Synthesis and biological properties of dicationic arginine–diglycerides

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A novel family of dicationic arginine–diglyceride surfactants, 1,2-diacyl-3-*O*-(L-arginyl)-*rac*-glycerol·2HCl (XXR) with alkyl chain lengths in the range of C_8–C_{14} was prepared. These new surfactants can be regarded as analogues of lecithins. They have two hydrophobic tails of identical fatty acid chains attached to the glycerol through ester bonds and a dicationic polar head from arginine instead of the zwitterionic on the lecithins. These new compounds can be classified as multifunctional surfactants with self-aggregation behaviour comparable to that of short-chain lecithins. They have antimicrobial activity similar to that of the conventional cationic surfactants and are as harmless as amphoteric betaines.

Introduction

Novel high-quality components of consumer friendly market products require surfactants with multifunctional characteristics such as: biodegradability, mildness, low potential toxicity, water solubility, wide hydrophilic–lipophilic balance (HLB), and ability to form lamellar phases and vesicles for potential use in drug-delivery systems with antimicrobial activity.

Naturally occurring amino acids have been of particular interest in the field of environmentally friendly surfactants. Surfactant molecules from renewable raw materials that mimic natural lipoamino acids are one of the preferred choices for food and cosmetic applications. Given their natural and simple structure, they show low toxicity and quick biodegradation. In this sense, our group has gained wide experience of the synthesis of amino acid-based surfactants obtained from the combination of natural saturated fatty acids, alcohols and amines with different amino acid head groups through ester, ether and amide linkages. Thus, saturated single-chain surfactants from arginine,[1] tryptophan,[2] double-chain ones from lysine,[3] glutamic acid and aspartic acid[4] and gemini from arginine[5] with different ionic characters have proved to be in all cases highly biodegradable, with low toxicity, ecotoxicity and irritation effects.[6,7] Water solubility and self-aggregation properties have been associated directly with the chemical structure of the molecule and only cationic lipoamino acids possessed antimicrobial activity.[6,8]

Polar glycerol-based lipids such as diacylglycerol derivatives or short-chain lecithins are environmentally friendly compounds with excellent dispersion properties. However, their low water solubility and absence of antimicrobial activity limit their uses as surfactants in many industrial applications.

In this paper, we describe a novel family of dicationic arginine based surfactants, 1,2-diacyl-3-*O*-(L-arginyl)-*rac*-glycerol·2HCl (XXR) (Fig. 1) with alkyl chain lengths in the range of C_8–C_{14}. Structurally, they can be considered analogues of lecithins (Fig. 1). These new surfactants have two hydrophobic tails of identical fatty acid chains attached to the glycerol backbone through ester bonds and a cationic polar head from the arginine amino acid instead of the phosphate derivative groups of lecithins.

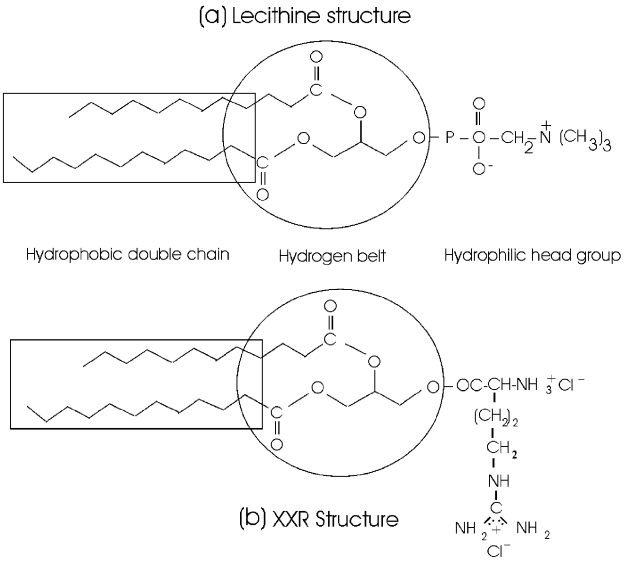


Fig. 1 (a) Molecular structure of lecithins. (b) Molecular structure of 1,2-diacyl-3-*O*-(*N*-L-arginyl)glycerol·2HCl (XXR), where X is the number of carbon atoms in the alkyl chains.

The novel family of compounds proposed in this work would combine in one molecule the physicochemical properties of the glycero derivatives and those of the polar arginine-based surfactants. It is also expected that they will be more hydrophilic than diacylglycerol derivatives and therefore will have higher water solubility properties. A dibasic amino acid such as arginine was selected to introduce antimicrobial activity into the molecule.

Preliminary basic surface active properties of XXR compounds[9] including adsorption and self-aggregation in aqueous solution reveal that they show a critical micelle concentration, CMC, in the range of 5–0.25 mM at 25 °C. Comparing these

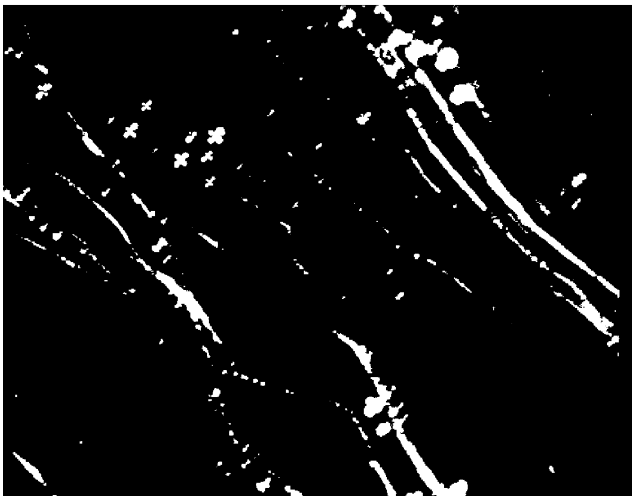


Fig. 2 Polarised optical microscopy image of the liquid crystal for the $88R/H_2O$ system at 35 °C.

values with those of the lecithins with the same alkyl chain length, the CMCs of the new compounds are one order of magnitude higher due to the better solubility properties of the hydrophilic head group for XXR. Furthermore, as in lecithins, the new compounds formed lamellar and nematic liquid crystal phases at room temperature (Fig. 2).

