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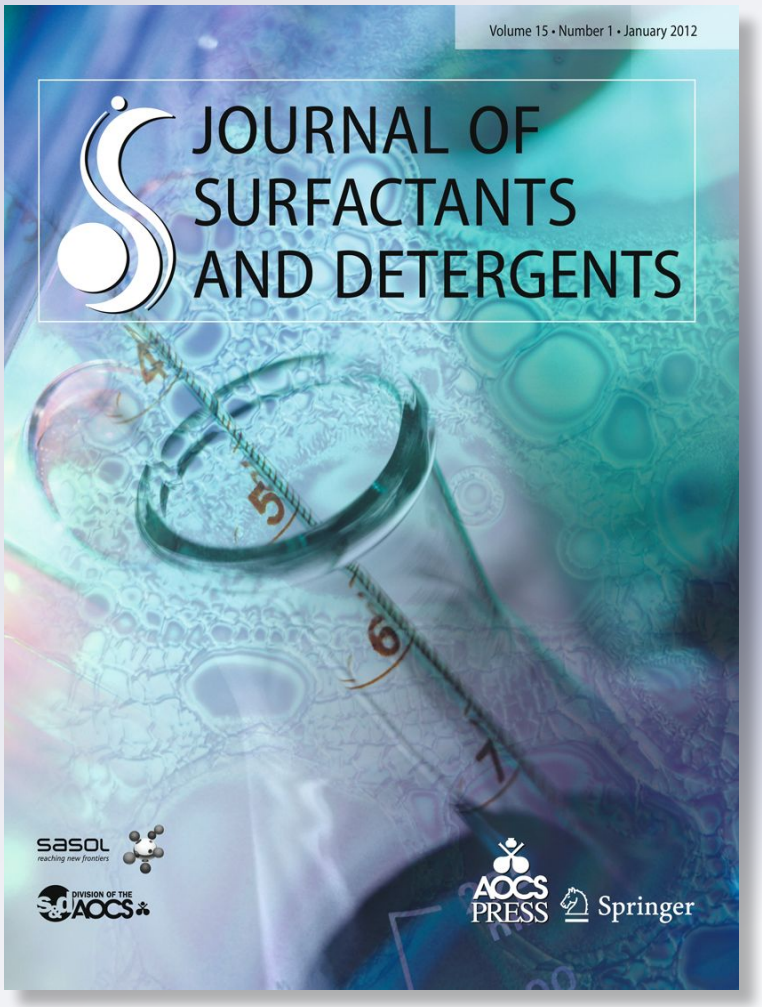

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ORIGINAL ARTICLE

Synthesis, Surface-Active Properties, and Anthelmintic Activities of New Cationic Gemini Surfactants Against the Gastrointestinal Nematode, *Heligmosomoides polygyrus bakeri*, In Vitro

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Abstract A novel series of cationic dimeric surfactants was prepared involving the ketalization reaction, Williamson etherification, and regioselective oxirane ring opening with tertiary alkyl amines. The synthesized compounds were obtained in high purity by a simple purification procedure using column chromatography. The critical micelle concentration (CMC), effectiveness of surface tension reduction (γ_{CMC}), surface excess concentration (Γ), and area per molecule at the interface (A) were determined and values indicate that the cationic series is characterized by good surface-active and self-aggregation properties. For the first time, we reported the anthelmintic activities against the rodent gastrointestinal nematode *Heligmosomoides polygyrus bakeri*, in vitro for cationic gemini compounds. In the series of five tested cationic compounds (**4a**–**e**), three of them (**4a**, **4b** and **4d**) were shown to have an excellent anthelmintic activity in vitro at different concentrations. The anthelmintic activity was found to be dependent on the type of cationic compound, concentration and incubation time. The cationic di-C_{12} (**4a**) derivate of the series was the best anthelmintic agent, its use was optimal at a minimum concentration of 50 ppm and with 60 min of incubation.

Keywords Cationic dimeric compounds · Anthelmintic activity · Surface-active properties · Gemini surfactants · *Heligmosomoides polygyrus bakeri*

Introduction

Over the last two decades, the growing demand for high-performance surface-active agents has multiplied the interest of the academic and industrial communities in the development of new gemini surfactants. Gemini surfactants consist of two hydrophobic chains and two polar headgroups covalently linked by a spacer. A considerable number of investigations have been carried out on gemini surfactants, focusing on their unique surface and bulk properties, such as high surface activity, low critical micelle concentration (CMC), unusual viscosity behavior, and specific aggregation structures [1–13].

Intestinal nematodes are extremely important pathogens of domestic livestock, especially sheep, goats and cattle. Collectively, they are responsible for severe losses to livestock agriculture throughout the world. The gastrointestinal (GI) nematode infections of small ruminants constitute the most important health-related impediment to productivity [14–16].

A range of different control strategies is available for gastrointestinal nematode infections including the use of anthelmintics, grazing management and improvements in sanitation, but these control methods are associated with many problems, the most important being the rapid

In memory of doctors Ricardo J. Grau and María I. Cabrera.

