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Synthesis, micellisation and interaction of novel quaternary ammonium compounds derived from L-Phenylalanine with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine as model membrane in relation to their antibacterial activity, and their selectivity over human red blood cells

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ABSTRACT

A series of quaternary ammonium compounds (QUATS) derived from L-Phenylalanine have been synthesized and their antibacterial efficiencies were determined against various strains of Gram-positive and Gram-negative bacteria. The antibacterial activity increased with increasing chain length, exhibiting a cut-off effect at C_{14} for Gram-positive and C_{12} for Gram-negative bacteria. The L-Phenylalanine QUATS displayed enhanced antibacterial properties with a higher cut-off point compared to their corresponding L-Phenylalanine ester hydrochlorides. The CMC was correlated with the MIC, inferring that micellar activity contributes to the cut-off effect in antibacterial activity. The hemolytic activities (HC_{50}) of the QUATS against human red blood cells were also determined to illustrate the selectivity of these QUATS for bacterial over mammalian cells. In general, the MIC was lower than the HC_{50}, and assessment of the micellar contribution to the antibacterial and hemolytic evaluation in TBS as a common medium confirmed that these QUATS can act as antibacterial, yet non-toxic molecules at their monomer concentrations. The interaction of the QUATS with the phospholipid vesicles (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, DPPC) in the presence of 1-anilino-8-naphthalene sulfonate (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) as fluorescence probes showed that the presence of the quaternary ammonium moiety causes an increase in hydrophobic interactions, thus causing an increase in antibacterial activity.

1. Introduction

In recent years, quaternary ammonium compounds (QUATS) have been widely used as biocides [1,2]. These compounds were found to exert antimicrobial activities against both Gram-positive and Gram-negative bacteria as well as against some pathogenic species of fungus and protozoa [3,4]. However, various bacteria have been found to develop resistance towards some QUATS derivatives, rendering these compounds ineffective as antibacterial agents [5]. Moreover, QUATS in general have been found to have toxic effects towards mammalian cells, thus some of them are acceptable only for topical applications as they are considered too toxic for systemic applications [6]. This brings the necessity for the search of new and more effective biocides.

A number of studies have demonstrated the relationship between surfactant hydrophobic chain lengths and its activity against microorganisms [7–10]. Head group architecture also profoundly affects antimicrobial activity, as the number, positioning and nature of cationic groups dictate the interaction with the cell surface [11–13]. Surfactants with large head groups and a single hydrophobic tail have been reported to have significant membrane lysing potential, allowing more efficient membrane disruption [14].

QUATS derived from amino acids have attracted much interest due to their lower toxicity profile. In literature, there are several works concerning the synthesis of cationic surfactants derived from amino acids such as lysine and arginine, but there are very few studies on QUATS derived from aromatic amino acids [15–18].

Surfactants containing phenylalanine residues have also been found to possess good antimicrobial activities with optimal effectiveness at some intermediate chain length, called the cut-off point, above and below which activity decreases [19]. In our previous study, we reported the effect of chain length on the bacterial activity of a series of the hydrochloride salts of L-Phenylalanine esters [20] (Fig. 1).

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This study is therefore intended to develop novel L-Phenylalanine QUATS esters by prompting the amino moiety with alkyl groups to investigate the effect of the quaternary ammonium moiety on the biocidal activities. In this paper, we report the synthesis, CMC and antibacterial activity of a series of novel quaternary ammonium surfactant analogues derived from L-Phenylalanine with varying *O*-alkyl ester chain length. The biological efficiencies of these quaternary ammonium surfactants of L-Phenylalanine esters were determined by bacterial growth inhibition assays. The hemolytic activity against red blood cells (RBCs) was undertaken to provide selectivity of these QUATS for bacterial over mammalian cells. In order to study the behaviour of the polar cationic head and lipophilic moiety of these QUATS with the phospholipid layer of the bacterial cell wall, the binding studies of selected L-Phenylalanine esters with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as model phospholipid membrane using 1-anilino-8-naphthalenesulfonate (ANS) and 1,6-diphenyl-1,3-5-hexatriene (DPH) as fluorescence probes were also undertaken.

2. Chemistry

With the aim to systematically study the influence of the quaternary ammonium moiety on the biological properties of the L-Phenylalanine QUATS, L-Phenylalanine esters were quaternised with methyl and ethyl groups. In this series, the quaternary ammonium moiety of L-Phenylalanine QUATS represent the polar head group while the alkyl ester chain represents the hydrophobic tail of the QUAT molecules (Fig. 2).

L-Phenylalanine QUATS of varying chain lengths were obtained in moderate yields and were soluble in water. The ^{1}H and ^{13}C NMR spectra were concordant with the expected structures.