The chemical synthesis, the minimum inhibitory concentrations against a series of microorganisms together with the hemolytic activity and irritation effects are described.

Experimental

All solvents were reagent grade and were used without further purification. Trifluoracetic acid (TFA) was obtained from Merck. *N*-Cbz-L-arginine·HCl (Cbz = benzyloxycarbonyl) was purchased from Bachem. Anhydrous glycerol and the long chain acid chlorides with 8, 10, 12 and 14 carbon atoms were supplied by Fluka. Boron trifluoroetherate ($BF_3{\cdot}O(C_2H_5)_2$) was from Sigma. The progress of the reactions and purity of the final products were monitored by analytical HPLC, model Merck-Hitachi D-2500 with a Lichrospher 100 CN (propylcyano) 5 μm, 250 × 4 mm column, using UV-VIS detector L-4250 at 215 nm. A gradient elution profile was employed from the initial composition of A/B 75/25 (by volume), changing over 24 min to a final composition of 5/95 where A is 0.1% (v/v) TFA in H_2O and B is 0.085% of TFA in H_2O/CH_3CN 1:4. The flow-rate through the column was 1.0 ml min^{-1}. The structures of the pure compounds were checked by ^1H and ^{13}C NMR analyses, which were recorded with a Varian 300 MHz spectrometer. Chemical shifts are reported in parts per million (δ, in ppm) downfield of tetramethylsilane (TMS) as internal reference. Mass spectroscopy (MS) spectra with fast atom bombardment (FAB) or electrospray techniques were also conducted with a VG-QUATTRO spectrometer from Fisons Instruments. Elemental analyses of the final compounds were recorded at the Microanalytical Service at the IIQAB-CSIC. Preparative reversed-phase (RP)-HPLC runs were performed on a Waters (Milford, MA, USA) Prep LC 4000 pumping system.

Synthesis of 1-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl (00RZ)

17.24 g of *N*-Cbz-L-arginine·HCl (50 mmol) were dissolved in 200 mL of glycerol. The solution was heated to 55 °C and 25 ml of $BF_3{\cdot}O(C_2H_5)_2$ was added to the stirred mixture dropwise. After 1 h the addition was completed and then the reaction mixture was stirred for 5 h. Then, 400 mL of 0.6 M sodium bicarbonate was added. The remaining amount of reactant *N*-Cbz-L-arginine (5.45 min) and the formation of the 00RZ (5.23 min) in the reaction media was followed by HPLC analysis. A conversion of 97% based on HPLC analysis was obtained. The purification of 00RZ was carried out by preparative HPLC and Ion Exchange Chromatography following the procedure described by Morán *et al.*[10] *M* 418.5; HPLC t_r = 5.23 min; Elemental Analysis calculated for $C_{17}H_{27}O_6N_4Cl{\cdot}2H_2O$ in %: C, 44.9; H, 6.8; N, 12.3; Cl, 7.8. Found: C, 44.6; H, 6.8; N, 12.2; Cl, 7.7. Spectral characteristics: MS: 382.9. ^1H NMR: Assignments corresponding to Scheme 1. δ_H (ppm) (CD_3OD), 1.6–2.0 [m, 4H,(9)]; 3.2 [t, 2H,(10)]; 3.5 [d, 2H,(1)]; 3.58 [m, 1H,(2)]; 4.0–4.6 [m, 3H,(3,5)]; 5.1 [s, 2H,(8)]; 7.3 [m, 5H,(15,16,17,18,19)]. ^{13}C NMR: Assignments corresponding to Scheme 1. δ_C (ppm) (CD_3OD), 26.23 and 29.76 (9), 41.86 (10), 55.08 (5), 63.88 (1), 67.22 (8), 67.75 (3), 70.95 (2), 128.79 (19), 129.04 (17,18), 129.48 (15,16), 138.04 (14), 158.58 (12,7), 173.60 (4).

