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Spermicidal, anti-HIV, and micellar properties of di- and trihydroxylated cationic surfactants

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Abstract—The syntheses of 2-hydroxy-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-1-alkanaminium **1**(*p*) and 2-hydroxy-*N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-1-alkanaminium **2**(*p*) bromides are described for the homologous series, tetradecanaminium (p=14) through octadecaminium (p=18). The critical micelle concentrations (cmc's), measured by conductivity at 25 and 37°C, of the even-numbered members of the series show the expected linear dependence on chain length. All compounds display spermicidal and virucidal activity. Plots of minimum effective concentration (MEC) versus chain length in the Sander–Cramer spermicidal assay of **1** and **2** show a cut-off effect and a parabolic relationship, respectively. The best compounds—**1**(17) and **1**(18)—have MECs ($\sim$0.12 mg mL^{-1}) slightly lower than that of the commercial spermicide nonoxynol-9 (N-9). Compounds **1**(16) and **2**(17) show the best anti-HIV activity in in-vitro, cell-free and cell-associated virus inactivation assays. Both are highly effective at 0.1 mg mL^{-1} in the cell-free assay and 0.5 mg mL^{-1} in the cell-associated assay. Compound **1**(16) shows the best combination of dual activity against sperm and HIV. In summary, hydroxylated cationic surfactants, **1** and **2**, show promise as inexpensive topical, contraceptive microbicides. © 2002 Published by Elsevier Science Ltd.

Many women want to control their fertility and reduce their risk of acquiring a sexually transmitted disease (STD).[1–4] Developing user-controlled, topical vaginal microbicides that provide protection against human immunodeficiency virus type 1 (HIV-1) and other sexually transmitted pathogens is an urgent global priority.[5]

Nonoxynol-9 (N-9), currently the most widely used spermicide,[6] functions as a detergent by disrupting cell walls and solubilizing membranes. It may increase the risk of HIV transmission[7] by activating interleukin-1-mediated NF-κB activation, which leads to cytokine-induced recruitment of HIV-1 host cells and increased HIV-1 replication.[8] N-9 increases the risk of urinary tract infections[9,10] vulvovaginal candidiasis[11] and genital ulcers.[12] Furthermore, N-9, a mixture of oligomers,[13–17] may not meet future regulations as the health-care industry increasingly uses pure compounds or mixtures whose individual components have met safety standards. The breakdown products of N-9 and related compounds pose health and environmental risks.[18] Several research groups are searching for agents that do not have these disadvantages.[19–23]

Quaternary ammonium salts have a long history as microbicides[24] and can act as spermicides; e.g. European and Canadian spermicidal products contain benzalkonium chloride, a popular microbicide. Although comparatively less spermicidal than N-9,[25] benzalkonium chloride has potential as an anti-HIV, topical agent if the protection of cynomolgus macaques against cervicovaginal transmission[26] of simian immunodeficiency virus (SIV) mac251 translates to humans and HIV. Another common microbicidal quaternary ammonium salt, *N*,*N*,*N*-trimethyl-1-hexadecanaminium bromide, is also spermicidal,[27] but has not been developed further. Although simple quaternary ammonium salts are quite microbicidal, they have some disadvantages in that they have modest solubility in water and can irritate sensitive tissue. Can one find or synthesize a quaternary ammonium salt that minimizes these shortcomings?

Many reasons support our choice to investigate the water-soluble quaternary ammonium salts, **1**(*p*) and **2**(*p*), as spermicides and anti-HIV agents. They can be easily formulated in hydrogels, common vehicles for the delivery of spermicides. The **1**(*p*) series, originally patented for anti-static and fabric-softening finishes for textiles,[28,29] is now

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Keywords: spermicidal activity; anti-HIV activity; critical micelle concentration; quaternary ammonium salts.

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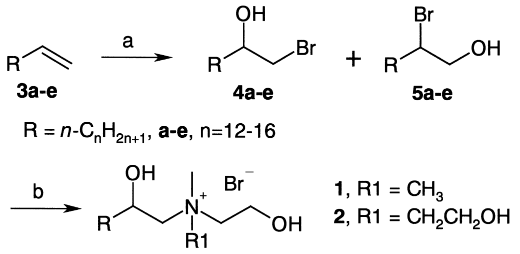
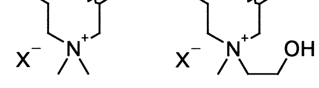


Scheme 1. (a) NBS, THF/H_2O (5:1), rt, 18 h; (b) R1N(CH_3)CH_2CH_2OH (R1=CH_3, CH_2CH_2OH), MeOH, heat, 3 d.

found in shampoo formulations,[30] suggesting that it is a safe for topical applications. A similar suggestion can be made for the **2**(p) series, which is patented for hair-conditioning formulations.[31] Furthermore, **1**(p) and **2**(p) are choline derivatives and somewhat resemble an alkylated sphingosine. Consequently, they might inhibit proteins involved in cellular signaling and, thus, operate as selective microbicides rather than indiscriminate detergents. Finally, these compounds are made from inexpensive starting materials, viz. hydroxyethylamines and 1-alkenes, which are commodity chemicals.



1(p) **2**(p)

(p = n+2, where n = # of carbons in R = n-C_nH_{2n+1})

In this paper, we report the experimental details for the syntheses of both series and apply the historic method of varying the chain-length (p=14–18) to probe structure-activity in spermicidal and anti-HIV assays. Furthermore, we have measured the critical micelle concentrations (cmcs) of the even-numbered members of each series to test whether these physicochemical properties affect the structure–activity relationships.

Table 2. A and B values for equation that correlates cmc with chain length (p) of **1** and **2** at 25 and 37°C

Compounds and temperature	*A*	*B*
1 at 25°C	1.60	0.279
1 at 37°C	1.73	0.286
2 at 25°C	1.53	0.274
2 at 37°C	1.56	0.275
N,*N*,*N*-Trimethyl-1-alkanaminium bromides[a] at 60°C	1.77	0.292
N,*N*,*N*-Trimethyl-1-alkanaminium bromides[b] at 25°C	2.01	0.317

[a] Refs. 36,34

[b] Ref. 37

1. Results

1.1. Chemistry

Reaction of an alkene **3a–e** with *N*-bromosuccinimide[32] in THF/H_2O gave a mixture of two bromohydrins **4** and **5** in ratio of ca. 4:1. (Scheme 1.) The two isomers could be separated by careful flash column chromatography. Separation of **4** and **5** was unnecessary, however, because refluxing a mixture of either isomer with *N*,*N*-dimethylethanolamine (R1=CH_3)[28,33] and *N*-methyldiethanolamine (R1=CH_2CH_2OH) in methanol gave quaternary ammonium salts, **1** and **2**, respectively. Pure quaternary ammonium salts were isolated after careful work-up and purification.

