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Short communication

Preparation and antimicrobial behaviour of gemini fluorosurfactants

Lionel Massi [a], Frédéric Guittard [a,*], Richard Levy [b], Yves Duccini [b], Serge Géribaldi [a]

[a] *Chimie des matériaux organiques et métalliques (CMOM), Université de Nice Sophia-Antipolis, faculté des sciences, parc valrose, 28, avenue de valrose, 06108, Nice cedex 2, France*

[b] *Rohm and Haas France S.A.S., European laboratories, 371, rue Ludwig Van Beethoven, 06560 Valbonne, France*

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Abstract

The introduction of perfluorinated chains in the molecular structure of quaternary ammonium gemini surfactants have led to particularly active antimicrobial agents evaluated in this work. Connectors and spacers were studied in relation with antimicrobial activity in order to determine which molecular parameters are 'critical' for biological activity.
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1. Introduction

Quaternary ammonium surfactants (QAS) are effective antimicrobial agents used in a number of domains such as cosmetics, common antiseptics, sanitizers in hospitals and disinfectants for contact lenses [1]. The efficacy of such agents is conditioned by the amphiphilic nature of the molecule [2] and consequently by its surfactant properties [3]. These products possess properties such as reduction of surface tension and a ready attraction for negatively charged surfaces like bacteria. These characteristics promote their adsorption onto bacteria surfaces. The mode of action cannot be reduced to surface activity only but cytolytic damage may be the primary lesion caused by such cationic surfactants and a major contribution to the cell death. There is a well-established relationship between cytolytic action and surface tension [4]. It is well known that the introduction of fluorinated chains in tensioactive structures leads to the enhancement of their surfactant properties, for instance lower critical micelle concentration and lower surface tension [5]. In view of the increasing resistance phenomenon occurring in antimicrobial agents [6] in general and particularly in QAS [7], a number of research programs are currently running on design of the new QAS as antimicrobial agents [8,9], notably the work on gemini surfactants [10–12]. We report here the antibacterial and antifungal properties of quaternary ammonium gemini fluorosurfactants in comparison to commercial available reference and to a hydrocarbon analogue.

2. Chemistry [13–15]

The synthetic pathways are described in Fig. 1. The formation of ester or thioester precursors (**1**,**2**) were obtained through the reaction of 2-(perfluorohexyl)ethane-1-thiol or alcohol with bromoacetic bromide (pathway i). Fluorinated (**3**) or hydrocarbon (**9**) amides are obtained by the reaction of primary amine with bromoacetic acid at room temperature in the presence of dicyclohexylcarbodiimide (DCC) (pathway ii). Gemini fluorosurfactants (**5-7**) or hydrocarbon analogue (**10**) are the result of the action of precursors previously prepared (bromothioacetate, bromoacetate or bromoacetamide) onto *N*,*N*,*N*′,*N*′-tetramethyl-α,ω-alkyldiamine (pathway iv). Preparation of **8** was carried out in two steps: the formation of *N*,*N*-dimethyl-2-(perfluorohexyl)ethylamine (**4**) consists in the dimethylation of fluorinated primary amine by the couple formaldehyde-formic acid (pathway iii) then the quaternization can occurs in the presence of 1,4-dibromobutane (pathway v). All compounds are colorless.

* Corresponding author.
E-mail address: guittard@unice.fr (F. Guittard).