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development and spread of resistance to the currently available chemotherapeutic anthelmintic drugs [16].

Resistance has already developed to the three drug classes in current use (the benzimidazoles/tetrahydropyrimidines and the macrocyclic lactones), despite each class having a different mode of action. Biological control or vaccines are unlikely to be available in the near future, so alternative strategies for the control of these parasite infections are urgently required [17–24].

In this paper, we present the preparation method, surface-active properties, and the anthelmintic activity of cationic dimeric compounds having a flexible, hydrophilic spacer and two varying alkyl chains. The anthelmintic properties were assayed using the rodent GI nematode *Heligmosomoides polygyrus bakeri*, in an in vitro assay using different aqueous solutions of cationic gemini compounds.

Experimental Procedures

Chemicals

All the chemicals used in synthetic procedures were of reagent grade quality and used as received. The purity and chemical structure of the synthesized compounds were checked by TLC, HRMS and NMR spectra.

Synthesis of 1,5-Dioxaspiro[5.5]undecane-3,3-dimethanol (**2**)

The reaction was carried out in a 100-mL three-neck round-bottomed flask equipped with a magnetic stirring bar and a Dean-Stark trap topped with a condenser. A catalytic amount of *p*-toluenesulfonic acid monohydrate (5% w/w relative to the cyclohexanone) was added to a pentaerythritol (2.995 g, 22 mmol) and benzene-dimethylformamide (40:60) mixture (50 mL) at room temperature. The well-stirred dispersion was warmed to 80 °C until complete dissolution, and cyclohexanone (1.571 g, 15 mmol) was then added dropwise while the reaction mixture was heated to 115 °C for 48 h. The water formed during the reaction was removed by distillation and collected in the Dean-Stark trap. The reaction was stopped when there was no further increase in the collected water. After cooling, the reaction mixture was poured into water (30 mL), neutralized with potassium carbonate and extracted three-fold with dichloromethane (30 mL). The organic layer was dried with $MgSO_4$ and evaporated in vacuo. After Kugelrohr distillation and column chromatography on silica gel by eluting with ether-light petroleum (80:20) mixture, the compound **2** was obtained as a white solid in good yield (90%) and good selectivity (92%). Physical data of **2**: White solid. Melting point (m.p.) 123–124 °C. IR (KBr): $\nu = 920.0$, 1,039.6, 1,062.7, 1,107.1, 1,369.4, 2,856.4, 2,922.0, 3,273.0 cm^{-1}. ^1H NMR [200 MHz, $(CD_3)_2SO$]: $\delta = 1.31–1.57$ (m, 6H), 1.61–1.78 (m, 4H), 3.27–3.48 (m, 4H), 3.61 (s, 4H), 4.47 (t, 2H $J = 6.5$). ^{13}C NMR [50 MHz, $(CD_3)_2SO$]: $\delta = 22.11$ (CH_2-4′), 25.19 (CH_2-3′ − CH_2-5′), 32.25 (CH_2-2′ − CH_2-6′), 40.36 (C), 60.63, 60.79, 61.19 (CH_2O − CH_2OH), 96.88 (C-1′). MS: spectra *m/z* (% rel. int.): 216 (M^+, 6), 187 (13), 173 (96), 160 (4), 125 (5), 101 (6), 99 (14), 83 (34), 71 (40), 55 (100), 41 (72). Anal. calc. for $C_{11}H_{20}O_4$ (Mol. wt. 216.278): C 61.08, H 9.32, O 29.59. Found: C 61.01, H 9.61, O 29.38. Already published [6].

Synthesis of 1,5-Dioxaspiro[5.5]undecane-3,3-dimethyl diglycidyl ether (**3**)

The Williamson etherification was carried out in solid–liquid reaction system with **2** and (±)-epichlorohydrin using tetrabutylammonium hydrogen sulfate (TBAB) as the phase-transfer catalyst yielding the diglycidyl ether **3**, as key intermediate. A 1:10:6:0.1 molar mixture of **2** (0.843 g, 3.90 mmol), (±)-epichlorohydrin (3.608 g, 39.0 mmol), NaOH (0.936 g, 23.40 mmol), and TBAB (0.132 g, 0.39 mmol), was heated at 30 °C under vigorous stirring (700 rpm) for 1.5 h. The solid material was filtered, washed with CH_2Cl_2 (150 mL) and the washing solvent evaporated to dryness. The residue was partitioned between Et_2O (2 × 150 mL) and brine (80 mL). The two organic layers were combined, dried over $MgSO_4$, filtered and the organic solvent evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel with acetone/hexane (3:7) as eluants. The compound **3** was obtained as a pale yellow oil, in good yield (92%) and excellent selectivity (98.9%). Physical data of **3**: Pale yellow oil. IR (KBr): $\nu = 1{,}100$, 1,460.0, 2,980.0 cm^{-1}. ^1H NMR [200 MHz, $(CDCl_3)$]: $\delta = 1.44–1.51$ (m, 10H), 2.45–2.60 (m, 4H), 2.96 (m, 2H), 3.46–3.64 (m, 4H), 3.80 (s, 4H), 4.13 (m, 2H), 4.23 (m, 2H). ^{13}C NMR [50 MHz, $(CDCl_3)$]: $\delta = 22.00$ (CH_2-4′), 24.26 (CH_2-3′ − CH_2-5′), 33.22 (CH_2-2′ − CH_2-6′), 41.58 (C), 44.73 ($CH\underline{CH_2}O$), 49.91 (CH), 63.19 ($C\underline{CH_2}O$), 86.08 ($CH\underline{CH_2}O$), 99.28 (C-1′). MS: spectra *m/z* (% rel. int.) = 328 (M^+, 3), 299 (4), 285 (12), 113 (10), 83 (42), 55 (95), 31 (100). Anal. calc. for $C_{17}H_{28}O_6$ (Mol. wt. 328.406): C, 62.17; H, 8.59; O, 29.24. Found: C, 62.28; H, 8.51; O, 29.21. Already published [6].