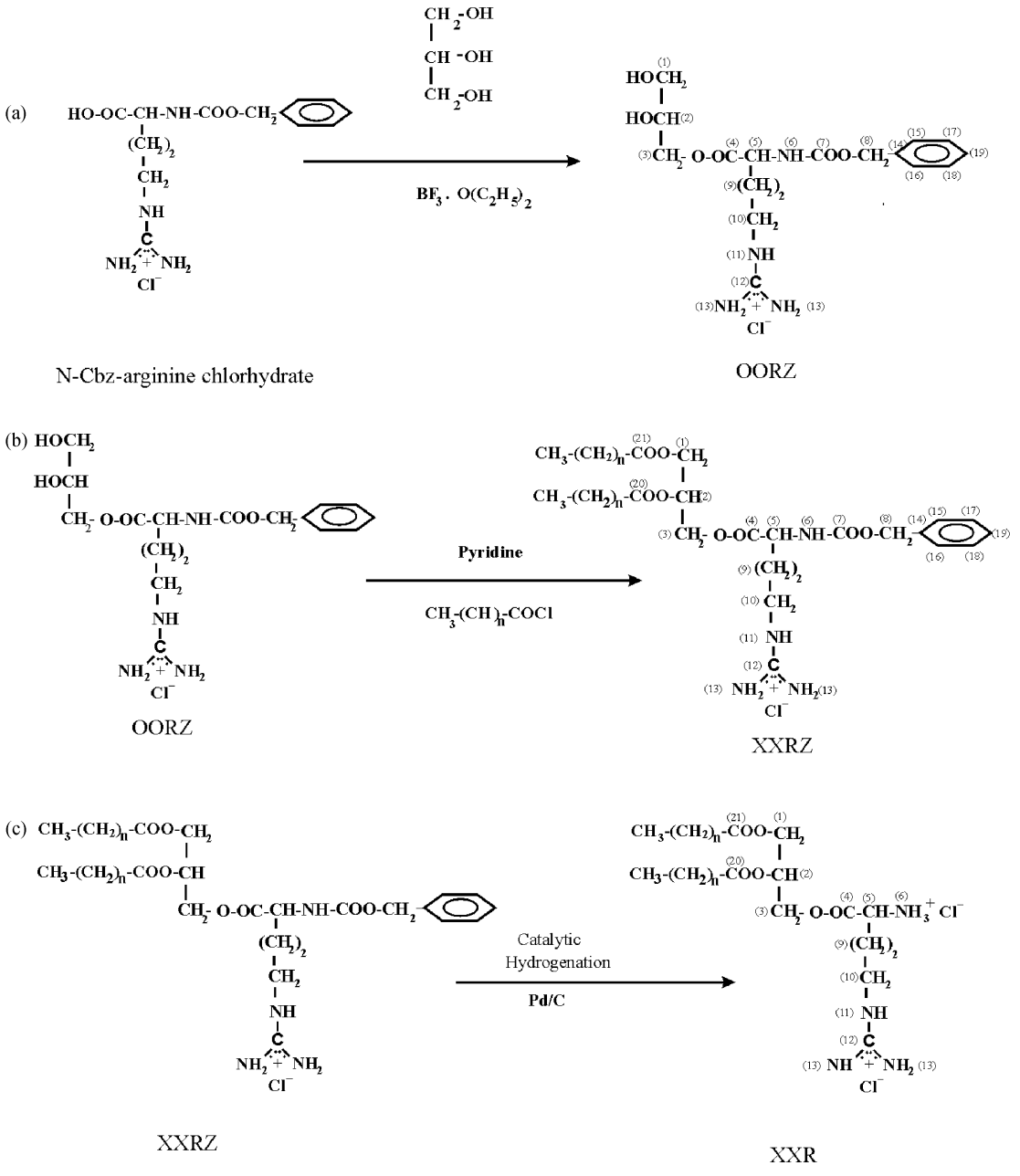
The stability of the 00RZ compound as a function of the pH and the temperature was evaluated by HPLC analysis to establish the experimental conditions for the synthesis of XXRZ. 10 mg of 00RZ were dissolved in an aqueous solution (5 mL) at the appropriate pH and temperature. HPLC analyses of these solutions were carried out every 24 hours, monitoring the decrease of the 00RZ peak and the formation and enlargement of those corresponding to the *N*-Cbz-L-arginine·HCl compound.

Synthesis of 1,2-diacyl-3-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl (XXRZ)

A solution of 40 mL of pyridine containing 00RZ (0.015 mol, 5 g) of 96% purity by HPLC was placed in a round-bottomed flask. To this solution, 0.05 mol of the corresponding long chain acid chloride was added dropwise, stirring continually at room temperature for 4 hours. After completion of the reaction, pyridine was removed under reduced pressure. The resulting solid was dissolved in methanol and washed three times with petroleum ether to remove the fatty acid excess. The solvent was evaporated and the solid was dissolved in chloroform. This solution was then extracted with water to eliminate water soluble impurities. After purification, transparent and viscous solids with 85% purity and yields exceeding 90% were obtained. Two chromatographic purification techniques were tested to obtain XXRZ compounds with purity higher than 95%: preparative RP-HPLC and silica acid flash chromatography.

Preparative RP-HPLC purification. 1 g of the product XXRZ was loaded into a preparative PrePack (Waters) column (47 × 300 mm) filled with DeltaPack C_4, 300 μm, 15 μm stationary phase. The product was eluted using a CH_3CN gradient (50 to 70% B in 30 minutes) in 0.1% aqueous TFA. The flow rate was 100 mL min^{-1} and detection at 220 nm. Analysis of the fractions was accomplished under isocratic conditions using a Lichrosphere 100 CN (propylcyano), 5 μm, 250 × 4 mm column, eluted with 67% of B (0.085% TFA in H_2O/CH_3CN 1:4) and 33% A (0.1% aqueous TFA), flow rate 1 mL min^{-1} and detection at 215 nm. The pure fractions were pooled and lyophilised.

Silica acid flash chromatography purification. Silica (100 mL Chromagel 60A CC, 70–230) was packed into a flash chromatography column. Then XXRZ (3 g, purity of 85%) dissolved in chloroform was loaded into the column and was eluted with a gradient from chloroform to chloroform/methanol, 90/10(v/v). The fractions containing the desired products were pooled and evaporated to dryness.



Scheme 1 Synthetic pathway for the preparation of XXR compounds.