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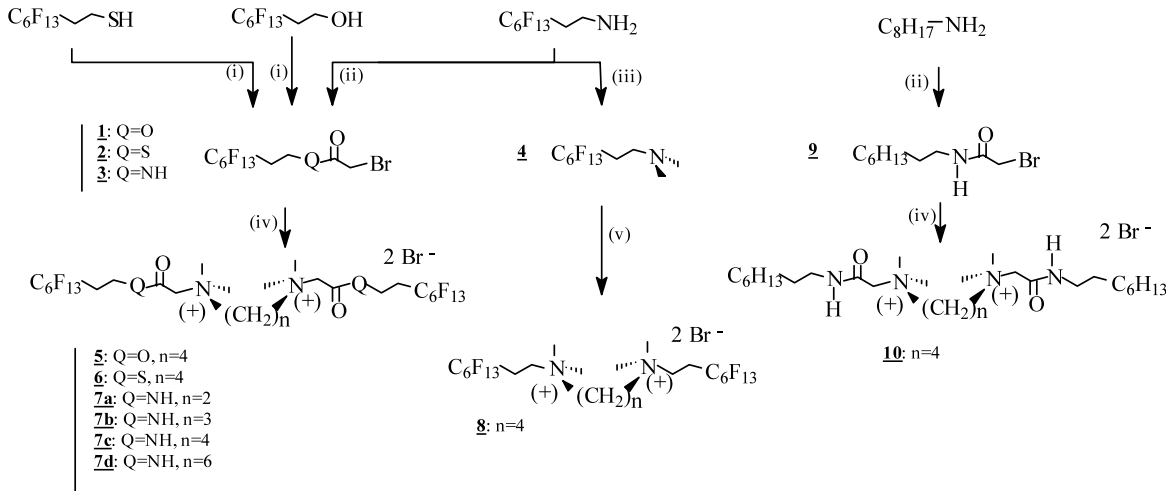

Fig. 1. Synthetic pathways to prepare highly fluorinated QAS; (i) $BrCH_2C(O)Br$, Et_2O, 0 °C → rt; (ii) $BrCH_2CO_2H$, DCC, CH_2Cl_2, rt; (iii) HCO_2H/$H_2C(O)$, 90 °C; (iv) $(CH_3)_2N(CH_2)_nN(CH_3)_2$, Et_2O, reflux; (v) iPrOH, $Br(CH_2)_nBr$, reflux.

3. Results and discussion

The results of minimal inhibitory concentration (MIC) evaluation are reported in Table 1. As a first step, we studied the effect of connector nature on antimicrobial agents activity (the connector is the chemical part bonding the perfluoroalkylethyle tail to the ammonium); the spacer length (which is the number of methylene between the two ammoniums heads) was kept constant and equal to four methylene units. We compared the activities of the compounds **5**, **6**, **7c**, and **8** [17]. The efficacy of these compounds was evaluated against bacterial and fungal strains. **6** was found to have a MIC above our detection limit i.e. 800 μmol L^{-1} whatever the micro-organism tested. **5** was above this limit for *Pseudomonas aeruginosa* and *Aspergillus niger*.

In view of the results, it appears that the connector type greatly influenced the antimicrobial activity. The variations of this factor can lead to properties ranging from inactive products e.g. **6** to products with strong biological activity such as **8** and **7c**. In this case, **7c** is the most active product showing that amide connector is the most suitable for enhancing the antimicrobial properties required. This result shows that the sole introduction of perfluorinated chains does not lead necessarily to an improvement of antimicrobial activity. Careful choices have to be made in the design of such molecules, noteworthy connector type and spacer length in this case.

Keeping the connector constant and corresponding to the amide function selected before, we have studied the variation on spacer length. In view of the results, it appears that in the range of spacer length studied, this factor influenced less the biological activity than the connector type; however it has a statistically significant effect on activity. Increasing the spacer length decreases the MIC indicating an enhanced activity. **7d**, corresponding to six methylene units in the spacer, was found to be statistically the most efficient product of this series (P value $< 1 \times 10^{-4}$, from two ways ANOVA analysis). As a result, connector nature is determinant for activity, but, it seems that, the spacer length has a modulating effect on antimicrobial activity.

A comparison to commercially available quaternary ammonium compounds was performed and shows a particularly strong activity against Gram negative bacteria. Whereas the references Empigen® BAC 50 and CPC (Cetyl Pyridinium Chloride purchased from ACIMA) have MIC against *Pseudomonas aeruginosa* of respectively 48 and 18 μmol L^{-1}, the active gemini surfactants synthesized have MICs from 5.2 to 7.3 μmol L^{-1}. We have also compared compound **7c** to its hydrocarbon analogue, **10** (c.f. also Table 1). It seems that the introduction of a fluorinated chain increase the activity noteworthy against *Candida albicans* and *Pseudomonas aeruginosa*.