Synthesis of Bis-alkyldimethylammonium salts (**4a–e**)

A 2.5:1:0.1 molar mixture of *N*,*N*-dimethylalkylamine (*N*,*N*-dimethyldodecylamine 0.815 g, *N*,*N*-dimethyloleylamine 1.129 g, *N*,*N*-dimethyloctadecylamine 1.138 g,

N,*N*-dimethylfarnesylamine 0.954 g, dimethyl-(4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoro-undecyl)-amine 1.932 g; i.e., 3.825 mmol), diglycidyl ether **3** (0.331 g, 1.530 mmol) and TBAB (0.049 g, 0.153 mmol) was added to absolute ethanol (12 mL), and the solution was stirred for 20 h at 30 °C. Then, the solvent was evaporated under reduced pressure at room temperature, and the residue was purified by column chromatography using a two-step procedure to afford the desired products. The unreacted tertiary amine was removed from the residue using ethyl acetate/acetone/NH_4OH (4:1:0.5) solvent system as eluant. This procedure was repeated using CH_2Cl_2/EtOH (1: 1) as eluants, and the eluates containing the desired products were evaporated under reduced pressure. Further addition of the HCl solution provides the corresponding salts rather than counteranions. The above mentioned general procedure gave **4a**–**e** in 97, 61, 46, 47, and 95% yields, respectively. The physical data of the synthesized products are as follows.

*1,5-Dioxaspiro[5.5]undecane-3,3-bis(1-methoxy-3-dodecyldimethylammonio-propan-2-ol) dichloride (**4a**)*

White waxy product. IR (KBr): ν = 1,110.0, 2,950.0, 3,640.0 cm^{-1}. ^1H NMR [200 MHz, ($CDCl_3$)]: δ = 0.83–0.91 (m, 10H), 1.21–1.40 (m, 32H), 1.37–1.60 (m, 10H), 1.95–2.01 (m, 4H), 3.12–3.16 (m, 6H), 3.32 (s, 12H), 3.33–3.41 (m, 4H), 3.77–4.05 (m, 8H), 4.13 (s, 2H), 4.23 (s, 2H). ^{13}C NMR [50 MHz]: δ = 14.04, 22.00, 22.62, 23.79, 24.26, 26.08, 26.87, 29.37, 29.45, 29.61, 29.62, 31.68, 34.22, 41.11, 59.43, 60.92, 63.50, 66.93, 68.57, 71.20, 98.27. Anal. calc. for $C_{45}H_{92}Cl_2N_2O_6$ (Mol. wt. 828.128): C, 65.27; H, 11.18; Cl, 8.55; N, 3.36; O, 11.64. Found: C, 65.26; H, 11.19; Cl, 8.56; N, 3.38; O, 11.61. Already published [6].

*1,5-Dioxaspiro[5.5]undecane-3,3-bis(1-methoxy-3-oleyldimethylammonio-propan-2-ol) dichloride (**4b**)*

White waxy product. IR (KBr): ν = 1,085.9, 2,940.0, 3,640.0 cm^{-1}. ^1H NMR [200 MHz, ($CDCl_3$)]: δ = 0.85–0.94 (m, 10H), 1.25–1.32 (m, 40H), 1.39–1.60 (m, 10H), 1.94–1.98 (m, 4H), 3.12–3.16 (m, 6H), 3.32 (s, 12H), 3.34–3.40 (m, 4H), 3.79–4.05 (m, 6H), 4.13 (s, 2H), 4.23 (s, 2H), 5.29–5.32 (m, 4H). ^{13}C NMR [50 MHz]: δ = 13.95, 22.00, 22.57, 23.79, 26.08, 27.88, 29.42, 29.69, 29.76, 31.60, 31.99, 34.22, 41.11, 59.43, 60.92, 63.07, 66.93, 68.57, 71.20, 98.28, 130.70. Anal. calc. for $C_{57}H_{112}Cl_2N_2O_6$ (Mol. wt. 992.415): C, 68.95; H, 11.39; Cl, 7.17; N, 2.80; O, 9.69. Found: C, 68.98; H, 11.37; Cl, 7.14; N, 2.82; O, 9.69. Already published [6].