1,2-Dioctyl-3-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol, 88RZ (as trifluoroacetate salt). Yield 40% (purified by RP-HPLC); HPLC $t_r = 19.23$ min, M 748.79: $C_{35}H_{55}O_{10}N_4F_3{\cdot}0.5H_2O$. Anal. calc. (%) C, 55.4, H, 7.4, N, 7.5. Found: C, 55.4; H, 7.65; N, 7.5. MS: 635.1; ^{1}H NMR: δ_H (CD_3OD), 0.89 [t, 6H, ($2CH_3$ of the alkyl chain)], 1.30 [s, 20H, ($10CH_2$ of the alkyl chain)], 1.40–2.00 [m, 8H, (–CH_2–CH_2–COO–), (9)], 2.29–2.35 [2t, (–CH_2–COO–)], 3.19 [t, (10)], 4.12–4.40 [m, 5H, (1)(3)(5)], 5.10 [s, 2H (8)], 5.23–5.32 [m, 1H, (2)], 7.29–7.35 [m, 5H, (15)(16)(17)(18)(19)]. ^{13}C NMR: δ_H (CD_3OD), 14.42 [CH_3–, alkyl chain], 23.68–41.85 [–CH_2– alkyl chain and (9), (10)], 55.02 [(5)], 63.26 [(1)], 64.04 [(3)], 67.79 [(8)], 70.45 [(2)], 128.82 [(19)], 129.05 [(17)(18)], 129.48 [(15)(16)], 138.08 [(14)], 158.63 [(12)], 173.17 [(4)], 174.47 [(20)], 174.83 [(21)].

1,2-Didecyl-3-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl, 1010RZ. Yield 62%. HPLC, $t_r = 20.54$ min. M 726.5: Anal. calc. (%) for $C_{37}H_{63}O_8N_{14}Cl{\cdot}H_2O$. C, 59.6; H,8.7; N, 7.52; Cl, 4.8. Found: C, 59.54; H, 9.1; N, 7.52; Cl,4.7. MS: 692.09; ^{1}H NMR: δ_H (CD_3OD), 0.83 [t, 6H, ($2CH_3$ of the alkyl chain)], 1.22 [s, 28H, ($14CH_2$ of the alkyl chain)], 1.42–1.86 [m, 8H, (–CH_2–CH_2–COO–), (9)], 2.22–2.28 [2t, 6H (–CH_2–COO–)], 3.07 [m, (10)], 4.03–4.29 [m, 5H, (1)(3)(5)], 5.02 [s, 2H (8)], 5.12–5.22 [m, 1H (2)], 7.22–7.37 [m, 9H, (13)(15)(16)(17)-(18)(19)]. ^{13}C NMR: δ_C (DMSO), 13.72 [CH_3–, alkyl chain], 21.93–33.34 [–CH_2– alkyl chain and (9), (10)], 53.47 [(5)], 61.68 [(1)], 62.29 [(3)], 65.48 [(8)], 68.55 [(2)], 127.54 [(19)], 127.66 [(17)(18)], 128.19 [(15)(16)], 136.72 [(14)], 155.97 [(12)], 157.05 [(7)], 171.64 [(4)], 172.05 [(20)], 172.33 [(21)].

1,2-Dilauroyl-3-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl, 1212RZ. Yield 70%; HPLC, $t_r = 21.71$ min. M 782.5: Anal. calc. (%) for $C_{41}H_{71}O_8N_4Cl$, C, 62.9; H, 9.1; N, 7.15; Cl, 4.5. Found: C, 62.65; H, 9.2; N, 7.1; Cl, 4.55. M/S: 748.14; ^{1}H NMR: δ_H (CD_3OD), 0.88 [t, 6H, ($2CH_3$ of the alkyl chain)], 1.29 [s, 32H, ($16CH_2$ of the alkyl chain)], 1.57–1.86 [m, 8H, (–CH_2–CH_2–COO–), (9)], 2.27–2.35 [2t, 6H (–CH_2–COO–)], 3.20 [t, (10)], 4.13–4.44 [m, 5H, (1)(3)(5)], 5.10 [s, 2H (8)], 5.22–5.26 [m, 1H (2)], 7.29–7.38 [m, 5H, (15)(16)(17)(18)(19)], 7.73 [d, 1H, (6)]. ^{13}C NMR: δ_C (CD_3OD), 14.50 [CH_3–, alkyl

chain], 23.76–41.88 [–CH_2– alkyl chain, (9), (10)], 55.03 [(5)], 63.30 [(1)], 64.02 [(3)], 67.77 [(8)], 70.44 [(2)], 128.84 [(19)], 129.06 [(17)(18)], 129.50 [(15)(16)], 138.09 [(14)], 158.57 [(12)], 173.17 [(4)], 174.42 [(20)], 174.78 [(21)].

1,2-Dimiristoyl-3-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl, 1414RZ. Yield 76%; HPLC, $t_r = 22.79$ min. *M* 835.5: Anal. calc. (%). for $C_{45}H_{79}O_8N_4Cl$, C, 64.6; H, 9.4; N, 6.6; Cl, 4.8. Found: C, 64.5; H, 9.2; N, 6.4; Cl, 4.4. M/S: 803.43; ^{1}H NMR: δ_H (CD_3OD), 0.90 [t, 6H, (CH_3 of the alkyl chain)], 1.28 [s, 40H, (20CH_2 of the alkyl chain)], 1.57–1.96 [m, 8H, (–CH_2–CH_2–COO–), (9)], 2.29–2.34 [2t, (–CH_2–COO–)], 3.21 [m, (10)], 4.12–4.43 [m, 5H, (1)(3)(5)], 5.11 [s, 2H (8)], 5.21–5.34 [m, 1H (2)], 7.24–7.41 [m, 5H, (15)(16)(17)(18)(19)]. ^{13}C NMR: δ_C (CD_3OD), 14.52 [CH_3–, alkyl chain], 23.77–41.88 [–CH_2– alkyl chain and (9), (10)], 55.04 [(5)], 63.31 [(1)], 64.02 [(3)], 67.75 [(8)], 70.44 [(2)], 128.79 [(19)], 129.04 [(17)(18)], 129.49 [(15)(16)], 138.07 [(14)], 158.56 [(12)], 173.13 [(4)], 174.38 [(20)], 174.78 [(21)].