4. Experimental protocols

4.1. Chemistry

The fluorosurfactants (**5–8**) reported in this work were synthesized using 2-perfluorohexylethylamine [15], alcohol and thiol as starting materials (from Atofina) or from octylamine (from Aldrich) for the preparation of hydrocarbon surfactant (**10**). Unless specified the solvents were of unpurified reagent grade. All gemini surfactants synthesized were analysed by ^{1}H-NMR spectroscopy and mass spectrometry (MS). The NMR spectra were recorded with a Brucker Advance 200 MHz NMR spectrometer. The Mass spectra were recorded on a Finnigan Mat LCQ Classic Electrospray source API1.

Table 1
MIC results

Cpd	Type [a]	Connector	Spacer	MIC (μmol L^{-1}) [b]				
				Staphylococcus aureus ATCC 9144	*Candida albicans* ATCC 2091	*Aspergillus niger* ATCC 6275	*Pseudomonas aeruginosa* CIP A22	
5	F	$OC(O)CH_2$	$(CH_2)_4$	190	560	> 800	> 800	
6	F	$SC(O)CH_2$	$(CH_2)_4$	> 800	> 800	> 800	> 800	
7a	F	$NHC(O)CH_2$	$(CH_2)_2$	6.7	6.7	4.0	5.8	
7b	F	$NHC(O)CH_2$	$(CH_2)_3$	4.5	6.6	4.6	5.2	
7c	F	$NHC(O)CH_2$	$(CH_2)_4$	3.1	7.6	4.9	5.9	
7d	F	$NHC(O)CH_2$	$(CH_2)_6$	3.0	4.4	3.8	5.5	
8	F	–	$(CH_2)_4$	4.3	63	21	7.3	
10	H	$NHC(O)CH_2$	$(CH_2)_4$	9.8	52	5.2	52	
CPC [c]	Ref	–	–	2.0	2.3	3.0	18	
Empigen® BAC 50 [d]	Ref	–	–	3.4	3.6	3.6	48	

[a] F, fluorosurfactant; H, hydrocarbon surfactant; Ref, commercial reference.
[b] Estimated precision of MIC of 10%.
[c] Cetyl pyridinium chloride purchased from ACIMA.
[d] Benzalkonium chloride.

The observation of *m*/2*z* -2Br, except for **10** where *m*/*z*-Br is observed, is in agreement with the structure. Satisfactory analysis were obtained with C ± 0.31, H ± 0.22, N ± 0.23.

4.1.1. Pathway (i) [14]

2-Perfluorohexylethanol or 2-perfluorohexylethanethiol (10 mmol) is added to bromoacetyl bromide in anhydrous diethylether (30 mL) under nitrogen atmosphere 2 h at 0 °C then at room temperature for 10 h. The mixture is hydrolysed with 10 mL of water at room temperature. The ether layer was separated and the aqueous layer was extracted with diethyl ether. The organic layers were washed with dilute $NaHCO_3$ solution and then concentrated to afford the crude product. The residue was distilled under reduced pressure to afford 2-perfluorohexylethylbromoacetate, 74 °C $mmHg^{-1}$, (**1**) in 93% yield and 2-perfluorohexylethylbromothioacetate, 89–90 °C $mmHg^{-1}$, (**2**) in 78% yield.

4.1.2. Pathway (ii) [13]

2-Perfluorohexylethylamine [15] or octylamine (10 mmol) dissolved in methylene chloride was added dropwise to a solution of DCC, (11 mmol) and bromoacetic acid (10 mmol) in methylene chloride (30 mL). The mixture is stirred 3 h at room temperature and then filtered. The filtrate is concentrated under reduced pressure and purified by column chromatography using silica gel 60 as adsorbent (eluent:methylene chloride) to afford the desired 2-perfluorohexylethylbromoacetamide (**3**) (yield: 88%) or octylbromoacetamide (**9**) (yield: 91%).