We used conductivity to measure the cmcs for selected quaternary ammonium bromides, **1** and **2**, respectively (Table 1). As we anticipated, both **1** and **2** show an increase in cmc with decrease in chain length.

One can determine the slope and intercept of the line generated in a log cmc versus alkyl chain length at a given set of conditions. The equation,[34]

$$\log \text{cmc} = A - BN,$$

where N represents the number of carbons in the long alkyl chain attached to the quaternary nitrogen, enables comparisons among related surfactants. For **1** and **2**, N=p. Intercept A depends on the ionic strength of the system and on

Table 1. Critical micelle concentrations (cmcs) and alpha (ratio of slopes before and after the cmc) for **1** and **2** as a function of temperature and chain length as measured by conductance in distilled water

Compound	10^3 cmc at 25°C (mol dm^{-3})	10^3 cmc at 37°C (mol dm^{-3})	Alpha at 25°C	Alpha at 37°C
1(14)	5.08±0.04	5.35±0.05	0.239±0.002	0.258±0.003
	5.10±0.02	5.38±0.03	0.2399±0.0009	0.292±0.005
1(16)	1.36±0.02	1.406±0.008	0.213±0.003	0.293±0.003
	1.34±0.02	1.411±0.006	0.264±0.006	0.288±0.002
1(18)	0.409±0.008	0.394±0.009	0.27±0.01	0.355±0.008
	0.38±0.01	0.378±0.007	0.26±0.01	0.338±0.008
	0.384±0.007		0.313±0.008	
2(14)	5.06±0.04	5.28±0.02	0.253±0.003	0.296±0.003
	5.07±0.02	5.26±0.02	0.260±0.002	0.299±0.002
2(16)	1.26±0.01	1.351±0.009	0.235±0.003	0.285±0.002
	1.262±0.009	1.37±0.05	0.252±0.002	0.31±0.01
2(18)	0.409±0.009	0.422±0.006	0.200±0.004	0.308±0.005
	0.401±0.007	0.413±0.006	0.254±0.003	0.332±0.003

Table 3. Spermicidal activity of **1** in the Sander–Cramer assay

Compound	Highest spermicidal dilution (HSD) (1/*X*)	MEC (mg mL^{-1})
1(14)	34.0±4.4	0.781±0.163
1(15)	117.3±14.8	0.202±0.023
1(16)	186.7±33.3	0.140±0.019
1(17)	192.0±18.5	0.117±0.011
1(18)[a]	192.0±18.5	0.117±0.011
N-9[b]	72.0±7.7	0.156±0.016

Sperm samples ($n=12$) were mixed with two-fold serial dilutions of the compounds in 0.9% NaCl (initial concentration of compound in distilled water, 20 mg mL^{-1}) and observed under the microscope for 20 s. Those dilutions that completely immobilized all screened spermatozoa were further diluted in excess buffer and incubated for 1 h to verify lack of motility recovery. The highest dilution that successfully passed both assessments was considered the HSD and was used to calculate the MEC.

[a] Requires warm water (40°C) to remain in solution at 20 mg mL^{-1}.

[b] Initial concentration, 10 mg mL^{-1}.

Table 4. Spermicidal activity of **2** in the Sander–Cramer assay

Compound	HSD (1/*X*)	MEC (mg mL^{-1})
2(14)	30.7±3.5	0.378±0.043
2(15)	45.3±4.6	0.248±0.022
2(16)	62.7±7.2	0.202±0.040
2(17)[a]	92.0±11.0	0.156±0.042
2(18)[a]	56.0±10.1	0.235±0.028
N-9	72.0±7.7	0.156±0.016

Sperm samples ($n=12$) were mixed with two-fold serial dilutions of the compounds in 0.9% NaCl (initial concentration of compound in distilled water, 10 mg mL^{-1}) and observed under the microscope for 20 s. Those dilutions that completely immobilized all screened spermatozoa were further diluted in excess buffer and incubated for 1 h to verify lack of motility recovery. The highest dilution that successfully passed both assessments was considered the HSD and was used to calculate the MEC.

[a] Requires warm water (40°C) to remain in solution at 10 mg mL^{-1}.

the type of hydrophilic head group, and slope B depends on the number of hydrophilic head groups and the number of hydrophobic alkyl chains present in the amphiphile.[35] Table 2 lists the A and B values for **1**, **2**, and *N*,*N*,*N*-trimethyl-1-alkanaminium bromides.[34,36,37]

For this narrow range, temperature has little effect on the cmc's of **1** and **2**; the values of A and B are very similar at 25 and 37°C. This limited comparison of three quaternary ammonium bromides suggests that increasing hydroxylation decreases A and B; both A and B decrease in the order *N*,*N*,*N*-trimethyl-1-alkanaminium bromides>**1**>**2**. For $p=16$, the values of cmcs rank in the following order: *N*,*N*,*N*-trimethyl-1-hexadecanaminium bromide, 1.00 mM< **2**(16) 1.26 mM<**1**(16), 1.35 mM. As replacement of methyl by hydroxyethyl has no significant effect on the cmc for $p=16$,[38] the cmc increases due to hydroxyl substitution on the long alkyl chain.[39] These equations also can accurately predict the cmcs of an odd-numbered chain, e.g. the cmc of **1**(15). The calculated value at 25°C—antilog(1.60+(0.279×15))=2.60 mM—matches our measured values—2.60±0.01 and 2.61±0.01 mM. At least for this limited homologous series ($p=14$–18) of **1**, the values of cmc's maintain linearity in log-cmc-versus-alkyl-chain-length plots.

1.2. Biology

Table 3 presents the minimum effective concentrations (MECs) for spermicidal activity in a modified Sander–Cramer assay[40] for **1** and N-9. MECs represent a measure of potency-the lower the MEC, the more potent the compound. The Sander–Cramer assay determines the minimum concentration required to stop all sperm motility in 20 s. The MEC decreases; i.e. the potency increases, as the chain length increases up to **1**(17). Compounds **1**(17) and **1**(18) have similar MECs, which are slightly lower than that of N-9. Compound **1**(18) has water-solubility problems; hence, the value of its MEC may represent an upper limit.

Table 4 presents MECs for spermicidal activity in a modified Sander–Cramer assay for **2** and N-9. The MECs of the series decrease as the chain length increases up to **2**(17). The MEC of **2**(17) equals that of N-9. Compound **2**(18) has solubility problems; hence, the value of its MEC may represent an upper limit.