Synthesis of 1,2-diacyl-3-*O*-(L-arginyl)-*rac*-glycerol·2(HCl) (XXR)

The corresponding pure XXRZ (0.0008 moles) was dissolved in 35 mL of methanol and hydrogenated (25 minutes) in the presence of Pd on activated charcoal (10% Pd) as catalyst. To control the pH of the reaction between 5–8, a solution of methanol with 0.0008 moles of HCl in water was added. The catalyst was filtered off on celite and rinsed with methanol. The filtrates were pooled and concentrated under reduced pressure. Pure compounds were obtained by several crystallizations from methanol/acetone. See Tables 1 and 2 for analytical and spectroscopic data.

Hemolytic activity

Erythrocytes preparation. Human blood was obtained from the Blood Bank of the Hospital Clinic (Barcelona). Blood was drain into heparinized tubs. The erythrocytes were washed three times in phosphate buffer isotonic saline (PBS), containing: 22.2 mmol L^{-1} Na_2HPO_4; 5.6 mmol L^{-1} KH_2PO_4, 123.3 mmol L^{-1} NaCl, glucose 10.0 mmol L^{-1} in distilled water

Table 1 Analytical data of 1,2-diacyl-3-*O*-(*N*-L-arginyl)-*rac*-glycerol·2(HCl) (XXR)

Compound	Molecular formula; molecular weight	Yield (%)	HPLC retention time (min.)	Elemental analysis calcd. (with 1 mol H_2O)/found			
				%C	%H	%N	%Cl
88R	$C_{25}H_{52}N_4O_6Cl_2$	60	12.77	48.5[a]	8.9[a]	9.05[a]	11.5[a]
	574			48.3	8.8	9.3	11.7
1010R	$C_{29}H_{58}N_4O_6Cl_2$	66	16.0	52.3[b]	9.3[b]	8.4[b]	10.7[b]
	629			52.8	9.5	8.7	10.5
1212R	$C_{33}H_{66}N_4O_6Cl_2$	71	18.0	56.3[c]	9.7[c]	8.0[c]	10.1[c]
	685			56.0	9.7	8.3	10.4
1414R	$C_{37}H_{74}N_4O_6Cl_2$	78	19.3	57.0[d]	10.0[d]	7.2[d]	9.2[d]
	741			57.4	9.9	6.8	9.2

[a] Calculated with 2.5 mol H_2O. [b] Calculated with 2 mol H_2O. [c] Calculated with 1 mol H_2O. [d] Calculated with 2 mol H_2O.

Table 2 Spectral assignments for 1,2-diacyl-3-*O*-(*N*-L-arginyl)-*rac*-glycerol·2(HCl) (XXR) compounds

Compound	*m/z* (M^+)	^{1}H NMR (DMSO), δ[a]	^{13}C NMR (DMSO), δ[a]
88R	501.2	0.90 [m, 6H, (2 CH_3 of the alkyl chain)], 1.31 [s, 16H, (8 CH_2 of the alkyl chain)], 1.59–2.01 [m, 8H, (–CH_2–CH_2–COO–), (9)], 2.31–2.38 [2t, 2 (–CH_2–COO–)], 3.25–3.31 [m, (10)], 4.12–4.61 [m, 5H, (1)(3)(5)], 5.32–5.36 [m, 1H (2)]	14.43 [CH_3–, alkyl chain], 23.68–41.75 [–CH_2– alkyl chain and (9), (10)], 53.57 [(5)], 63.17 [(1)], 65.42 [(3)], 70.31 [(2)], 158.64 [(12)], 170.10 [(4)], 174.45 [(20)], 174.81 [(21)]
1010R	557.4	0.86 [m, 6H, (2 CH_3 of the alkyl chain)], 1.26 [s, 24H, (12 CH_2 of the alkyl chain)], 1.55–2.02 [m, 8H, (–CH_2–CH_2–COO–), (9)], 2.27–2.33 [2t, (–CH_2–COO–)], 3.25 [m, (10)], 4.02–4.55 [m, 5H, (1)(3)(5)], 5.28–5.37 [m, 1H (2)]	14.47 [CH_3–, alkyl chain], 23.73–41.79 [–CH_2– alkyl chain and (9), (10)], 53.68 [(5)], 63.197 [(1)], 65.25 [(3)], 70.34 [(2)], 158.64 [(12)], 170.63 [(4)], 174.46 [(20)], 174.81 [(21)]
12,12,R	613.7	0.90 [m, 6H, (2 CH_3 of the alkyl chain)] 1.29 [s, 32H, 16 CH_2 of the alkyl chain)] 1.57–2.03 [m, 8H, (–CH_2–CH_2–COO–), (*9*)] 2.31–2.38 [2t, (–CH_2–COO–)] 3.27 [m, (*10*)], 4.12–4.60 [m, 5H, (*1*)(*3*)(*5*)] 5.31–5.39 [m, 1H (*2*)]	14.48 [CH_3–, alkyl chain] 23.75–41.73 [–CH_2– alkyl chain and (*9*), (*10*)] 53.56 [(*5*)], 63.19 [(*1*)], 65.30 [(*3*)] 70.29 [(*2*)], 158.62 [(*12*)], 170.01 [(*4*)], 174.36 [(*20*)], 174.76 [(*21*)]
14,14,R	669.7	0.90 [m, 6H, (2 CH_3 of the alkyl chain)], 1.29 [s, 40H, (20 CH_2 of the alkyl chain)], 1.57–2.31 [m, 8H, 2 (–CH_2–CH_2–COO–), (9)], 2.31–2.37 [2t, (–CH_2–COO–)], 3.27 [m, (10)], 4.12–4.60 [m, 5H, (1)(3)(5)], 5.31–5.39 [m, 1H (2)]	14.49 [CH_3–, alkyl chain], 23.76–41.74 [–CH_2– alkyl chain and (9), (10)], 53.57 [(5)], 63.21 [(1)], 65.39 [(3)], 70.31 [(2)], 158.64 [(12)], 170.06 [(4)], 174.44 [(20)], 174.78 [(21)]

[a] Assignments corresponding to Scheme 1C.

