská,[2,5] J. Valentová,[1] and I. Lacko[1]

[1]*Department of Chemical Theory of Drugs, Faculty of Pharmacy, Comenius University, Kalinčiakova 8, 832 32 Bratislava, Slovakia*

[2]*NMR Laboratory, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava, Slovakia*

[3]*Department of Zoology, Faculty of Natural Sciences, Comenius University, Mlynská Dolina B-1, 842 15 Bratislava, Slovakia*

[4]*Department of Parasitology, Microbiological Laboratory, HPL (Ltd), Istrijská 20, 841 07 Bratislava, Slovakia*

[5]*Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Kalinčiakova 8, 832 32 Bratislava, Slovakia*

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ABSTRACT: *A series of four heterogemini surfactants have been synthesized: 2-decyl [dodecyl(dimethyl)ammonio]ethyl phosphate, decyl 2-(1-dodecylpyrrolidinio-1-yl)ethyl phosphate, decyl 2-(1-dodecylpiperidinio-1-yl)ethyl phosphate, and decyl 2-(1-dodecylmorpholinio-1-yl)ethyl phosphate. Antiprotozoal activity of prepared compounds against* Acanthamoeba lugdunensis *was investigated. Decyl 2-(1-dodecylpyrrolidinio-1-yl)ethyl phosphate exhibited the best trophocidal activity. The activities of heterogemini surfactants were compared with their aggregation properties.* © 2010 Wiley Periodicals, Inc. Heteroatom Chem 21:203–209, 2010; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/hc.20587

Correspondence to: M. Lukáč; e-mail: lukac@fpharm.uniba.sk.
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INTRODUCTION

The term gemini surfactant was coined by Menger and Littau in 1991 [1]. The vast majority of work on gemini surfactants has been made with symmetrical geminis, i.e., gemini surfactants, which contain identical polar head groups and identical hydrophobic tails [2]. Geminis can be divided into anionic [3,4], cationic [5–7], or nonionic surfactants [8,9]. Unsymmetrical geminis, the surfactants that have different head groups, such as anionic—nonionic or anionic—cationic, have been referred to as heterogemini surfactants [10]. Probably, the first example of a true heterogemini surfactant is an anionic–cationic species reported in 1996 by Jaeger et al. [11]. One of the hydrophilic groups is a carboxylate anion, and the other one is a quaternary ammonium cation. Other types of anionic–cationic gemini surfactants (zwitterionic gemini surfactants) are represented by the phosphate-quaternary ammonium heterogemini surfactants with branched, unbranched, or fluorinated alkyl chains [12–15]. These

geminis have interesting physicochemical properties [16–19]. Depending on the length of alkyl chains, they form gels, micelles, vesicles, tubules, or coacervates in water [12,13]. These zwitterionic gemini surfactants are a special type of alkylphosphocholines (APC), where one methyl group of choline is replaced with a long alkyl chain.

APCs possess a wide range of biological activities. Miltefosine (hexadecylphosphocholine, HPC) is the main representative of APCs. HPC exhibits antineoplastic [20], antiprotozoal [21], antibacterial, antimycotic [22], and antivirotic activities [23]. Antiprotozoal activity of HPC and other APCs is the most studied in recent time. HPC affects many types of protists such as *Leishmania* spp. [24], *Trypanosoma* spp. [25], *Balamuthia mandrillaris*, *Naegleria fowleri* [26], *Trichomonas vaginalis* [27], *Entamoeba histolytica* [28], and *Acanthamoeba* spp. [29].