3. Critical micellar concentration (CMC)

Selected CMCs were determined in deionised water/Tris buffered saline (TBS) solution (0.05 M, pH 7.4) by using measured conductivity values as a function of L-Phenylalanine QUATS concentration at 25 °C. The CMCs were determined by plotting the conductivity values against the concentration of the compounds in aqueous solutions. The CMCs were noted by the sharp change in conductivity values as concentrations are increased.

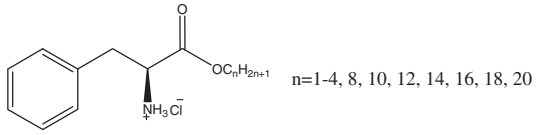
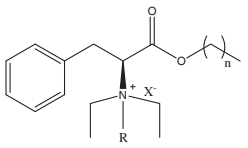


Fig. 1. Hydrochloride salts of L-Phenylalanine esters.



Compound	1b	2b	3b	4c	5b	6b	7b	8b
n	0	1	2	3	9	11	13	15
R	C_2H_5	C_2H_5	C_2H_5	CH_3	CH_3	CH_3	CH_3	CH_3
X	Br	Br	Br	I	I	I	I	I

Fig. 2. Molecular structure of L-Phenylalanine QUATS.

4. Pharmacology

4.1. Antimicrobial activity

Antimicrobial tests were performed against five Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213, ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and *Bacillus cereus* (ATCC 11778, ATCC 10876) and four Gram-negative bacteria *Escherichia coli* (ATCC 22922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028) and *Klebsiella pneumoniae* (ATCC 13883).

4.2. Hemolytic activity

The determination of the hemolytic activity was made on human red blood cells obtained from a healthy donor by venipuncture and collected in sodium-EDTA coated tubes.

4.3. Phospholipid binding assay

Binding studies of the C_{12}, C_{14} and C_{16} derivatives of L-Phenylalanine QUATS (6b–8b) with phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were carried out in TBS (0.01 M, pH 7.0) by a fluorescence probe technique using the protocol described previously [20].

5. Experimental protocol

5.1. Chemistry

L-Phenylalanine was obtained from HiMedia Laboratories (India). The fluorescence probes, 1-anilino-8-naphthalene sulfonate (ammonium salt; ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH), Tris Buffered saline (0.05 M, pH 7.4), bromoethane, decan-1-ol, dodecan-1ol, tetradecan-1-ol, hexadecan-1-ol, octadecan-1-ol and eicosan-1-ol, 0.65 mM phosphorus standard solution, ammonium molybdate(VI) tetrahydrate, hydrogen peroxide (30%) and streptomycin were obtained from Sigma–Aldrich (St. Louis, USA). *p*-Toluene sulfonic acid (PTSA) was obtained from Merck (Germany). Silica gel (60–120 Mesh) obtained from Alpha Chemika (India) was used for column chromatography. Mueller Hinton agar and Mueller Hinton broth were obtained from Oxoid Ltd. (United Kingdom). Cetyl trimethyl ammonium bromide (CTAB) was obtained from BDH Laboratory Supplies (England). L-Ascorbic acid was obtained from s.d fine chemicals (India). The synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polarlipids, Inc (USA). The different bacterial strains were obtained from Microbiologics® (St. Cloud, MN, USA) and Oxoid Ltd. (United Kingdom).

^{1}H NMR and ^{13}C NMR spectra were recorded at 250 MHz and 62.9 MHz on a Bruker electro spin NMR spectrometer using $CDCl_3$, D_2O and DMSO-d_6 as solvents. Mass spectra were recorded on a TOF mass spectrometer with electrospray ionization in the positive mode. Fluorescence intensities were recorded on a LS 55 Perkin Elmer fluorescence spectrophotometer. UV absorbances were recorded on a Biochrom Libra S22 UV–VIS spectrophotometer. Conductivity measurements were made using a Jenway 4320 conductivity meter and sonification was carried out using a Soniprep 150 sonifier. The *clog P* values were predicted by Chemdraw ultra 8.0.

The X-ray data were recorded on a Bruker Apex Duo diffractometer equipped with an Oxford Instruments Cryojet operating at

Table 1
Crystal data and structure refinement details for 1b.

Crystal data	1b
Chemical formula	$C_{16}H_{26}BrNO_2{\cdot}2H_2O$
Molar mass (g mol^{-1})	362.29
Crystal system, space group	Monoclinic, $P2_1$
Temperature (K)	100(2)
a, b, c (Å)	10.0997(5), 10.8334(6), 16.8425(9)
α, β, γ (°)	$\alpha = \gamma = 90$, $\beta = 104.852(2)$
V (Å^3)	1781.24(16)
Z	4
Radiation type	Mo$K\alpha$
μ (mm^{-1})	2.32
Crystal size (mm)	0.52 × 0.20 × 0.11
Data collection	
Diffractometer	Bruker Apex Duo CCD diffractometer
Absorption correction	Multi-scan, *SADABS*, Bruker 2012
T_{min}, T_{max}	0.379, 0.785
No. of Measured, independent and observed [$I > 2\sigma(I)$] reflections	14068, 6937, 6561
R_{int}	0.023
Refinement	
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.022, 0.045, 0.84
No. of reflections	6937
No. of parameters	403
No. of restraints	0
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement.
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.29, −0.20
Absolute structure	Flack, H.D. (1983), ActaCryst. A39, 876–881
Flack parameter	0.011(4)

100(2) K and an Incoatecmicrosource operating at 30 W power. Crystal and structure refinement data are given in Table 1.