Table 5 presents the data for anti-HIV activity of **1** in two in-vitro assays: cell-free and cell-associated virus inactivation. All chain lengths are effective, i.e. ≥3 log units of infectious titer reduction at the two higher concentrations in both assays. At the lower concentrations, **1**(16) emerges as the most potent against cell-free and cell-associated HIV. Compound **1**(16) has comparable activity to that of N-9 in both assays.

Table 6 presents the data for anti-HIV activity of **2** in the cell-free and cell-associated HIV inactivation assays. All chain lengths are effective, i.e. ≥3 log units of infectious titer reduction at the two higher concentrations in both

Table 5. HIV cell-free and cell-associated inactivation assays for **1** and Nonoxynol-9 (N-9). Reduction of viral infectivity (in logs)

	Cell-free inactivation assay concentration (mg mL^{-1})				Cell-associated inactivation assay concentration (mg mL^{-1})			
Compound	1.0	0.5	0.1	0.05	1.0	0.5	0.1	0.05
1(14)	>3.7	>3.7	2.0	1.5	>4.5	>4.5	<1.0	<1.0
1(15)	>3.7	>3.7	1.6	1.3	>4.5	4.0	<1.0	<1.0
1(16)	>3.7	>3.7	3.0	2.3	3.7	3.2	3.0	<1.0
1(17)	>3.7	>3.7	2.3	1.3	>4.5	>4.5	<1.0	<1.0
1(18)	>3.7	>3.7	2.0	1.6	>4.5	>4.5	1.0	<1.0
N-9	>3.7	>4.7	3.3	1.5	>4.5	>4.5	2.0	1.4

Cell-free or cell-associated HIV-1 was incubated with multiple concentrations of the test agents for 2 min at 37°C. Compound effect was then terminated by 10-fold serial dilutions, and the treated virus was further incubated with infection-susceptible MT-2 cells for 6 d. Virus-induced cytopathic effects (formation of cell syncytium) were recorded under microscopic observation. The values presented in the body of the table represent the reduction in the infectious titer of the untreated virus (in logs) effected by each compound concentration.

Table 6. HIV cell-free and cell-associated inactivation assays for **2** and nonoxynol-9 (N-9). Reduction of viral infectivity (in logs)

	Cell-free inactivation assay concentration (mg mL^{-1})				Cell-associated inactivation assay concentration (mg mL^{-1})			
Compound	1.0	0.5	0.1	0.05	1.0	0.5	0.1	0.05
2(14)	>4.5	>5.5	<1.0	<1.0	>3.8	3.6	<0.3	<0.3
2(15)	>4.5	>4.5	<1.0	<1.0	>3.8	3.3	0.3	<0.3
2(16)	>4.5	>4.5	3.3	<1.0	>3.8	>3.8	<0.3	<0.3
2(17)	>4.5	>4.5	4.0	1.0	>3.8	>3.8	<0.3	<0.3
2(18)	>4.5	>4.5	1.8	<1.0	>3.8	3.8	<0.3	<0.3
N-9	>4.5	>5.5	4.0	1.3	>4.8	2.6	0.8	<0.3

Cell-free or cell-associated HIV-1 was incubated with multiple concentrations of the test agents for 2 min at 37°C. Compound effect was then terminated by 10-fold serial dilutions, and the treated virus was further incubated with infection-susceptible MT-2 cells for 6 d. Virus-induced cytopathic effects (formation of cell syncytium) were recorded under microscopic observation. The values presented in the body of the table represent the reduction in the infectious titer of the untreated virus (in logs) effected by each compound concentration.

assays. At 0.5 mg mL^{-1}, **2**(16) and **2**(17) have a higher anti-HIV activity than N-9 against the cell-associated virus. Compounds **2**(17) and **2**(16) also represent the most potent of the series against cell-free HIV, with comparable activity to that of N-9.

2. Discussion

2.1. Spermicidal activity and micellar concentration

Fig. 1 presents the MECs for spermicidal activity and cmcs for **1** plotted as a function of chain length. The cmcs are expressed as mg mL^{-1} to facilitate comparison with the bioassays. The cmcs, which were measured in distilled water, represent an upper limit of the cmc under the conditions of the Sander–Cramer assay. That is, ~1% bovine serum albumin (BSA), 0.9% saline, and Baker's (phosphate and glucose) solution might decrease the cmc. BSA absorbs cationic surfactants, e.g. benzalkonium;[41] this absorption should increase the total surfactant needed to form a micelle. Salt significantly decreases the cmc of hydroxy-functionalized quaternary ammonium bromides.[42] In the presence of salt, the linear relationship between cmc and chain length would likely shift to lower values.

In the curvilinear relationship between MEC and chain length, the curve flattens for **1**(17) and **1**(18). This flattening suggests chain length plays a role in the mechanism of action. Three explanations come to mind. First, the result might arise from two opposing effects—(1) long chains facilitate entry to the cell and lead to disruption of cellular function and (2) long chains tend to form micelles at very low concentrations. Such a decrease in cmc lowers the concentration of monomer in solution. If the micelle does not attack the cell, a higher total concentration of surfactant is needed to have enough monomer to produce the biological result. A second explanation—a chain-length selectivity for incorporating the surfactant into the sperm membrane—is pure speculation. A third explanation is a solubility problem, which is invisible to the naked eye, with **1**(18); there is less of it in solution and the MEC should be lower.

Fig. 2 presents the MECs for spermicidal activity and cmcs for **2** plotted as a function of chain length. As before, the cmcs in water decrease linearly with increasing chain length. When the cmcs are expressed in mg mL^{-1}, compounds **2** have higher cmcs than **1**; the reverse occurs when expressing cmcs in mol×dm^{-3} (Tables 1 and 2). The curvilinear relationship between MEC and chain length

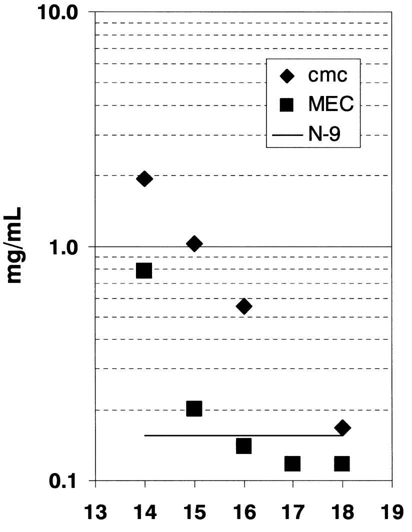


Figure 1. Comparison of spermicidal activities (MECs) for **1** (■) and N-9 (—) with cmcs for **1** (◆) in water.