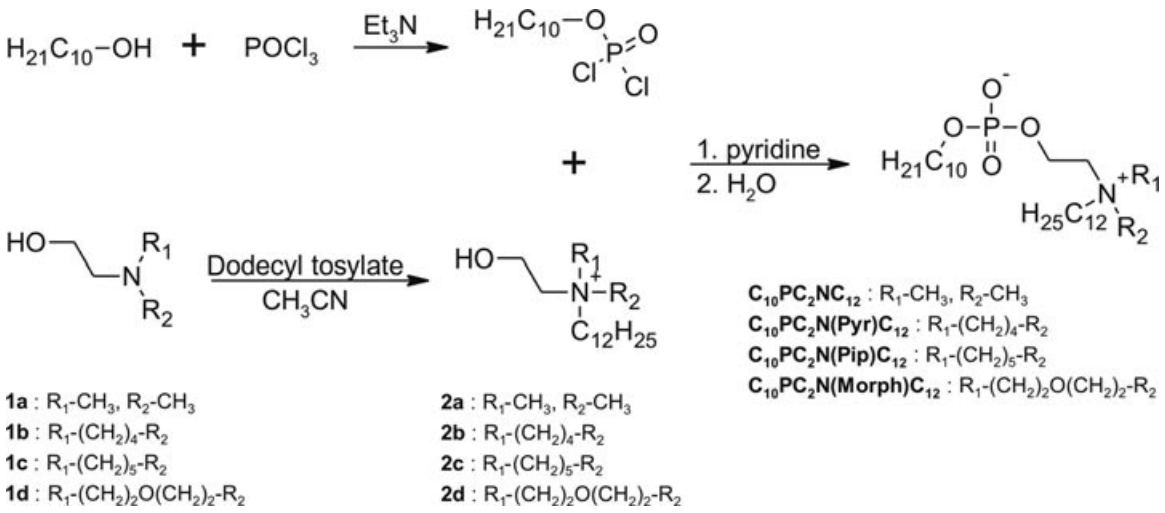
The last named genus is the causative agent of keratitis, granulomatous amoebic encephalitis, or nasopharyngeal and cutaneous infections. No efficient and easily manageable treatments are available for these diseases. Often, the same drugs that have been successfully used for treatment in one patient have a little or no effect in the treatment of other cases of acanthamoebiasis [30]. Therefore, the susceptibility of several *Acanthamoeba* spp. to APCs was investigated [26,29,31–37].

The aim of the study was to prepare the novel heterocyclic heterogemini surfactants and to evaluate their potential efficacies against free-living amoeba of the genus *Acanthamoeba*. The trophocidal activities of four different APCs against *Acanthamoeba lugdunensis* were evaluated in vitro. The activity of heterogemini surfactants was compared with their aggregation properties.

RESULTS AND DISCUSSION

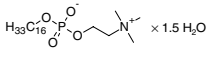
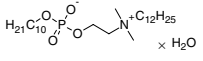
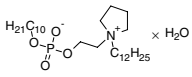
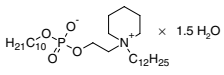
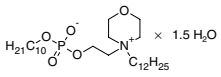
Four heterogemini surfactants based on various types of cations were synthesized. The compounds were designed as analogues of decyl 2-[dodecyl(dimethyl)ammonio]ethyl phosphate ($C_{10}PC_2NC_{12}$) with different polar head groups. The dimethylamino group was replaced with heterocyclic rings, containing nitrogen as a heteroatom (in the case of morpholine analogue, an oxygen atom was incorporated into the heterocyclic ring) to establish the influence of chemical structure on antiprotozoal activities and physicochemical properties.

The synthetic strategy for the preparation of the heterogemini surfactants is depicted in Scheme 1. Representative phosphate analogues were prepared to determine the importance of the phosphocholine moiety for bioactivity according to modified procedures [37]. The strategy involves phosphorylation of decanol using $POCl_3$ and subsequent attachment of the desired choline analogue. $C_{10}PC_2NC_{12}$, $C_{10}PC_2N(Pyr)C_{12}$, $C_{10}PC_2N(Pip)C_{12}$, and $C_{10}PC_2N(Morph)C_{12}$ were prepared by reaction of decyl dichloridophosphate with choline tosylate analogue and subsequently hydrolyzed with H_2O. Heterogemini surfactants were prepared as hydrates. The amount of crystal water was estimated by ^1H NMR. A single ^{31}P NMR signal indicated the purity of the compound. The choline analogues were obtained by quaternization of **1a, 1b, 1c**, and **1d** with dodecyl *p*-toluenesulfonate in acetonitrile. Tertiary 2-aminoethanols **1b** and **1d** were prepared by the reaction of a secondary amine with 2-chloroethanol and potassium carbonate in the presence of a catalytic amount of KI [38]. Dodecyl *p*-toluenesulfonate was prepared according to the procedure described



SCHEME 1 Preparation of heterogemini surfactants.