*1,5-Dioxaspiro[5.5]undecane-3,3-bis(1-methoxy-3-octadecyldimethylammonio-propan-2-ol) dichloride (**4c**)*

White waxy product. IR (KBr): ν = 1,085.9, 2,940.0, 3,640.0 cm^{-1}. ^1H NMR [200 MHz, ($CDCl_3$)]: δ = 0.85–0.94 (m, 10H), 1.25–1.32 (m, 44H), 1.39–1.60 (m, 10H), 1.94–1.98 (m, 4H), 3.12–3.16 (m, 6H), 3.32 (s, 12H), 3.34–3.40 (m, 4H), 3.79–4.05 (m, 6H), 4.13 (s, 2H), 4.23 (s, 2H), 5.29–5.32 (m, 4H). ^{13}C NMR [50 MHz]: δ = 13.95, 22.00, 22.57, 23.79, 26.08, 27.88, 29.42, 29.69, 29.76, 31.60, 31.99, 34.22, 41.11, 59.43, 60.92, 63.07, 66.93, 68.57, 71.20, 98.28, 130.70. FAB-HRMS (M—2Cl–2CH_3)$^+$: Calcd. for $C_{57}H_{116}Cl_2N_2O_6$: 895.8442. Found: 895.8421.

*1,5-Dioxaspiro[5.5]undecane-3,3-bis[1-methoxy-3-(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl-dimethylammonio-propan-2-ol] dichloride (**4d**)*

White waxy product. IR (KBr): ν = 1,085.9, 2,940.0, 3,640.0 cm^{-1}. ^1H NMR [200 MHz, ($CDCl_3$)]: δ = 1.40–1.57 (m, 10H), 1.63 (s, 12H), 1.69 (s, 6H), 1.94–2.18 (m, 16H), 3.32 (s, 12H), 3.34–3.56 (m, 4H), 3.70–4.25 (m, 8H). ^{13}C NMR [50 MHz]: δ = 15.23, 17.56, 22.00, 23.22, 24.26, 25.56, 27.03, 34.22, 38.48, 39.95, 41.11, 53.58, 60.92, 62.77, 63.07, 66.95, 68.83, 71.20, 98.28, 124.17, 124.90, 129.89, 135.27, 144.63. FAB-HRMS (M—2Cl–2CH_3)$^+$: Calcd. for $C_{51}H_{92}Cl_2N_2O_6$: 799,6564. Found: 799.6535.

*1,5-Dioxaspiro[5.5]undecane-3,3-bis[1-methoxy-3-(4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11)-heptadecafluo roundecyldimethylammonio-propan-2-ol] dichloride (**4e**)*

White waxy product. IR (KBr): ν = 1,085.9, 2,940.0, 3,640.0 cm^{-1}. ^1H NMR [200 MHz, ($CDCl_3$)]: δ = 1.41–1.58 (m, 10H), 2.19–2.37 (m, 8H), 3.25 (dd, 4H), 3.32 (s, 12H), 3.34–3.37 (m, 4H), 3.85 (m, 10H), 4.13 (s, 2H), 4.23 (s, 2H). ^{13}C NMR [50 MHz, ($CDCl_3$)]: δ = 14.00, 22.80, 25.95, 28.93, 29.23, 29.55, 29.60, 29.90, 32.20, 42.24, 62.74, 64.09, 70.11, 71.14, 72.71, 73.77, 98.28, 110.36, 115.24, 126.65, 130.50. FAB-HRMS (M–2Cl)$^+$ Calcd. for $C_{43}H_{54}F_{34}Cl_2N_2O_6$: 1340.3438. Found: 1340.3421.

Analytical Methods

Melting points (m.p.) were determined on a Büchi 510 micro melting point apparatus and were not corrected. Infrared (IR) spectra were recorded on a Shimadzu 8201 PC spectrophotometer; ^1H-, ^{13}C- and ^{19}F-NMR spectra on a Bruker FT-200 spectrometer, using $(CD_3)_2SO$ and $CDCl_3$

as solvent. Chemical shifts (δ) were reported in ppm related to internal tetramethylsilane. Mass spectra (MS) were obtained using a Shimadzu GCMS-QP 5000 spectrometer. Elemental analyses were performed at the Galbraith Laboratories, Inc., Knoxville, United States. Gas–liquid chromatography (GLC) analyses were performed on a Shimadzu GC-17AATF chromatograph equipped with a methyl silicone capillary column (30 m × 0.32 mm, 0.25 μm film thickness) with a flame ionization detector. Column Chromatography was performed on silica gel (70–230 mesh ASTM). Isolated and authenticated compounds were used as internal standards to perform quantitative GC analyses.