4.1.3. Pathway (iii)

This pathway corresponds to the Eschweiler and Clarke methylation reaction of amines. 2-Perfluorohexylethylamine (10 mmol) is added dropwise to a solution of acetic acid (50 mmol) and formaldehyde (30 mmol). The mixture is stirred 3 h at 90 °C. Concentrated NaOH is added to the resulting solution, the mixture is extracted three times with diethyl ether and the organic layer is concentrated. The residue is distilled under reduced pressure (76 °C 25 mmHg) to afford *N*,*N*-dimethyl-2-perfluorohexylethylamine (**4**) (yield: 85%).

4.1.4. Pathway (iv)

This procedure was used to obtain gemini surfactants from precursors **1**, **2**, **3** and **9**. One of these precursors (18 mmol) is added to *N*,*N*,*N*′,*N*′-tetramethyl-α,ω-alkanediamine (9 mmol) dissolved in 35 mL of diethyl ether providing gemini surfactants, i.e., **5**, **6**, **7a–d** [13,14] and **10**, respectively. The gemini surfactants precipitate, the mixture was filtered and the salt obtained was washed several times with diethyl ether. The yield range is from 80 to 93%.

4.1.5. Pathway (v)

α,ω-Dibromobutane (7.7 mmol) is added to a solution of *N*,*N*-dimethyl-2-perfluorohexylethylamine in isopropanol (35 mL). The mixture is stirred at reflux for 72 h. The solvent is evaporated, the residue is washed with diethyl ether. This procedure affords the gemini surfactants **8** (yield: 55%).

*4.1.6. Compound **5***

^{1}H-NMR (CD_3OD) δ ppm, 1.90 (4H, m, $[N^+–CH_2–CH_2]_2$), 2.80 (4H, tt, $[C_6F_{13}–CH_2]_2$), 3.35 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.80 (4H, t, $[^+N–CH_2–CH_2]_2$), 4.60 (4H, t, $[CH_2–O–C(O)]_2$), 5.85 (4H, s, $[C(O)–CH_2–N^+]_2$). MS, *m*/2*z* (%): 477.2 (100).

*4.1.7. Compound **6***

^{1}H-NMR (CD_3OD) δ ppm, 1.9 (4H, m, $[^+N–CH_2–CH_2]_2$), 2.65 (4H, tt, $[C_6F_{13}–CH_2]_2$), 3.35 (4H, m, $[CH_2–S]_2$), 3.40 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.75 (4H, t, $[^+N–CH_2–CH_2]_2$), 4.90 (4H, s, $[C(O)–CH_2–N^+]_2$). MS, *m*/2*z* (%): 493.3 (100).

*4.1.8. Compound **7a***

^{1}H-NMR (CD_3OD) δ ppm, 2.55 (4H, tt, $[C_6F_{13}–CH_2]_2$), 3.4 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.6 (4H, t, $[CH_2–NH–C(O)]_2$), 3.7 (4H, m, $[^+N–CH_2]_2$), 4.25 (4H, s, $[C(O)–CH_2–N^+]_2$). MS, *m*/2*z* (%): 462.2 (100).

*4.1.9. Compound **7b***

^{1}H-NMR (CD_3OD) δ ppm, 2.55 (6H, m, $[C_6F_{13}–CH_2]_2+[^+N–CH_2–CH_2]$), 3.4 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.6 (4H, t, $[CH_2–NH–C(O)]_2$), 3.8 (4H, t, $[^+N–CH_2–CH_2]_2$), 4.35 (4H, s, $[C(O)–CH_2–N^+]_2$). MS, *m*/2*z* (%): 469.2 (100).

*4.1.10. Compound **7c***

^{1}H-NMR (CD_3OD) δ ppm, 1.95 (4H, m, $[^+N–CH_2–CH_2]_2$), 2.55 (4H, tt, $[C_6F_{13}–CH_2]_2$), 3.4 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.6 (4H, t, $[CH_2–NH–C(O)]_2$), 3.8 (4H, t, $[^+N–CH_2–CH_2]_2$),4.25 (4H, s, $[C(O)–CH_2–N^+]_2$), MS, *m*/2*z* (%): 476.2 (100).