TABLE 1 Characterization of Prepared Compounds, Their Trophocidal Activity, and Diameter of Aggregates Formed in Aqueous Suspensions

Formula	*Abbreviation*	*MTC (μM)*	*d (μm)*
	HPC	400 [37]	–
	$C_{10}PC_2NC_{12}$	>400 [36]	<15
	$C_{10}PC_2N(Pyr)C_{12}$	200	<128
	$C_{10}PC_2N(Pip)C_{12}$	>800	<20
	$C_{10}PC_2N(Morph)C_{12}$	>800	<12.5

MTC: Minimal trophocidal concentration; *d*: diameter of aggregates.

by Marvel and Sekera [39]. The synthesis of choline *p*-toluenesulfonate analogues **2a, 2b, 2c**, and **2d** is depicted in Scheme 1.

The antiprotozoal efficiencies of the prepared compounds were evaluated against a strain of *Acanthamoeba lugdunensis* (Table 1), which was isolated from a patient suffering from amoebic keratitis [40]. The trophocidal activities of heterogemini surfactants were compared with the activity of HPC. HPC is considered a standard in the series of APCs. Its minimal trophocidal concentration (MTC) was 400 μM [37]. This strain of *Acanthamoeba lugdunensis* is relatively resistant to the action of APCs. Other species of *Acanthamoeba* seem to be more sensitive to HPC. Walochnik et al. [29] studied three species of *Acanthamoeba*: *A. castellanii*, *A. polyphaga*, *A. lenticulata*, and they observed lower values of MTC (MTC = 20–40 μM). Lower values of MTC were also obtained by McBride et al. [31]. They studied the influence of HPC on two strains of *Acanthamoeba*: *A. polyphaga* (MTC = 31.25 μM), *A. castellanii* (MTC = 31.25 μM). In our case, HPC was about 10 times less active against *A. lugdunensis* [37] in comparison with its activity against *A. castellanii*, *A. polyphaga*, and *A. lenticulata*, which was observed by other authors [29,31]. $C_{10}PC_2NC_{12}$, $C_{10}PC_2N(Pip)C_{12}$, and $C_{10}PC_2N(Morph)C_{12}$ exhibited lower trophocidal activity than HPC. Their MTCs were higher than 400 μM. The amoebae were not affected by these heterogemini surfactants. The trophozoites were viable, and their cells were of typical shapes without any pathological signs. Only $C_{10}PC_2N(Pyr)C_{12}$ was about twice as active as HPC (MTC = 200 μM). It may be a promising new candidate for the topical treatment of *Acanthamoeba* keratitis. Recently, we reported [36] trophocidal activities of a series of 15 similar heterogemini surfactants against *A. lugdunensis*. Decyl 3-[dodecyl(dimethyl)ammonio]propyl phosphate ($C_{10}PC_3NC_{12}$, MTC = 100 μM) and decyl 4-[dodecyl(dimethyl)ammonio]butyl phosphate ($C_{10}PC_4NC_{12}$, MTC = 200 μM) possess comparable activities as $C_{10}PC_2N(Pyr)C_{12}$ but compounds with longer spacers between the phosphate group and ammonium cation (decyl 6-[dodecyl(dimethyl)ammonio]hexyl phosphate ($C_{10}PC_6NC_{12}$), MTC > 400 μM; and decyl 10-[dodecyl(dimethyl) ammonio]decyl phosphate ($C_{10}PC_{10}NC_{12}$) MTC > 400 μM) were practically inactive. This observation indicates that changes in the polar group of $C_{10}PC_2NC_{12}$ can increase the activity, but such modifications have limitations. Incorporation of a five-membered ring to the ammonium cation and extension of the spacer between the phosphate group and the ammonium cation from two carbon atoms to three or four carbon atoms proved useful.