(pH = 7.4). The cells were then suspended in the PBS at a cell density of 8×10^9 cell mL^{-1}.

Hemolysis assay. Different volumes from 10 to 80 μL of surfactant solution (1 mg mL^{-1}) were pipetted into polystyrene tubs. Aliquots of 25 μL of erythrocyte suspension were added to the tubs and incubated for 10 minutes, whilst shaking, at room temperature. Following incubation, the tubes were centrifuged (5 min at 5000 rpm) and the percentage of hemolysis was determined by comparing the absorbance (540 nm) of the supernatant with that of control samples totally hemolysed with distilled water.

Protein denaturation. Aliquots of 100 μL of test sample were added to 875 μL of PBS and 25 μL of erythrocyte suspension were added to the tubs and incubated for 10 minutes. The tubes were centrifuged and the absorbance was determined at the supernatant at 575 nm and 540 nm in a dual-beam UV/VIS spectrophotometer. The ratio of absorbance at 575 nm and 540 nm allowed the calculation of the hemoglobin denaturation index (DI) which was compared with the SDS as the internal standard.

L/D ratio. The relationship between hemolysis (HC50) and the denaturation index (DI) is defined as the lysis/denaturation quotient or L/D ratio used to predict the potential ocular irritation of the surfactant.[11,12] The surfactants were classified in accordance with the following criteria: L/D < 0.1 very irritant, L/D from 0.1 to 1 irritant, L/D from 1 to10 moderately irritant, L/D from 10 to 100 slightly irritant and L/D > 100 non irritant.

Antimicrobial activity

The antimicrobial activities were determined "*in vitro*" on the basis of the minimum inhibitory concentration (MIC) values,[13] defined as the lowest concentration of antimicrobial agent which inhibits the development of visible growth after 24 h of incubation at 37 °C. The micro-organisms used (15 bacteria and one yeast) were the following: gram-negative bacteria: *Alcaligenes faecalis* ATCC 8750, *Bordetella bronchiseptica* ATCC 4617, *Citrobacter freundii* ATCC11606, *Enterobacter aerogenes* ATCC 10938, *Salmonella typhimurium* ATCC 14028, *Streptococcus faecalis* ATCC 1054, *Escherichia coli* ATCC 27325, *Klebsiella pneumoniae* ATCC 9721, *Pseudomonas aeruginosa* ATCC 9721, *Arthrobacter oxydans* ATCC 8010; gram-positive bacteria: *Bacillus cereus var. mycoides* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Staphylococcus aereus* ATCC 25178, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 9341; yeast: *Candida albicans* ATCC 10231.

Results and discussion

The synthesis of the XXR compounds was achieved following a three step procedure, starting from commercially available *N*-Cbz-L-arginine·HCl. The synthetic pathway for the preparation of these compounds is outlined in Scheme 1.

The first step 1A (Scheme 1) consists of the preparation of 1-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl (00RZ) by chemical esterification of the α-carboxyl group of *N*-Cbz-L-arginine·HCl HCl with the primary hydroxyl function of glycerol using boron trifluoroetherate as catalyst. Boron trifluoroetherate is widely used in the esterification of various kinds of carboxylic acids with alkyl alcohols[14] and in the synthetic preparation of amides from carboxylic acids.[15] This reagent has also been employed to synthesise glycerol esters of several amino acids.[16] In our case the use of boron trifluoroetherate as catalyst allows us to develop the esterification reaction under mild conditions yielding a fast, clean and high (95%) conversion of *N*-Cbz-L-arginine·HCl to the glycerol arginine ester. Purification of the 00RZ product was carried out by two chromatographic techniques, preparative HPLC (yield = 45%) and ion exchange chromatography (yield = 84%).

The next synthetic step 1B (Scheme 1) consists of the preparation of 1,2-diacyl-3-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl (XXRZ) by acylation of the two remaining free hydroxyls groups of 1-*O*-(*N*-Cbz-L-arginyl)-*rac*-glycerol·HCl (00RZ) with the corresponding long chain acid chloride. Several methods of preparation described in the literature for the acylation of the hydroxyl groups of glycerol use high temperatures[17] and basic or acid catalyst. Under these conditions hydrolysis of the ester bond between the arginine and the glycerol may occur.

In order to assess the appropriate experimental conditions for the acylation of the 00RZ compound without hydrolysis of the ester bond of this compound, the stability of 00RZ as a function of pH and temperature was studied (Figs. 3, 4). Figs. 3(a) and (b) show that the compound 00RZ is stable in aqueous solution over a wide range of pH (3.0–10.0) at room temperature. At this pH range, percentages of decomposition lower than 5% in 144 hours were observed. Alkaline aqueous solution (pH > 10) or acidic aqueous solution (pH < 3) of the 00RZ at room temperature decomposed in a few minutes as a result of hydrolysis. Fig. 4 shows the percentage of the 00RZ hydrolysis *versus* temperature. At 25 °C the compound remains stable during the whole period of study. At higher temperatures, 40 and 50 °C, percentages of hydrolysis of 5% are found.