The surface tension values of aqueous solutions (pH 7) were measured at 20 °C using a semiautomatic tensiometer apparatus (Cole-Parmer Surface Tensiomat 21) by the Du Nouy ring method. Calibration was performed against a range of standard liquids obtaining an excellent agreement with the reference values. A time-dependent surface tension behavior was observed by an increase of the experimental values over successive measurements at each concentration. This behavior has been related to the difficulties of gemini in organizing at the air/water interface [8]. The surface tension was then measured three times for each sample within a 40-min interval between each reading to ensure equilibrium data. The critical micelle concentration (CMC) values were determined using a series of aqueous solutions at various concentrations, and estimated from the break point of each surface tension versus concentration (on a log scale) curves. The ability of these compounds to lower surface tension at the CMC (γ_{CMC}) and reduced by 20 mN/m (C_{20} or pC_{20}) were calculated therefrom. The optimal cross-section surface area A occupied by the surfactant headgroup at the air/water interface was estimated from the surface excess concentration Γ. Results are summarized in Table 1.

Animals

Two C57 black male mice of 10 weeks of age were used. The animals were provided with food and water ad libitum.

Parasites

Mice were infected with a suspension of 150 *H. Polygyrus bakeri* L3 in 0.1 mL of distilled water. After 14 days post-infection, mature male and female worms were available for use in vitro. The mice were euthanized humanely by inhalation anesthesia, then, opened longitudinally with a pair scissors and the small intestine was removed. The intestine was placed in a Petri dish containing pre-warmed (37 °C) Hanks′ Balanced Salt Solution (HBSS) for 5–10 min to allow the worms to get out of the intestine. The adult male and female worms were identified by optic-microscope, separated and placed in pre-warmed HBSS before use.

Effects of Gemini Compound on the Motility of Adult Worms

One adult male and one adult female worm were transferred to each well of a 3-well plate containing Hanks′ saline, pH 7.2 (without phenol red) and one of the following gemini preparations: 0, 10, 50, 100 and 500 ppm of **4a–e**. Control wells were incubated at the same time.

These plates were incubated at 37 °C and the worms state was assessed visually every 15 min for 2 h, using a standard 0–5 motility scale, where 0 is motionless and 5 is fully active [15]. The figures shows the mean motility for specific treatments at given times [±SE of the mean (SEM)]. In separate experiments, 2 male and 2 female worms were removed from plates containing either 500 ppm **4a**, 500 ppm **4d** o HBSS. These worms were then prepared for scanning electron microscopy (SEM) following fixation in 2.5% glutaraldehyde in 0.1 M phosphate

Table 1 Surface activity and aggregation data of synthesized dimeric cationic compounds

C4aompound	CMC (mM)	C_{20} (mM)	γ_{CMC} (mN/m)	pC_{20}	CMC/C_{20}	$\Gamma \times 10^6$ (mol/m^2)	$A \times 10^{20}$ (m^2)
4a	1.54	0.146	35.93	3.84	10.55	2.59	64.17
4b	0.38	0.038	34.61	4.42	10.00	2.87	57.82
4c	0.05	0.021	40.95	4.68	2.38	4.72	35.18
4d	1.30	0.262	33.78	3.58	4.96	5.07	32.74
4d (NaCl 0.1 M)	1.08	0.093	31.75	4.03	11.61	3.73	44.51
4e	0.10	0.006	27.34	5.18	16.67	3.60	46.12

Experimental conditions: temperature 20 ± 0.5 °C, aqueous solution at pH 7, experimental uncertainties are estimated to be ±0.03 mM on CMC and ±1 mN/m on γ_{CMC} values

buffer, pH 7.2, washing in ethanol 50% and (GA 2.5%) and critical point drying from Polaron apparatus. Dried specimens were sputter-coated with gold before examination in a LEO EVO40 scanning electron microscope.

Statistics

The data obtained from the in vitro motility experiments were analyzed using factorial ANOVA with Statgraphics Plus 5.1. For analysis of changes in mobility with time, we fitted motility as dependent variable. Sex (male/female) of worm, compound (**4a–c**) and concentration of gemini compounds were fitted as factors. All tests were performed with a confidence level of 95%. For determining which means were significantly different from each other, we applied the Tukey method.

Results and Discussion

Synthesis

The new series of cationic dimeric surfactants were easily prepared following the three-step procedure outlined in Scheme 1. The intermediate compound **2** was obtained by ketalization of pentaerythritol **1** with cyclohexanone, with a benzene-DMF mixture as solvent and in the presence of *p*-toluensulfonic acid monohydrate as a homogeneous catalyst (step a). The diglycidyl ether **3** was obtained by etherification of **2** with (±)-epichlorhydrine via solid–liquid phase-transfer catalysis using tetrabutylammonium bromide (TBAB) as the catalyst (step b). The *β*-hydroxy amino or ammonium alkyl functionalization was performed by regioselective oxirane ring opening with tertiary amines to prepare the cationic compounds **4a–e** (step c). The nucleophilic substitutions involving tertiary amines were assisted by TBAB as phase transfer catalyst.