We assume that the different activity of $C_{10}PC_2N(Pyr)C_{12}$ in comparison with other heterogemini surfactants can be partly explained by their physicochemical properties. HPC formed clear aqueous solution at the concentration $c = 1 \times 10^{-3}$ mol L^{-1}. No aggregates were observed under an optical microscope. Heterogemini surfactants formed cloudy aqueous suspensions at the same concentrations. Clouds were caused by the presence of self-assembled vesicles; solid insoluble particles of the compounds were not observed. $C_{10}PC_2NC_{12}$, $C_{10}PC_2N(Pip)C_{12}$, and $C_{10}PC_2N(Morph)C_{12}$ form aggregates in water suspensions with diameters up to 20 μm (Figure 1). $C_{10}PC_2N(Pyr)C_{12}$ formed similar aggregates but also giant aggregates with diameters

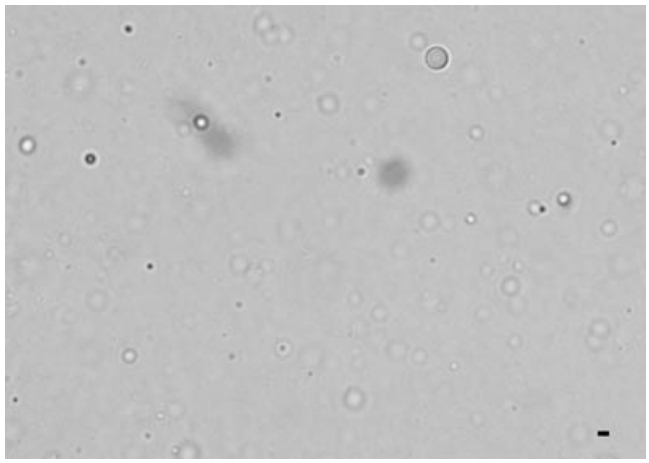


FIGURE 1 Aggregate of an aqueous suspension of compound $C_{10}PC_2NC_{12}$ (bar is 5 μm).

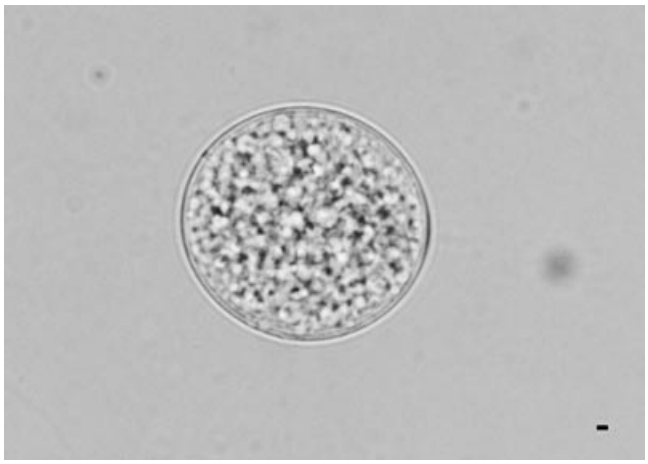


FIGURE 2 Aggregate of an aqueous suspension of compound $C_{10}PC_2N(Pyr)C_{12}$ (bar is 5 μm).

up to 128 μm (Figure 2). Smaller aggregates could be unilamellar or multilamellar vesicles. Compound $C_{10}PC_2NC_{12}$ formed a lamellar phase in water (Figure 3) ($C_{10}PC_2NC_{12}$: H_2O 1:2). The lamellar phase is stable in a range of temperatures from 25°C to 70°C. The similarity of the chemical structures of the prepared compounds and their behavior in aqueous suspensions indicate that the heterocyclic analogues might form the same phases. The occurrence of this phase in compound/water mixtures justifies the assumption that vesicles could be formed. The giant aggregates seem to be multicomponent, multilamellar vesicles. Small unilamellar and/or multilamellar vesicles are enclosed in the larger vesicles (Figure 2). When we tested the trophocidal activity, we observed that the giant aggregates of $C_{10}PC_2N(Pyr)C_{12}$ bound