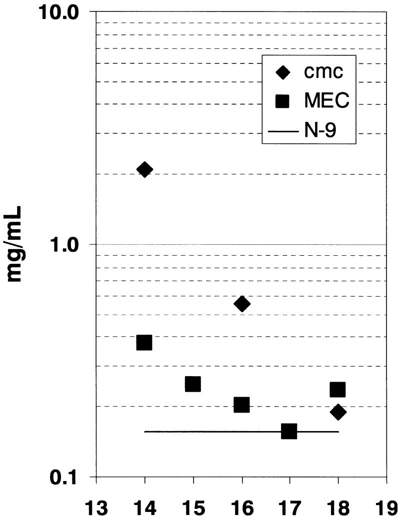


Figure 2. Comparison of Spermicidal Activities (MECs) for **2** (■) and N-9 (—) with cmcs for **2** (◆) in water.

differs from that in Fig. 1. One must be careful, however, in over-interpreting the MEC data because of potential solubility problems. In Fig. 2, the MEC increases for **2**(18) compared to **2**(17). The MEC for **2**(17) represents a minimum in the plot. This 'parabola' in Fig. 2 contrasts with the leveling off seen in Fig. 1 for the MECs of **1**(17) and **1**(18). Compounds **1** and **2** might have different mechanisms for biological activity, but an explanation based on micellar effects or limited solubility cannot be excluded.

2.2. Anti-HIV properties

For anti-HIV properties, (Tables 5 and 6) both **1** and **2** show more activity against cell-free than the cell-associated associated virus, an expected finding given the addition 'protection' offered by the cell carrier. Compounds **1**(16), **2**(16), and **2**(17) are the best compounds in this study with activities similar to or slightly better than that of N-9. The data in Table 5 imply that **1**(16) represents an optimum for antiviral activity, as longer chain lengths are less active. The data in Table 6 imply that **2**(17) represents an optimum for anti-HIV activity, as longer chain lengths are less active. These results could arise from a biological effect related to preferential membrane intercalation or a micellar effect.

2.3. Comparisons with other work

As Shelton et al.[43] found over a half century ago, the microbicidal activity of a homologous series of *N*,*N*,*N*-trimethyl-1-alkanaminium salts increases with increasing chain length until a maximum occurs for the hexadecanaminium homolog. More recent bioassays with a homologous series of *N*,*N*,*N*-trimethyl-1-alkanaminium salts[44,45] show similar results. As Cella et al. suggested,[46] this structure–activity relationship may result from surface-active properties and the tendency of these salts to form aggregates. Although the mechanism of action is unknown, Hueck et al.'s[47] early suggestion that surface interactions of the quaternary ammonium salt at the plasma membrane disrupt cellular processes still holds promise. A recent study[48] suggests that quaternary ammonium salts do not reach the inner monolayer of a cholesterol-rich bilayer membrane, e.g., the sperm plasma membrane. Incorporated surfactants change the fluidity and porosity of a membrane; such changes might have profound effects on cellular function.

The curvilinear relationships in Figs. 1 and 2 between log MEC and chain length reveal the well-documented cut-off effect[49] and parabolic relationship,[50] respectively. Kopecký's recent review[51] of these relationships might apply to our spermicidal data. Kopecký asserts, "[cmc]… represents the maximal available concentration of the monomer…[. This] may account for both the observed decrease of the antimicrobial activity above [cmc] and…the cut-off effect of [very] hydrophobic [quaternary ammonium salts] with low [cmcs]".

3. Conclusions

From these results, we draw three conclusions: (1) **1**(17), **1**(18), **1**(16) and **2**(17) display potent antispermatic activity and are just as spermicidal as, or in some cases even more than, N-9. (2) The structural differences among the compounds do not lead to large variations in anti-HIV activity; these compounds inhibit HIV by similar mechanisms. (3) Compound **1**(16), which has excellent spermicidal and anti-HIV properties, merits further investigation. In summary, hydroxylated cationic surfactants, **1** and **2**, show promise as topical microbicides. A chain length of 16 carbons appears optimal for concomitant expression of spermicidal and anti-HIV activity. Further structure-activity studies on these compounds are warranted.

4. Experimental

4.1. General

Reagents were used as received. The solvents were HPLC grade and used as received. Flash chromatography was performed with silica gel, 60 X, 230–400 mesh. TLC was performed with PE SIL G/UV 250 μm plates. Apparent coupling constants, J_{app}, were the measured peak widths. Atlantic MicroLab Inc. performed the elemental analyses.

4.2. General method for synthesis of bromohydrins 4a–e and 5a–e

To a cooled solution of a 1-alkene (10–15 mmol) in THF/H_2O (5:1) (ca. 40 mL g^{-1} of alkene), was added slowly *N*-bromosuccinimide (1.05 equiv.). The reaction mixture was stirred at rt overnight. Hexanes (ca. same volume as THF/H_2O) was added, and the organic layer was washed with water (3×25 mL), and then brine (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a colorless oil. The crude product was purified by flash column chromatography, eluting with hexanes (2000 mL), then hexanes/ether (20:1; 400 mL; 10:1, 400 mL; 5:1, 400 mL, 2:1, until both bromohydrins eluted), to give a mixture of the two isomeric bromohydrins **4** and **5** (65–72% combined yield, about 4:1 ratio of **4**:**5**). Small amounts of pure **4** and **5** were isolated for analytical purposes.

4.2.1. 1-Bromo-2-tetradecanol (4a). ^1H NMR (400 MHz, $CDCl_3$) δ 3.77 (m, 1H), 3.54 (dd, 1H, J_{app}=10.2, 3.0 Hz), 3.38 (dd, 1H, J_{app}=10.2, 7.2 Hz), 2.11 (d, 1H, J_{app}=5.0 Hz), 1.56–1.25 (comp, 22H), 0.88 (t, 3H, J_{app}=6.9 Hz); (lit.[32] 60 MHz ^1H NMR) ^{13}C NMR (100 MHz, $CDCl_3$) δ 71.0, 40.7, 35.1, 31.9, 29.6, 29.58, 29.50, 29.45, 29.44, 29.3, 25.6, 22.6, 14.1; mass spectrum (CI) *m/z* 294, 291.1327 (M−H, $C_{14}H_{28}BrO$ requires 291.1324).