*4.1.11. Compound **7d***

^{1}H-NMR (CD_3OD) δ ppm, 1.4 (4H, m, $[N^+–CH_2–CH_2–CH_2)]_2$), 1.95 (4H, m, $[^+N–CH_2–CH_2]_2$), 2.55 (4H, tt, $[C_6F_{13}–CH_2]_2$), 3.4 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.6 (4H, t, $[CH_2–NH–C(O)]_2$), 3.8 (4H, t, $[^+N–CH_2–CH_2]_2$), 4.25 (4H, s, $[C(O)–CH_2–N^+]_2$). MS, *m*/2*z* (%): 490.3 (100).

*4.1.12. Compound **8***

^{1}H-NMR (CD_3OD) δ ppm, 2.0 (4H, m, $[^+N–CH_2–CH_2]_2$), 2.9 (4H, tt, $[C_6F_{13}–CH_2]_2$), 3.3 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.6 (8H, t, $[CF_2CH_2CH_2–N^+]_2+[N^+–CH_2–CH_2]_2$). MS, *m*/2*z* (%): 419.2 (100).

*4.1.13. Compound **10***

^{1}H-NMR (CD_3OD) δ ppm, 1.0 (6H, t, $[CH_3–CH_2]_2$), 1.4 (20H, m, $[CH_3(CH_2)_5]_2$), 1.6 (4H, m, $[CH_2CH_2NH]_2$), 2.0 (4H, m, $[N^+–CH_2CH_2]_2$), 3.3 (4H, t, $[CH_2–NHC(O)]_2$), 3.4 (12H, s, $[CH_3–N^+–CH_3]_2$), 3.9 (4H, m, $[^+N–CH_2–CH_2]_2$), 4.1 (4H, s, $[C(O)–CH_2–N^+]_2$). MS, *m*/*z* (%): 563.4,565.3 (M–Br, 100).

4.2. Antimicrobial evaluation

Antibacterial and antifungal evaluations of our molecules were run using MIC measurements on four micro-organisms i.e. *Staphylococcus aureus* (ATCC 9144), *Pseudomonas aeruginosa* (CIP A22), *Aspergillus niger* (ATCC 6275), and *Candida albicans* (ATCC 2091). Three independent experiments were undertaken for each compound. The choice of these strains allows us to study a broad spectrum of micro-organisms including Gram-positive and Gram-negative bacteria, fungi and yeasts. The MICs were taken as the minimal concentration showing no growth (turbidity) after 24 h of incubation at 30 °C for bacteria and after 72 h of incubation at 25 °C for fungis. MICs were determined using a serial dilution method [16]. The Biomek® 1000 (Beckman®) apparatus used for our experiments performed automatically dilutions of the tested antimicrobial agents solutions in 96 wells micro-titration plates. Bacteria were incubated on Trypticase Soy Agar slants for 18 h at 30 °C. MIC tests are run with the third generation of bacteria; samples are taken during the exponential phase of bacterial growth. This third generation of bacterial strains obtained is incubated in M9G (minimal phosphate medium) as a suspension for *Pseudomonas aeruginosa* and Trypticase Soy Broth for *Staphylococcus aureus*. In case of fungal strains, the incubating protocol is similar, except for the incubation time (7 days in case of fungal strains). The third generation was incubated exclusively in M9G suspension. The inocula were prepared at a concentration of 5×10^6–5×10^7 cfu mL^{-1} (cfu, colony forming unit). Initial solutions of preservatives were prepared at a 500 ppm concentration of active ingredient in Milli-Q water. Fungal inocula were prepared at 5×10^6–5×10^7 spores mL^{-1}. Adjustment was made by optical density measurement for bacteria (absorbance 0.05 at a wavelenght of 660 nm) and by count under microscope using a Neubauer cell for fungi and yeast. The antimicrobial behaviour were evaluated in comparison with two efficient commercial references i.e. Empigen® BAC 50 and CPC.

5. Conclusion

Antimicrobial properties of gemini fluorosurfactants are reported. When care is taken in the design of the

molecule as regards connector choice and spacer length, the enhancement of surfactant properties obtained by introducing perfluorinated chains into quaternary ammonium compounds, leads to particularly active antimicrobial agent, which have peculiar bacteriostatic properties as compared to commercial available references and to a hydrocarbon analogue.

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