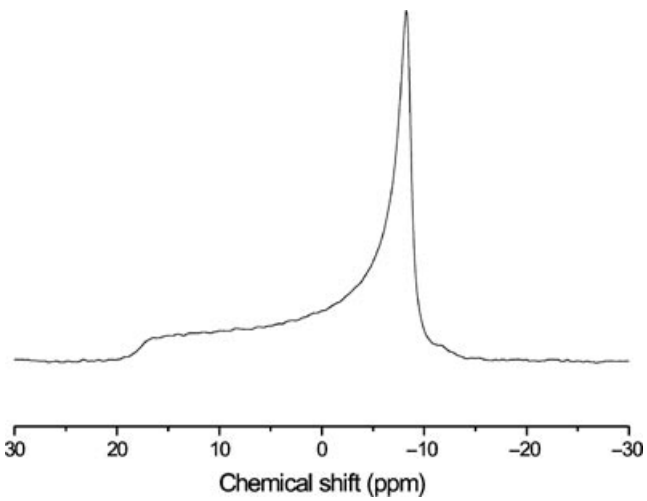


FIGURE 3 ^{31}P NMR spectrum of $C_{10}PC_2NC_{12}$ (25°C).

some artifacts, which can be considered as parts of destroyed cells of amoebae. We suppose that the giant aggregates affect the trophozoites more than the vesicles with smaller diameters.

MATERIALS AND METHODS

Materials

All chemicals used for synthesis were purchased from commercial suppliers. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian Gemini 2000 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 $CDCl_3$ relative to the internal standard TMS for ^1H and ^{13}C NMR spectra and to external standard 85% H_3PO_4 for ^{31}P NMR spectra. Infrared spectra were recorded on a FT-IR Impact 400 D spectrophotometer as potassium bromide disks.

Chemistry

Synthesis of decyl 2-[dodecyl(dimethyl)amonio] ethyl phosphate ($C_{10}PC_2NC_{12}$) and its pyrrolidinium ($C_{10}PC_2N(Pyr)C_{12}$), piperidinium ($C_{10}PC_2N(Pip)C_{12}$), and morpholinium ($C_{10}PC_2N(Morph)C_{12}$) analogues is shown in Scheme 1.

General Procedure for the Quaternization of Aminoalcohols with Dodecyl p-Toluenesulfonate. Aminoalcohol (22 mmol) was added to solution of dodecyl p-toluenesulfonate (20 mmol) in acetonitrile (20 mL). The resulting mixture was refluxed for 4 h. After cooling down, the acetonitrile was evaporated in vacuum. The resulting mixture was crystallized from acetone. The quaternary salts were obtained as white, hygroscopic solids.

N-(2-Hydroxyethyl)-N,N-dimethyldodecane-1-ammonium Tosylate (**2a**). Yield 73.9%; ^1H NMR ($CDCl_3$, TMS) δ: 0.88 (t, 3H, J = 6.6 Hz), 1.17–1.50 (m, 18H), 1.60–1.69 (m, 2H), 2.34 (s, 3H), 3.27 (s, 6H), 3.36–3.45 (m, 2H), 3.64–3.74 (m, 2H), 4.09–4.20 (m, 2H), 5.51 (t, 1H, J = 5.7 Hz), 7.15 (d, 2H, J = 7.9 Hz), 7.74 (d, 2H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.1, 21.3, 22.7, 22.8, 26.2, 29.1, 29.3, 29.4, 29.5, 29.6, 31.9, 51.8, 56.4, 65.8, 66.0, 125.8, 128.7, 139.6, 143.0.

N-Dodecyl-N-(2-hydroxyethyl)pyrrolidinium Tosylate (**2b**). Yield 35.8%; ^1H NMR ($CDCl_3$, TMS) δ: 0.88 (t, 3H, J = 6.7 Hz), 1.18–1.42 (m, 18H), 1.58–1.67 (m, 2H), 1.98–2.32 (m, 4H), 2.34 (s, 3H), 3.26–3.40 (m, 2H), 3.50–3.63 (m, 4H), 3.73–3.85 (m, 2H), 3.98–4.05 (m, 2H), 7.15 (d, 2H J = 8.1 Hz), 7.73 (d, 2H, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.1, 21.3, 21.6, 22.7, 22.9, 23.4, 26.4, 29.2, 29.3, 29.4, 29.5, 29.6,

31.9, 54.7, 56.5, 57.4, 57.6, 63.4, 125.8, 128.7, 139,7, 142.9.