4.2.2. 2-Bromo-1-tetradecanol (5a). ^1H NMR (400 MHz, $CDCl_3$) δ 4.14 (m, 1H), 3.81 (m, 1H), 3.74 (m, 1H), 2.09 (br s, 1H), 1.82 (m, 2H), 1.57–1.20 (comp, 20H), 0.88 (t, 3H, J_{app}=6.9 Hz); (lit.[32] 60 MHz ^1H NMR) ^{13}C NMR (100 MHz, $CDCl_3$) δ 67.2, 60.1, 34.8, 31.9, 29.6, 29.58, 29.56, 29.48, 29.35, 29.29, 29.0, 27.4, 22.6, 14.1; mass spectrum (CI) *m/z* 291.1329 (M−H, $C_{14}H_{28}BrO$ requires 291.1324).

4.2.3. 1-Bromo-2-pentadecanol (4b). ^1H NMR (400 MHz, $CDCl_3$) δ 3.77 (br m, 1H), 3.54 (dd, 1H, J_{app}=10.3, 3.2 Hz), 3.38 (dd, 1H, J_{app}=10.3, 7.1 Hz), 2.13 (d, 1H, J_{app}=3.5 Hz), 1.56–1.25 (comp, 24H), 0.88 (t, 3H, J_{app}=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 71.0, 40.6, 35.1, 31.8, 29.63, 29.60, 29.58, 29.50, 29.45, 29.44, 29.3, 25.6, 22.6, 14.1; mass spectrum (CI) *m*/*z* 307, 306, 305.1484 (M−H, $C_{15}H_{30}BrO$ requires 305.1480), 227, 213.

4.2.4. 2-Bromo-1-pentadecanol (5b). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 (m, 1H), 3.82 (m, 1H), 3.75 (m, 1H), 2.01 (dd, 1H, J_{app}=7.9, 5.8 Hz), 1.84 (m, 2H), 1.55–1.26 (comp, 22H), 0.89 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 67.2, 60.2, 34.8, 31.9, 29.63, 29.60, 29.56, 29.48, 29.35, 29.30, 29.0, 27.4, 22.6, 14.1; mass spectrum (CI) *m*/*z* 307, 305.1469 (M−H, $C_{15}H_{30}BrO$ requires 305.1480).

4.2.5. 1-Bromo-2-hexadecanol (4c). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.77 (m, 1H), 3.54 (dd, 1H, J_{app}=10.3, 3.3 Hz), 3.37 (dd, 1H, J_{app}=10.3, 7.0 Hz), 2.19 (d, 1H, J_{app}=5.0 Hz), 1.57–1.19 (comp, 26H), 0.87 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 71.0, 40.6, 35.1, 31.9, 29.65, 29.63, 29.62, 29.59, 29.51, 29.46, 29.44, 29.3, 25.6, 22.6, 14.1. (lit.[52] 90 MHz ^{1}H NMR and 22.6 ^{13}C NMR).

4.2.6. 2-Bromo-1-hexadecanol (5c). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 (m, 1H), 3.82 (m, 1H), 3.74 (m, 1H), 2.00 (dd, 1H, J_{app}=7.9, 5.8 Hz), 1.84 (m, 2H), 1.58–1.20 (comp, 24H), 0.88 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 67.3, 60.2, 34.8, 31.9, 29.63, 29.60, 29.57, 29.49, 29.35, 29.32, 29.0, 27.4, 22.6, 14.1. (lit.[53] *R* and *S* enantiomers 400 MHz ^{1}H NMR and 75 MHz ^{13}C NMR).

4.2.7. 1-Bromo-2-heptadecanol (4d). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (m, 1H), 3.55 (dd, 1H, J_{app}=10.3, 3.2 Hz), 3.38 (dd, 1H, J_{app}=10.3, 7.1 Hz), 2.09 (d, 1H, J_{app}=5.0 Hz), 1.57–1.25 (comp, 28H), 0.88 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 71.0, 40.7, 35.1, 31.9, 29.64, 29.61, 29.58, 29.50, 29.43, 29.44, 29.3, 25.6, 22.6, 14.1.

4.2.8. 2-Bromo-1-heptadecanol (5d). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 (m, 1H), 3.82 (m, 1H), 3.74 (m, 1H), 2.00 (dd, 1H, J_{app}=7.9, 5.8 Hz), 1.84 (m, 2H), 1.56–1.25 (comp, 26H), 0.88 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 67.3, 60.2, 34.8, 31.9, 29.63, 29.60, 29.56, 29.48, 29.35, 29.31, 29.0, 27.4, 22.6, 14.1.

4.2.9. 1-Bromo-2-octadecanol (4e). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.77 (m, 1H), 3.54 (dd, 1H, J_{app}=10.4, 3.2 Hz), 3.38 (dd, 1H, J_{app}=10.4, 7.1 Hz), 2.10 (d, 1H, J_{app}=5.0 Hz), 1.58–1.25 (comp, 30H), 0.88 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 71.0, 40.6, 35.1, 31.9, 29.64, 29.60, 29.58, 29.5, 29.45, 29.43, 29.3, 25.6, 22.6, 14.1.

4.2.10. 2-Bromo-1-octadecanol (5e). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.14 (m, 1H), 3.81 (m, 1H), 3.74 (m, 1H), 2.07 (dd, 1H, J_{app}=7.8, 5.8 Hz), 1.84 (m, 2H), 1.57–1.22 (comp, 28H), 0.88 (t, 3H, J_{app}=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 67.2, 60.2, 34.8, 31.9, 29.65, 29.61, 29.57, 29.5, 29.4, 29.3, 29.0, 27.4, 22.6, 14.1.

4.3. General methods for synthesis of quaternary ammonium bromides, 1(14–18)

A solution of bromohydrin (**4** or **5** or a mixture of **4** and **5**) and *N*,*N*-dimethylethanolamine (2 equiv.) in CH_3OH (about 1 mL/100 mg of the bromohydrin) was heated to reflux overnight. The solvent was removed under reduced pressure to give a crude product, which was purified by recrystallization (conditions are specified for individual compounds.).

4.3.1. 2-Hydroxy-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-1-tetradecanaminium bromide, 1(14). Recrystallized in EtOAc/CH_2Cl_2 (5:1) to give a white solid (85%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.78 (t, 1H, J_{app}=5.3 Hz), 4.58 (d, 1H, J_{app}=7.0 Hz), 4.27 (br m, 1H), 4.10 (br m, 2H), 3.85 (m, 2H), 3.61 (dd, 1H, J_{app}=13.7, 10.1 Hz), 3.42 (comp, 7H), 1.57–1.23 (comp, 22H), 0.86 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.2, 66.7, 65.6, 55.9, 53.6, 53.3, 35.9, 31.9, 29.7, 29.64, 29.62, 29.57, 29.3, 25.2, 22.6, 14.1. Anal. calcd for $C_{18}H_{40}NO_2Br$: C, 56.53; H, 10.54; N 3.66. Found: C, 56.44; H, 10.64; N, 3.70.