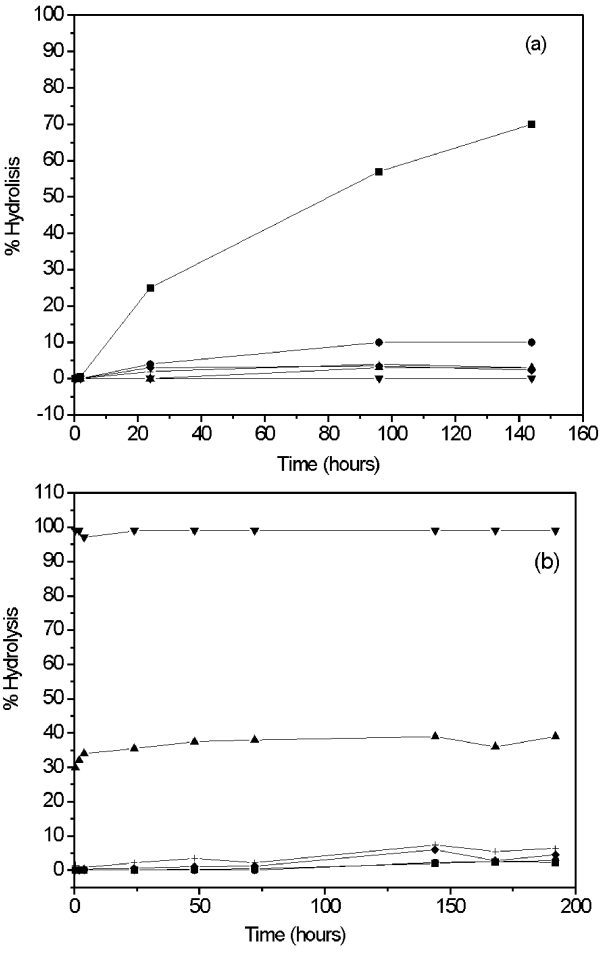


Fig. 3 (a) Stability of the 00RZ compounds at room temperature and pH 1 (■); pH 2 (●); pH 3 (▲); pH 4 (▼); pH 5 (◆); pH 6 (+). (b) Stability of the 00RZ compounds at room temperature and pH 12 (▼); pH 11 (▲); pH 10 (+); pH 9 (●); pH 8 (◆); pH 7 (■).

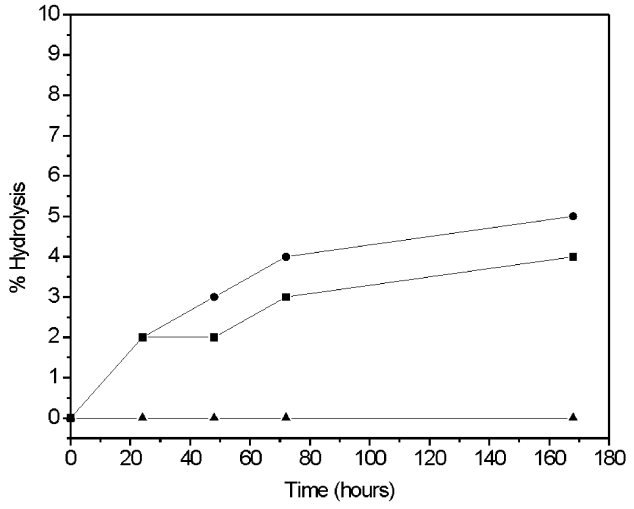


Fig. 4 Stability of the OORZ compounds with the temperature; 25 °C (s); 40 °C (■); 50 °C (●).

Accordingly, the reaction for the esterification of the two hydroxyl groups of 00RZ was carried out with long chain acid chloride in pyridine at room temperature (Scheme 1B). Under these conditions, the reaction mixture was kept in the pH range 5–7 and the starting compound 00RZ remained stable. The desired glycerol ester was obtained in an overall yield exceeding 90%.

The main advantage of this reaction was the simple procedure of the product purification owing to the clean reaction mixtures obtained. The crude reaction mixture consisted basically of the product (XXRZ) and the excess of acid chloride.

By successive hexane and water extractions, products with a purity of 85–90% (by HPLC) with a 80–90% yield were achieved. Products with purity exceeding 98% were obtained by both preparative RP-HPLC and silica gel chromatography. By preparative RP-HPLC yields of 45% were obtained. Better yields, 60–80%, were obtained by purification of the crude reaction with silica gel column chromatography.

For the four compounds synthesized, the HPLC chromatogram shows two peaks at the end of the reaction, a main peak and a minor peak constituting 5–10% of the product. After careful separation by silica gel column chromatography, by ^{13}C NMR the main peak was identified as the desired products (XXRZ) and the minor peak was identified as the 2-*O*-regioisomer 1,3-diacyl-2-*O*-(*N*-Cbz-L-arginyl)glycerol·HCl (XRZX).

The ^{13}C NMR spectrum of the main compound provides four signals, corresponding to the three carbons of the glycerol skeleton [70.397 ppm, C(2); 63.994 ppm, C(3); 63.266 ppm, C(1)], and C(8) [67.716 ppm] (see Scheme 1B). The same spectrum region for the 2-*O*-regioisomer only gives three signals. The C(1) and C(3) of the glycerol have the same chemical shift (62.975 ppm), which means that these carbons are magnetically and chemically equivalent. This is only possible if the arginine is linked to C(2) of glycerol.

It has been described for monoglycerides that acyl migration takes place easily giving an equilibrium mixture of the two regioisomers:[18] 2-monoglyceride (10%) and 1-monoglyceride (90%). In the case of 1-*O*-(*N*-Cbz-L-arginyl)glycerol·HCl (00RZ) this type of migration occurs probably when it is dissolved in pyridine to carry out the acylation of the hydroxyl groups of this compound. Because of this, the 2-*O*-regioisomer 1,3-diacyl-2-*O*(*N*-Cbz-L-arginyl)glycerol·HCl (XRZX) in percentages of 5–10% was obtained.