N-Dodecyl-N-(2-hydroxyethyl)piperidinium Tosylate (**2c**). Yield 49.4%; ^{1}H NMR ($CDCl_3$, TMS) δ: 0.88 (t, 3H, J = 6.7 Hz), 1.20–1.32 (m, 18H), 1.58–2.01 (m, 8H), 2.34 (s, 3H), 3.38–3.50 (m, 4H), 3.60–3.76 (m, 2H), 4.03–4.15 (m, 2H), 5.60 (t, 1H, J = 5.8 Hz), 7.14 (d, 2H, J = 7,9 Hz), 7.75 (d, 2H, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.3, 20.0, 21.0, 21.5, 21.9, 22.8, 26.6, 29.3, 29.5, 29.6, 29.7, 29.8, 32.1, 55.9, 58.6, 60.1, 61.6, 128.0, 128.8, 139.6, 143.4.

N-Dodecyl-N-(2-hydroxyethyl)morpholinium Tosylate (**2d**). Yield 24.2%; ^{1}H NMR ($CDCl_3$, TMS) δ: 0.88 (t, 3H, J = 6.5 Hz), 1.18–1.42 (m, 18H), 1.58–1.65 (m, 2H), 2.34 (s, 3H), 3.28–3.62 (m, 6H), 3.71–4.17 (m, 8H), 5.51 (m, 1H), 7.16 (d, 2H, J = 7.8 Hz), 7.18 (d, 2H, J = 7.9 Hz); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.2, 21.3, 21.7, 22.7, 26.3, 29.2, 29.4, 29.5, 29.7, 31.9, 52.7, 55.7, 56.1, 59.0, 59.4, 60.5, 61.1, 63.8, 125.8, 128.9, 139.9, 142.8.

General Procedure for the Preparation of Alkylphosphocholines. Solution of decanol (9 mmol) in chloroform (20 mL) was added dropwise at 0°C to a stirred solution of phosphorous oxychloride (10 mmol) and triethylamine (20 mmol) in chloroform (10 mL). The resulting mixture was stirred at room temperature (r.t.) for 2 h. This intermediate was used immediately without any purification. Pyridine (15 mL) was added dropwise at t = 0°C to the resulting solution, followed by the addition of choline tosylate analogue (12.5 mmol). The reaction mixture was stirred at r.t. 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–water (5:1 V/V). To the stirred solution, exchange resin 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 resulting crude solid was purified by crystallization from a mixture of chloroform and acetone or chloroform and diethyl ether. ALPs were dried in vacuo over P_4O_{10}.

Decyl 2-[Dodecyl(dimethyl)ammonio]ethyl Phosphate × H_2O ($C_{10}PC_2NC_{12}$). Yield 22.2%; ^{1}H NMR ($CDCl_3$, TMS) δ: 0.84–0.92 (m, 6H), 1.18–1.41 (m, 32H), 1.55–1.78 (m, 4H), 2.18 (s, 2H), 3.35 (s, 6H), 3.41–3.50 (m, 2H), 3.76–3.89 (m, 4H), 4.27–4.35 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.1, 22.7, 22.9, 26.0, 26.3, 29.3, 29.4, 29.5, 29.6, 29.7, 31.0, 31.1, 31.9, 51.9, 58.8, 64.2, 65.5, 65.6, 65.9; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ: 0.76; IR ν_{max} (cm^{-1}): 3427, 2919, 2851, 1635, 1469, 1242, 1100, 1067, 822.

Decyl 2-(1-Dodecylpyrrolidinio-1-yl)ethyl Phosphate × H_2O ($C_{10}PC_2N(Pyr)C_{12}$). Yield 4.3%; ^{1}H NMR ($CDCl_3$, TMS) δ: 0.8–0.98 (m, 6H), 1.18–1.52 (m, 32H), 1.57–1.74 (m, 4H), 1.98–2.18 (m, 2H), 2.29–2.47 (m, 2H), 2.77 (s, 2H), 3.35–3.50 (m, 2H), 3.63–3.91 (m, 6H), 3.94–4.07 (m, 2H), 4.20–4.31 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.1, 21.5, 22.7, 23.4, 25.9, 26.5, 29.4, 29.5, 29.6, 29.7, 31.1, 31.9, 58.7, 59.1, 60.2, 63.0, 63.5, 65.6; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ: 0.26; IR ν_{max} (cm^{-1}): 3416, 2921, 2851, 1645, 1468, 1260, 1101, 1069, 826.