Surface Properties

The cationic compounds **4a–c** and **4e** exhibited clear solutions and displayed a sharp break of the surface tension versus concentration (on log scale) curves and a final plateau indicating a well-defined CMC and surface tension at the CMC (γ_{CMC}), as shown in Fig. 1 (**4a** and **4b** are not shown because they had already been published [6]). The surface tension versus concentration curve for the **4d** compound shows a particular behavior due to the presence of three unsaturations into the hydrophobic group, these unsaturations modify the solubility of the surfactant in water and causes looser packing of the surfactant molecules at the interface, the *cis* isomer is particularly loosely packed; the *trans* isomer is packed almost as closely as the saturated isomer [7]. The surface excess, Γ, at the air–water interface was calculated by applying the Gibbs adsorption isotherm equation. The area per molecule at the interface was estimated

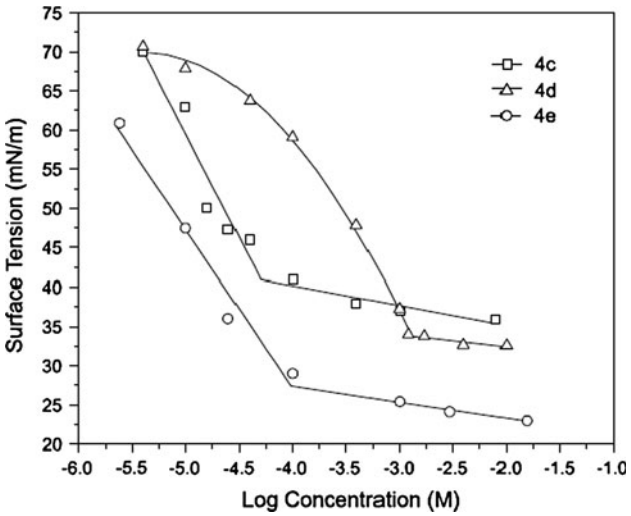
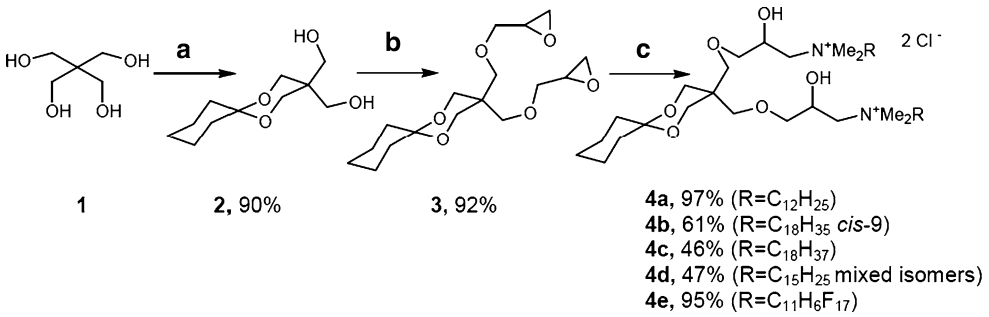


Fig. 1 Surface tension versus logarithm of the aqueous molar concentration (log C) of synthesized dimeric cationic compounds at pH 7 and 20 °C



Scheme 1 Synthetic steps to cationic dimeric compounds: (**a**) pentaerythritol (*1*), cyclohexanone, *p*-toluensulfonic acid monohydrate, benzene-DMF (40:60), 115 °C, 48 h; (**b**) Diol (*2*), (±)-epichlorhydrine, NaOH, TBAB, 30 °C, 1.5 h; (**c**) Diglycidyl ether (*3*), *N*,*N*-dimethylalkylamine, TBAB, ethanol, 30 °C, 20 h

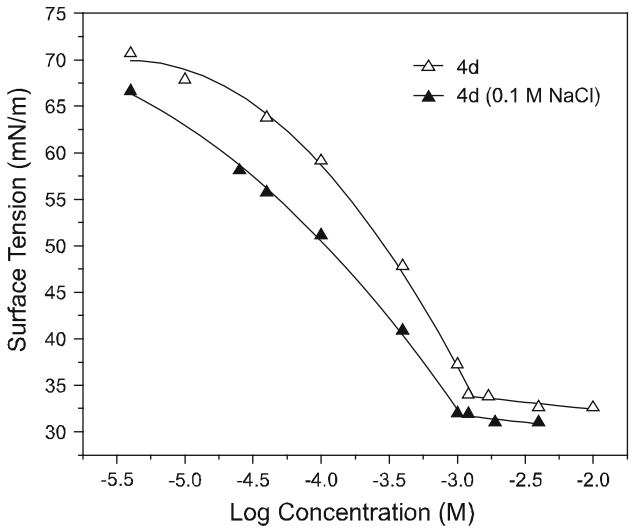


Fig. 2 Dependence of surface tension on concentration of the gemini compound **4d**. Curves are related to the absence of NaCl and the presence of 0.1 M NaCl at pH 7 and 20 °C

from the corresponding value of Γ. Table 1 summarizes the CMC, C_{20}, γ_{CMC}, pC_{20}, CMC/C_{20}, Γ, and A values, which are in agreement with those characterizing the proper quantitative behavior of good surfactants. The pC_{20} values indicate that the five-cationic compounds are good surfactants, especially **4e**. The surface excess Γ and the area per molecule A vary for the compounds of the cationic series. In fact, the area per molecule at air/water interface of headgroup in the surfactants of the cationic series was found to be within a range 32.74–64.17 Å^2, showing a smaller area per molecule with increasing tail length (**4a**–**e**). This would be attributable to the flexibility of the spacing group and stronger intermolecular Van der Waals forces at increasing chain lengths.