The target compounds XXR were obtained by catalytic hydrogenation of the Cbz group using Pd over charcoal (Scheme 1C). The reaction was carried out controlling the pH between 4–7 by the addition of a solution of HCl in methanol. This allowed us to obtain the products as dihydrochloride salts and avoid the hydrolysis of the ester linkages. Hydrogenation was controlled by HPLC by monitoring the disappearance of the protected compounds. ^{1}H NMR and ^{13}C NMR of all the final compounds showed the characteristic peaks corresponding to the expected compounds (Table 2). The mass spectra of XXR compounds showed a molecular ion peak corresponding to $[M + H]^+ - 2Cl$.

Antimicrobial activity

The dilution antimicrobial susceptibility test was carried out and the minimum inhibitory concentration (MIC) values were determined (Table 3). In contrast to short-chain lecithins, XXR compounds exhibited antimicrobial activity. As expected, the gram-negative bacteria were more resistant than the gram-positive bacteria. This antimicrobial activity can make them suitable for the subsequent biodegradation of these surfactants. It is well known that some gram-negative bacteria are relatively insensitive because their outer membranes are impermeable to some hydrophobic–hydrophilic compounds.[19]

The maximum antimicrobial activity corresponds to the compound with 8 carbon atoms in the alkyl chains, then, the activity decreases with increasing alkyl chain length. This

Table 3 Minimum inhibitory concentrations (mg L^{-1}) of the XXR compounds

	Microorganism	88R	1010R	1212R	1414R
Gram-positives	*Bacillus cereus var. mycoide* ATCC 11778	64	16	> 256	> 256
	Bacillus subtillis ATCC 6633	64	2	> 256	> 256
	Staphylococcus aereus ATCC 6538	4	16	> 256	256
	Staphylococcus epidermidis ATCC 12228	8	> 256	> 256	16
	Micrococcus luteus ATCC 9341	16	1	4	1
	Candida albicans ATCC 10231	16	32	64	> 256
Gram-negatives	*Salmonella typhimurium* ATCC 14028	16	32	> 256	> 256
	Pseudomonas aeruginosa ATCC 9027	64	> 256	> 256	> 256
	Escherichia coli ATCC 8793	8	64	> 256	> 256
	Arthrobacter oxidans ATCC 8010	32	16	> 256	> 256
	Streptoccocus faecalis ATCC 19434	8	4	2	0.5
	Bortedella bronchiseptica ATCC 4617	0.25	0.25	0.25	0.25
	Citrobacter freundii ATCC 22636	32	> 256	> 256	256
	Alcaligenes faecalis ATCC 8750	8	4	> 256	256
	Enterobacter aerogenes CECT 689	32	> 256	> 256	16
	Klebsiella pneumoniae v. preumonial CIP 104216	8	16	> 256	4

Table 4 Hemolytic activity of the XXR compounds, lauroyl arginine methyl ester (LAE), hexadecyltrimethylammonium bromide (HTAB) and commercial betaine (Tego-Bet)

	1414R	1212R	1010R	88R	HTAB	LAE	Tego-Bet[a]
HC50/mg L^{-1}	64.3	60.1	20.5	24.0	11.6	14.7	14.4
ID (%)	12.8	11.7	11.9	19.1	46.5	38.4	34.4
L/D	5.0	5.6	1.7	1.3	0.2	2.6	2.4
Classification	Moderate	Moderate	Moderate	Moderate	Irritant	Moderate	Moderate

[a] From Goldschmidt (Essen, Germany).

maximum for 88R can be attributed to the combination of a number of physicochemical parameters: hydrophobicity, adsorption, CMC, and aqueous solubility, the solubility being the limiting step for the transport. A loss of activity occurs for the surfactants with longer alkyl chain lengths because they are less water-soluble.

On the other hand, the MIC values of the 1010R and 88R compounds are similar to those reported for HTAB[20] (hexadecyltrimethylammonium bromide), a known biocide product. Thus, these compounds can be regarded as good antimicrobial agents.

Hemolysis

Hemolytic activity tests were performed on human red blood cell suspensions. The results are given in Table 4. The hemolytic activity is of the same order for the four compounds studied. This is similar to the other arginine based cationic surfactants synthesised in our lab[1] and is also similar to some betaines which are considered in some cases to be soft amphoteric surfactants. The potential ocular irritation predicted by the red blood cell method indicates that these surfactants are moderate irritants such as betaine in contrast to the very irritant action of other commercially available surfactants (SDS, benzalkonium chloride, cetyltrimethylammonium chloride).[21] There is a considerable difference between the hemolytic potency of these new surfactants and that of those with a quaternary group in the polar head.[21] MonoQuats are capable of inducing lysis of erythrocytes at low concentrations (0.05–0.1 mg L^{-1})[21] whereas these surfactants show hemolytic effects at concentration ranges between 19–60 mg L^{-1}.

In summary the novel synthesized compounds can be classified as multifunctional surfactants with a self aggregation behaviour comparable to that of short-chain lecithins.[9] Moreover, they showed a low toxicity profile and an antimicrobial activity similar to those of conventional cationic surfactants. Furthermore, given that both the hydrophobic and hydrophilic parts of the molecule are attached to glycerol by ester bonds, the resulting surfactant is expected to be readily biodegradable. In support of this idea, Puchta *et al.*[22] have shown that the incorporation of ester functionality in a cationic surfactant considerably accelerates biodegradation. This has also been demonstrated in the case of glucoside esters, which exhibited very rapid biodegradation.[23]

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