Decyl 2-(1-Dodecylpiperidinio-1-yl)ethyl Phosphate × 1.5H_2O ($C_{10}PC_2N(Pip)C_{12}$). Yield 16.3%; ^{1}H NMR ($CDCl_3$, TMS) δ: 0.81–0.95 (m, 6H), 1.18–1.54 (m, 32H), 1.54–2.05 (m, 10H), 2.91 (s, 3H), 3.44 (m, 4H), 3.75–3.92 (m, 6H), 4.22–4.36 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.1, 20.0, 20.8, 21.9, 22.7, 26.0, 26.5, 29.3, 29.4, 29.5, 29.6, 29.7, 31.1, 31.2, 31.9, 57.3, 58.2, 58.3, 59.9, 60.8, 65.4, 65.5; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ: 0.78; IR ν_{max} (cm^{-1}): 3417, 2922, 2852, 1634, 1469, 1222, 1100, 1066, 823.

Decyl 2-(1-Dodecylmorpholinio-1-yl)ethyl Phosphate × 1.5H_2O ($C_{10}PC_2N(Morph)C_{12}$). Yield 1.7%; ^{1}H NMR ($CDCl_3$, TMS) δ: 0.84–0.96 (m, 6H), 1.18–1.45 (m, 32H), 1.54–1.65 (m, 2H), 1.65 (m, 2H), 2.70 (s, 3H), 3.46–3.69 (m, 4H), 3.76–3.86 (m, 2H), 3.90–4.11 (m, 6H), 4.13–4.25 (m, 2H), 4.25–4.36 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ: 14.1, 21.9, 22.7, 25.9, 26.5, 29.3, 29.4, 29.5, 29.6, 29.7, 31.0, 31.1, 31.9, 58.3, 58.4, 59.1, 60.0, 60.1, 60.6, 65.6; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ: 0.15; IR ν_{max} (cm^{-1}): 3420, 2922, 2852, 1636, 1468, 1249, 1087, 827.

Testing Heterogemini Surfactants for Trophocidal Susceptibility

These procedures were carried out using the modified methods described by Walochnik et al. [29]. The cultures of amoeba were grown axenically in 100-mL Erlenmeyer flasks. Axenic culture was obtained from the monoxenic culture of clinically isolated *Acanthamoeba lugdunensis* as reported previously [40]. Trophozoites were harvested from 2-day monoxenic plate cultures and transferred to Bacto-Casitone/Serum (BCS) medium with penicillin and ampicillin. Actively growing trophozoites were harvested by centrifugation at 500×g for 7 min, and subsequently they were incubated at 37°C for 3 days. Thereafter, trophozoites were transferred to BCS medium without antibiotics and cultivated at 37°C for 3 days. The cultivation in the BCS medium was repeated five times, then the trophozoites were transferred to peptone–yeast extract–glucose (PYG) medium and afterwards cultivated in this medium. Experiments were carried out in 96-well microtiter

plates at 37°C under sterile conditions. Each well was seeded with 100 μL (2×10^5 cells) of a trophozoite suspension. Then 100 μL of test substance solution in PYG medium was added and mixed with the suspension of trophozoites. APCs were tested at five concentrations (50, 100, 200, 400, and 800 μM). Viability was determined by trypan blue exclusion. One hundred percent eradication was confirmed by transferring 50 μL of the suspension to a PYG medium and by recording the amoeba growth for 14 days.

Measurement of Some Physicochemical Properties

Diameter Measurements of Aggregates of Compounds in Water. The samples were prepared by simple soaking of solids in deionized water. Compounds were dissolved by shaking in a volumetric flask. The concentrations of solutions/suspensions were 1×10^{-3} mol L^{-1}. The samples were investigated 2 weeks after preparation. The aggregates were observed under Nikon Labophot optical microscope. The diameter was measured by an ocular micrometer.

Measurement of Aggregates of $C_{10}PC_2NC_{12}$ in Water by ^{31}P NMR. The sample was prepared in an NMR tube. 100 μL of deionized water was added to 50 mg of $C_{10}PC_2NC_{12}$. The mixture was homogenized by several cycles of heating to about 50°C and cooling to −18°C. ^{31}P NMR spectra were measured on the prepared sample at 25 and 70°C.

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