4.3.2. 2-Hydroxy-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-1-pentadecanaminium bromide, 1(15). Recrystallized in EtOAc/CH_2Cl_2 (5:1) to give a white solid (89%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.80 (t, 1H, J_{app}=4.7 Hz), 4.59 (d, 1H, J_{app}=7.2 Hz), 4.27 (br m, 1H), 4.11 (br m, 2H), 3.85 (m, 2H), 3.62 (dd, 1H, J_{app}=13.4, 9.8 Hz), 3.42 (comp, 7H), 1.58–1.24 (comp, 24H), 0.87 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.2, 66.7, 65.6, 56.0, 53.6, 53.3, 35.9, 31.9, 29.7, 29.64, 29.57, 29.3, 25.2, 22.6, 14.1. Anal. calcd for $C_{19}H_{42}NO_2Br$: C, 57.56; H, 10.68; N, 3.53. Found: C, 57.25; H, 10.52; N, 3.67.

4.3.3. 2-Hydroxy-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-1-hexadecanaminium bromide, 1(16). Recrystallized in EtOAc/CH_2Cl_2 (2:1) to give a white solid (85%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79 (br t, 1H), 4.57 (d, 1H, J_{app}=6.9 Hz), 4.28 (br m, 1H), 4.11 (br m, 2H), 3.86 (m, 2H), 3.62 (dd, 1H, J_{app}=13.5, 10.2 Hz), 3.43 (comp, 7H), 1.58–1.24 (comp, 26H), 0.89 (t, 3H, J_{app}=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.2, 66.7, 65.6, 56.0, 53.6, 53.3, 35.9, 31.9, 29.7, 29.64, 29.57, 29.3, 25.2, 22.6, 14.1. Anal. calcd for $C_{20}H_{44}NO_2Br$: C, 58.52; H, 10.80; N, 3.41. Found: C 58.34, H 10.70, N 3.47.

4.3.4. 2-Hydroxy-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-1-heptadecanaminium bromide, 1(17). Recrystallized in EtOAc/CH_2Cl_2/CH_3OH (25:1:1) to give a white solid (83%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79 (t, 1H, J_{app}=5.3 Hz), 4.56 (d, 1H, J_{app}=7.3 Hz), 4.29 (br m, 1H), 4.12 (br m, 2H), 3.87 (m, 2H), 3.63 (dd, 1H, J_{app}=13.4, 10.2 Hz), 3.43 (comp, 7H), 1.57–1.24 (comp, 28H), 0.87 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.2, 66.7, 65.6, 56.0, 53.7, 53.3, 35.9, 31.9, 29.7, 29.63, 29.61, 29.55, 29.3, 25.2, 22.6, 14.1. Anal. calcd for $C_{21}H_{46}NO_2Br$: C, 59.42; H, 10.92; N, 3.30. Found: C, 59.38; H, 10.98; N, 3.35.

4.3.5. 2-Hydroxy-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-1-octadecanaminium bromide, 1(18). Recrystallized in EtOAc to give a white solid (88%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.80 (t, 1H, J_{app}=5.4 Hz), 4.57 (d, 1H, J_{app}=7.0 Hz), 4.30 (br m, 1H), 4.12 (br m, 2H), 3.87 (m, 2H), 3.63 (dd, 1H, J_{app}=13.4, 9.9 Hz), 3.43 (comp, 7H), 1.59–1.19 (comp, 30H), 0.87 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.2, 66.7, 65.6, 56.0, 53.6, 53.3, 35.9, 31.9, 29.7, 29.6, 29.5, 29.3, 25.2, 22.6, 14.1). Anal.

calcd for $C_{22}H_{48}NO_2Br$: C, 60.26; H, 11.03; N, 3.19. Found: C, 60.12; H, 10.87; N, 3.26.

4.4. General method for the synthesis of quaternary ammonium bromides, 2(14–18)

A solution of bromohydrin (a mixture of **4** and **5**) and *N*-methyldiethanolamine (1.8 equiv.) in CH_3OH (about 1 mL/100 mg of the bromohydrin) was heated to reflux for 3 d. The reaction mixture was concentrated to give pale yellow oil. Repeated trituration of the oil with EtOAc (25 mL g^{-1} of oil/treatment) eventually produced a white solid. (Care was taken to break up lumps during trituration!) The crude solid was purified by recrystallization (conditions are specified for individual compounds.).

4.4.1. 2-Hydroxy-*N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-1-tetradecanaminium, bromide, 2(14). The solid (~200 mg) was dissolved in warm $CHCl_3$ (~0.5 mL). The solution turned milky after addition of CH_3CN (~0.5 mL) and then EtOAc (7 mL). Crystals were formed after 4 h; the solution was allowed to stand at rt overnight. The crystals were collected by vacuum filtration, washed with EtOAc (20 mL), air-dried on the Buchner funnel for 10 min, and then dried under high vacuum for 3 d at rt. ^1H NMR (400 MHz, $CDCl_3$) δ 4.64 (two overlapping triplets, 2H), 4.43 (d, 1H, J_{app}=7.2 Hz), 4.28 (m, 1H), 4.12 (m, 4H), 3.96–3.86 (comp, 4H), 3.68 (dd, 1H, J_{app}=13.7, 9.9 Hz), 3.47 (d, 1H, J_{app}=13.7 Hz), 3.41 (s, 3H), 1.54–1.25 (comp, 22H), 0.88 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 68.4, 65.7, 65.3, 65.0, 55.9, 51.2, 36.1, 31.9, 29.8, 29.7, 29.6, 29.4, 25.3, 22.7, 14.1. Anal. calcd for $C_{19}H_{42}NO_3Br$: C, 55.33; H, 10.26; N, 3.40. Found: C, 55.20; H, 10.32; N, 3.35.