Figure 2 shows the surface tension versus log surfactant concentration in the absence and presence of 0.1 M NaCl. The values of CMC obtained from Fig. 2 and Table 1 confirm the results obtained by Rabinovich et al. They conclude that the CMC decreases with increasing electrolyte concentration for gemini surfactants [25].

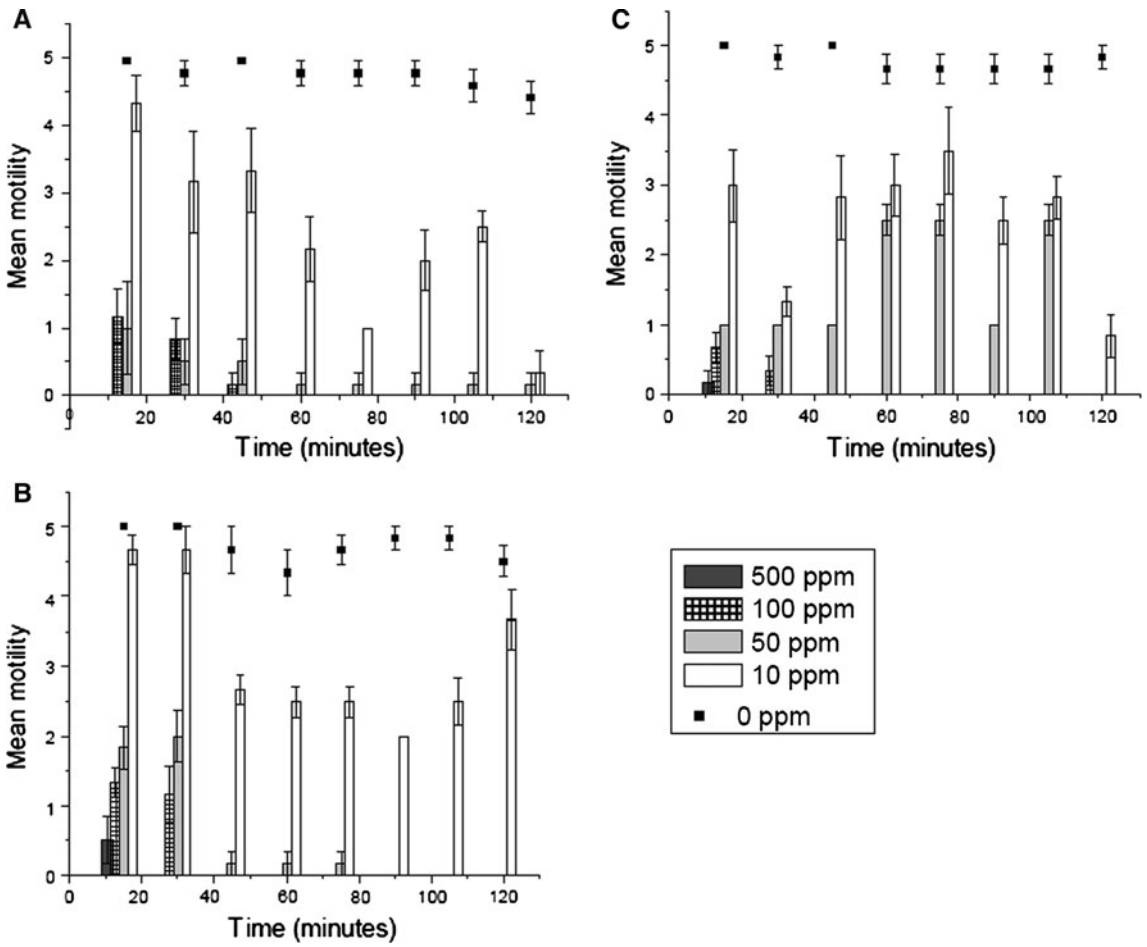


Fig. 3 The motility of *Heligmosomoides polygyrus bakeri* adult worms' exposed to various gemini compounds in vitro. Data for male and female worms were combined because there was no significant effect of sex. **a** Gemini compound **4a** (**b**) gemini compound **4b** and **c** gemini compound **4d**

Incubation of Worms in Varying Concentrations of Gemini Compounds

The gemini compounds **4c** and **4e** showed no activity against *H. polygyrus bakeri* already showing a highly variable behavior. For the three cationic compounds (**4a**, **4b** and **4d**) that showed significant in vitro activity against *H. polygyrus bakeri* factorial analysis of variance was performed also including interactions between factors. The motility of male and female adult worms exposed to various gemini compounds showed no significant difference between the sexes (p value = 0.1983). Moreover, the concentration, the type of compound and incubation time were factors with a statistically significant effect on mobility (p values <0.05).