4.4.2. 2-Hydroxy-*N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-1-pentadecanaminium, bromide, 2(15). The white solid (~200 mg) was dissolved in warm $CHCl_3$ (~0.3 mL) and CH_3CN (about 0.5 mL) and EtOAc (10 mL) were added. The resulting milky solution remained at rt for 12 h. The white solid was collected by vacuum filtration, and washed with EtOAc/CH_3CN (5:1, 10–15 mL). The white solid was air-dried on the Buchner funnel for 10 min, but the white solid was still too sticky to scrap out of the filter paper. The whole filter paper with the solid was dried in vacuo overnight in a dessicator. The solid was scrapped out carefully, and dried under high vacuum for 3 d at rt. ^1H NMR (400 MHz, $CDCl_3$) δ 4.67 (two overlapping triplets, 2H), 4.46 (d, 1H, J_{app}=6.4 Hz),4.26 (m, 1H), 4.11 (m, 4H), 3.91–3.86 (comp, 4H), 3.67 (dd, 1H, J_{app}=13.0, 9.3 Hz), 3.47 (d, 1H, J_{app}=13.0 Hz), 3.41 (s, 3H$_3$), 1.53–1.25 (comp, 24H), 0.88 (t, 3H, J_{app}=6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 68.4, 65.7, 65.3, 65.0, 55.9, 51.2, 36.1, 31.9, 29.8, 29.74, 29.70, 29.66, 29.4, 25.3, 22.7, 14.1. Anal. calcd for $C_{20}H_{44}NO_3Br$: C, 56.33; H, 10.40; N, 3.28. Found: C, 56.19; H, 10.45; N, 3.28.

4.4.3. 2-Hydroxy-*N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-1-hexadecanaminium, bromide, 2(16). The white solid (~400 mg) was dissolved in warm $CHCl_3$ (1 mL) and CH_3CN (1 mL) was added. The solution was warmed to dissolve the entire solid and was filtered through a small amount of flash silica gel in a 6″ pipet into a wide-mouth vial; rinsed with $CHCl_3$ (about 0.2 mL). EtOAc (10 mL) was added and the solution turned milky. After standing for 1 h at rt, fine crystals formed. The white crystals were collected by vacuum filtration and dried under high vacuum for 3 d at rt. ^1H NMR (400 MHz, $CDCl_3$) δ 4.72 (two overlapping triplets, 2H), 4.48 (d, 1H, J_{app}=7.2 Hz),4.27 (m, 1H), 4.12 (m, 4H), 3.94–3.86 (comp, 4H), 3.67 (dd, 1H, J_{app}=13.3, 10.1 Hz), 3.46 (d, 1H, J_{app}=13.3 Hz), 3.41 (s, 3H$_3$), 1.58–1.20 (comp, 26H), 0.88 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 68.3, 65.7, 65.2, 64.9, 55.9, 51.2, 36.1, 31.9, 29.82, 29.79, 29.76, 29.69, 29.4, 25.3, 22.7, 14.1. Anal. calcd for $C_{21}H_{46}NO_3Br$: C, 57.26; H, 10.53; N, 3.18. Found: C, 57.29; H, 10.53; N, 3.17.

4.4.4. 2-Hydroxy-*N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-1-heptadecanaminium, bromide, 2(17). The solid (~200 mg) was dissolved in warm $CHCl_3$ (~0.5 mL). The solution turned milky after addition of CH_3CN (~0.5 mL) and then EtOAc (7 mL). Crystals were formed after 4 h; the solution was allowed to stand at rt overnight. The crystals were collected by vacuum filtration, washed with EtOAc (20 mL), air-dried on the Buchner funnel for 10 min, and then dried under high vacuum for 3 d at rt. ^1H NMR (400 MHz, $CDCl_3$) δ 4.76 (two overlapping triplets, 2H), 4.52 (d, 1H, J_{app}=6.6 Hz),4.26 (m, 1H), 4.11 (m, 4H), 3.93–3.84 (comp, 4H), 3.66 (dd, 1H, J_{app}=12.6, 10.5 Hz), 3.46 (d, 1H, J_{app}=12.6 Hz), 3.39 (s, 3H$_3$), 1.58–1.20 (comp, 28H), 0.88 (t, 3H, J_{app}=6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 68.3, 65.7, 65.3, 65.0, 55.9, 55.8, 51.2, 36.1, 31.9, 29.81, 29.77, 29.75, 29.67, 29.4, 25.3, 22.7, 14.1. Anal. calcd for $C_{22}H_{48}NO_3Br$: C, 58.13; H, 10.64; N, 3.08. Found: C, 57.99; H, 10.59; N, 3.14.

4.4.5. 2-Hydroxy-*N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-1-octadecanaminium, bromide, 2(18). The solid was dissolved in boiling CH_2Cl_2/$CHCl_3$/EtOAc (20/10/10 mL), filtered through a filter paper. The filtrate was cooled at rt for 30 min; a white solid was collected by vacuum filtration, and washed with CH_2Cl_2 (20 mL). ^1H NMR (400 MHz, $CDCl_3$) δ 4.72 (two overlapping triplets, 2H), 4.48 (d, 1H, J_{app}=7.0 Hz), 4.27 (m, 1H), 4.11 (m, 4H), 3.97–3.85 (comp, 4H), 3.67 (dd, 1H, J_{app}=13.2, 10.1 Hz), 3.45 (d, 1H, J_{app}=13.2 Hz), 3.40 (s, 3H$_3$), 1.58–1.20 (comp, 30H), 0.88 (t, 3H, J_{app}=6.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 68.3, 65.7, 65.3, 65.0, 55.9, 55.8, 51.2, 36.1, 31.9, 29.80, 29.76, 29.67, 29.4, 25.3, 22.7, 14.1. Anal. calcd for $C_{23}H_{50}NO_3Br$: C, 58.96; H, 10.76; N, 2.99. Found: C, 58.66; H, 10.55; N, 3.06.

4.5. Determination of critical micelle concentrations by conductivity

Conductivity measurements were made with a conductance meter connected to a conductance cell, containing platinum–iridium electrodes coated with platinum black and gold soldered to platinum lead wires, a dip-type cell made from ABS plastic with a cgs cell constant of 1.0 cm^{-1}. The meter gave readings in siemens cm^{-1} (S cm^{-1}). A temperature probe connected directly to the meter measured the temperature of the sample solution before and after each set of data was collected. To prevent sample polarization inside the cell, the conductance meter operated with a 50–60 Hz AC circuit. The factory-set calibration of the

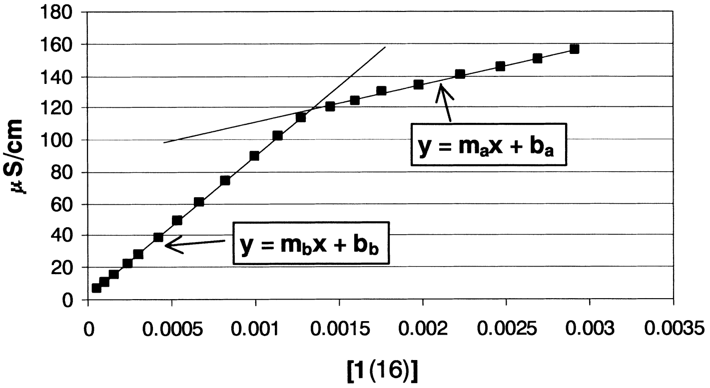


Figure 3. Typical Plot of conductivity versus concentration of **1**(16). Lines represent linear regressions through consecutive data. The equation $y=m_bx+b_b$ are data below the cmc, $y=m_ax+b_a$ are data above the cmc.

conductance meter and the integrity of the conductivity cell were checked periodically by with two NIST traceable conductivity standards (13.61 and 102.3 μS cm^{-1}).