Representative graphs of the time-course of anthelmintic activity for different concentrations of the gemini compounds are shown in the Fig. 3. It is evident that, with the exception of the 10 ppm concentration, all the solutions produced a rapid detrimental effect on *H. polygyrus bakeri* adult male and female worms. In contrast, the worm motility was high in Hanks′ solution alone (controls).

The motility of the worms declined in all experiments, over the period of observation, but the loss of motility was significantly greater among worms exposed to high concentrations.

Statistics: Tukey′s Test

The Tukey method resulted in two homogeneous groups for the three active cationic gemini compounds. We concluded that the gemini compound of 12 carbon saturated (**4a**) presented the best performance, while the products **4b** and **4d** were equivalent in their biological activity. To determine the optimal concentration and incubation time needed to obtain the best results for biological activity, for each of the gemini compounds we used Tukey ′s test. For the gemini compound **4a**, it was shown that concentrations of 500, 100 and 50 ppm constituted a homogeneous group, like that for 60, 75, 90, 105 and 120 min. Therefore we recommend the use of compound **4a** at a concentration of 50 ppm for 60 min. In the case of the **4b** and **4d** compounds, the optimal values of concentration and incubation time obtained were 100 ppm, 45 min and 100 ppm and 120 min respectively.

SEM of Worms Incubated in Gemini Compounds

The cuticle was observed using SEM. Figure 4 shows that there is no damage in the cuticle of the nematode. These results suggest that the mechanism of action of gemini tested is not obtained at the cuticle surface level of the parasite. In contrast, Stepek et al., examined crude and

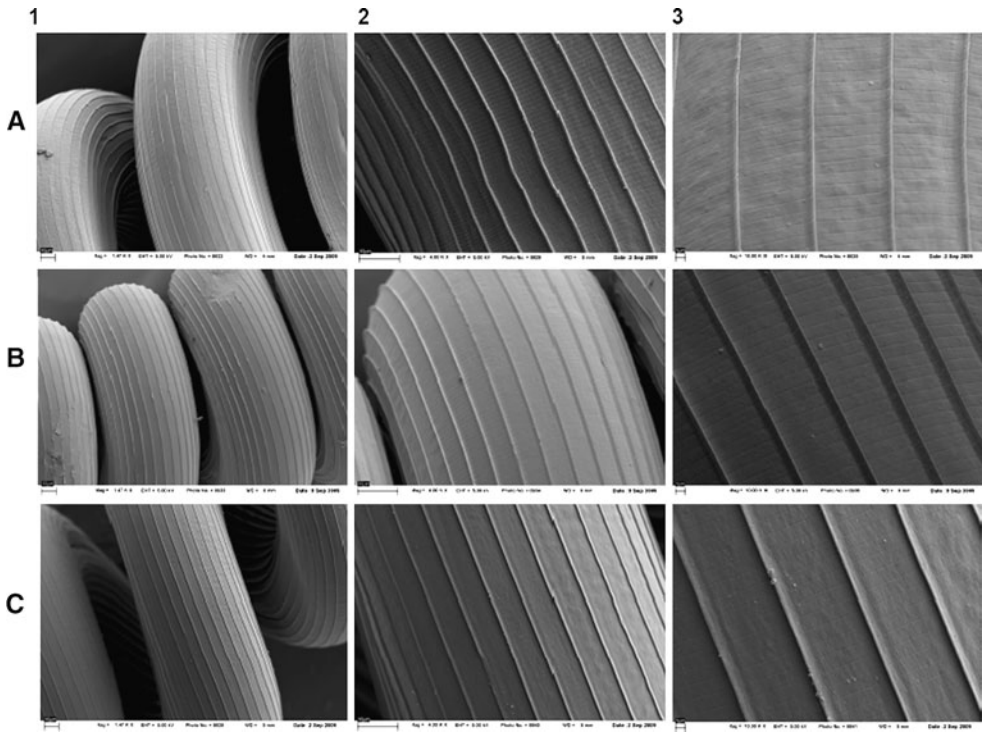


Fig. 4 Scanning electron micrographs of a *Heligmosomoides polygyrus bakeri* adult worm exposed to a gemini compound in vitro. There is no evidence of damage to the cuticle that can be seen from (**a**) worms incubated in Hanks′ saline (**b**) worms incubated in **4a** 500 ppm for 30 min. **c**. Worms incubated in **4d** 500 ppm for 30 min. In **1** Mag = 1.47 k x; en **2** Mag = 4.00 k x y en **3** Mag = 10 kx. All the photos are EHT = 5.00 kv

purified enzymes from papaya, fig, pineapple, kiwi fruit and Egyptian milkweed, using *Heligmosomoides polygyrus* and *Trichuris muris*, in an in-vitro assay employing preparations that had been standardized for the number of active enzyme molecules [16]. Their observations clearly indicate that the mechanism of action of all the efficacious plant cysteine proteinases is similar, and probably identical, involving digestion and removal of the cuticle.

Observations by scanning electron microscopy suggest that the mechanism of action of this compound does not take place at the cuticle of the parasite since there was no damage in the outer surface thereof. Apparently, the active compound enters the parasite by an undetermined mechanism causing its effect.

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