Conductivity measurements were made on a 25-mL, sample-volume scale. Prior to use, all glassware was washed and rinsed with distilled H_2O and acetone, then dried in an oven at 100°C for 6 h. The sample beaker was immersed in a water bath whose temperature was controlled with an immersion circulator. The sample was continuously stirred with a magnetic stir bar. All weights were measured with an analytical balance (readability 0.01 mg) that was internally calibrated before use.

Weighing out the compound in volumetric flasks and diluting to the mark with distilled H_2O provided stock solutions, whose concentrations ranged from 0.756 to 0.0708 M; however, the majority of the stock solution concentrations were between 0.1 and 0.2 M. The weight of the solution was recorded; the apparent density was calculated for the current rt, typically between 19 and 24°C. (There are errors associated with these density calculations because the glassware was calibrated for 20°C. However, because of the 0.11% difference between the density of water at 19 and 24°C, we neglected the errors in the volume measurements associated with variations in rt.) A volumetric pipette was used to add distilled H_2O to the sample beaker, which was then placed into a water bath. The conductance cell was placed in the H_2O and the system equilibrated for ~5 min. The purity of the H_2O was evaluated by the conductivity, giving readings no higher than 3.00 μS cm^{-1}.

The amount of stock solution delivered to the beaker was measured by weighing a 250-μL syringe before and after sample delivery. The solution equilibrated for ~10 min before recording the conductivity reading and adding the next aliquot of stock solution. The procedure was repeated until 20 data points (typically 10 below and 10 above the cmc) were collected. Data points up to but not exceeding five times the cmc were collected.

The weight of the stock solution added to the sample was calculated by taking the difference of the syringe weights before and after injection. These weights were entered into a Microsoft® Excel spread sheet where the results were calculated by the following method: (1) dividing the weight by the determined apparent density gave the volume of added stock solution. (2) Adding the total volume of the added sample to the original sample volume gave the volume of the sample solution. (3) Multiplying the total delivered volume by the stock-solution concentration, and dividing by the sample volume gave the concentration of the sample. In a plot of conductivity versus sample concentration, the cmc was taken as the point where the slope of the line drastically changes. Fig. 3 is a plot of typical data.

The graph of concentration vs. conductivity can be thought of as two separate straight lines, illustrated above. The equations $y=m_ax+b_a$ and $y=m_bx+b_b$, where the subscripts 'b' and 'a', respectively, stand for 'before' and 'after' the break in slope, describe these lines. At the cmc, taken as the point where the two lines intersect, $(y_b,x_b)=(y_a,x_a)$. Therefore, the equations for the two lines can be set equal to one another and the x-coordinate of the intersection point (the cmc) solved, $m_bx + b_b=m_ax + b_a$ or $x=(b_a-b_b)/(m_b-m_a)$.

Linear regression analyses were performed to determine b_a, b_b, m_b, and m_a. As the cmc is actually a narrow concentration range and not a single specific point, the lines curved slightly before and after the cmc. To increase the robustness of the results, several linear-regression analyses were performed. The initial regressions used the five data points furthest from the inflection, and then, adding one point at a time, approaching the inflection. To determine which regression data were retained, the standard error of each regression analysis was calculated. If the standard error for a regression was more than three times the initial five-point regression's standard error, that set of data was rejected. In this way variations due to the gradual curving of the lines near the cmc were avoided. Then every possible combination of the regression data was used to calculate all possible cmc's. For example, if lines calculated with 5, 6, 7, and 8 data points fitted the criteria both before and after the cmc, then 16 (4×4) calculations of the cmc resulted. The means of these calculations, along with the standard deviation from the mean, were reported in Table 1.

4.6. Semen samples

Semen samples were collected by healthy volunteers by masturbation and allowed to liquefy for 30 min at rt. If completely liquefied, they were evaluated for sperm concentration and motion parameters with a computer-assisted semen analyzer (Hamilton Thorne Research, Beverly, MA) Only specimens with $>60\times10^6$ motile sperm/mL and >50% motility were used.

4.7. In vitro spermicidal activity

Spermicidal activities of the compounds were evaluated at once using a modified version to the protocol originally described by Sander and Cramer.[40] Semen samples were adjusted with a phosphate-buffered glucose solution (Baker's buffer) supplemented with 0.5–1.0% BSA in order to contain 60×10^6 motile sperm/mL; two-fold serial dilutions of the compounds were prepared in 0.9% NaCl (saline). Fifty microliters of semen were pipetted into 250 μL of compound/saline dilutions and gently vortexed for 3 s. A drop of this mixture was placed on a pre-warmed glass slide, coverslipped, and analyzed under a dark-field microscope with a 10× objective during 30 s. If any motile spermatozoon was seen, the dilution was considered as negative. Positive dilutions, i.e. all observed sperm immotile, were further incubated at 37°C for 1 h with two volumes of Baker's buffer, and then re-examined for sperm motility. If no motile sperm were seen, the positive score was maintained. The MEC of a compound was calculated with the highest sperm-immobilizing dilutions and the initial concentration of the compound. Several different donor semen samples were assayed to ensure consistency of results. Nonoxynol-9 (N-9) was used as positive control in all assays.

4.8. Statistical analysis

Comparisons between pairs of spermicidal results were done with the non-parametric Mann–Whitney test.

4.9. In vitro anti-HIV activity

Following the method of Resnick et al.[54] cell-free (RF strain) and cell-associated (RF-infected H9 cells) HIV-1 stocks, displaying titers around 6−log 10 TCID_{50}, were incubated with four dilutions of the test agents (starting at 0.1%) for 2 min. Compound effect was terminated by 10-fold serial dilutions, which were then incubated with plated MT-2 cells for 4–6 d (in quadruplicates). Endpoint: syncytium formation and compound cytotoxicity (agents+MT-2 cells without virus). Results were calculated by the Reed–Muench method.[55] N-9 was used as positive control.

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