Selected bond lengths and angles are given in Table 2. The data were collected with Mo $K\alpha$ (λ = 0.71073 Å) radiation at a crystal-to-detector distance of 50 mm. The data collection was performed using omega and phi scans with exposures taken at 30 W X-ray power and 0.50° frame widths using APEX2 [21]. The data were reduced with the programme SAINT [21] using outlier rejection, scan speed scaling, as well as standard Lorentz and polarisation correction factors. A SADABS semi-empirical multi-scan absorption correction [21] was applied to the data. Direct methods, SHELXS-97 [22] and WinGX [23] were used to solve the data. All non-hydrogen atoms were located in the difference density map and refined anisotropically with SHELXL-97 [22]. All hydrogen atoms were included as idealized contributors in the least squares process. Their positions were calculated using a standard riding model with C–$H_{aromatic}$ distances of 0.95 Å and U_{iso} = 1.2 U_{eq}, C–$H_{methine}$ distances of 1.00 Å and U_{iso} = 1.2 U_{eq}, C–$H_{methylene}$ distances of 0.99 Å and U_{iso} = 1.2 U_{eq} and C–H_{methyl} distances of 0.98 Å and U_{iso} = 1.5 U_{eq}. The hydrogen atoms of the water solvate molecules were located in the difference density map, and refined isotropically.

Table 2
Selected bond lengths (Å) and bond angles (°) of 1b[a].

Bond	Molecule A	Molecule B
N–$C_{methylene}$[b]	1.53(5)	1.53(4)
N–C7	1.54(3)	1.54(3)
C15–O1	1.21(3)	1.21(2)
C15–O2	1.33(2)	1.33(2)
C16–O2	1.47(2)	1.46(2)
Angle		
C7–N1–$C_{methylene}$	108.4(2)	110.1(2)
O1–C15–O2	124.4(2)	124.2(2)
C15–O2–C16	115.4(2)	115.5(2)

[a] Standard uncertainties given in parentheses.
[b] Mean of the three N–$C_{methylene}$ bonds per molecule.

5.1.1. General synthesis of N,N,N-triethyl ammonium L-Phenylalanine esters (1b–3b)

L-Phenylalanine esters were synthesized according to a modified procedure [24]. L-Phenylalanine (1.00 g, 6 mmol) was stirred with the corresponding alcohol (15 mL) at 0 °C. Thionyl chloride (1 mL, 13.8 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 48 h. Excess alcohol was evaporated in *vacuo* and the residue washed with diethyl ether to yield the compound as a white solid.

Bromoethane (4 mL) was added to a mixture of L-Phenylalanine ester (3 mmol) and K_2CO_3 (1.2 g) in acetonitrile (10 mL) and the mixture was heated to 90 °C in a sealed tube for 18 h. The reaction mixture was filtered to remove excess K_2CO_3 and the excess solvent was then removed under *vacuo*. Diethyl ether was added and the mixture was filtered. The residue was collected and washed with ether to yield the *N,N,N*-triethyl derivative as a white solid, which was recrystallised in acetonitrile. Evaporation of the filtrate yielded the *N,N*-diethyl derivative as a colourless oil.

5.1.2. General synthesis of N,N-diethyl,N-methyl phenylalanine esters(4c, 5b–8b)

The C_{10}–C_{16} esters of L-Phenylalanine were synthesized using the modified procedure reported by Bazcko et al. [25]. A mixture of the amino acid (12.1 mmol), PTSA (2.30 g, 12.1 mmol) and alcohol (14.5 mmol) in toluene (100 mL) was heated for 48 h instead of 24 h using a Dean–Stark apparatus. The crude products were purified by column chromatography on silica gel with EtOAC/Hexane (1:2) to afford the corresponding esters as oils.

The esters were then converted to the *N,N*-diethyl derivatives using EtBr/K_2CO_3 in a sealed tube at 90 °C. The QUATS (5b–8b) were then synthesised by heating the corresponding *N,N*-diethyl derivatives with excess MeI in a sealed tube at 90 °C for 18 h. The solvent was removed under *vacuo* and on addition of diethyl ether the desired quaternary ammonium compound precipitated out.

5.1.3. Analytical data and spectral assignments of N,N,N-trialkyl ammonium L-Phenylalanine esters

5.1.3.1. Compound 1a. Yield: 63%. ^{1}H NMR: δ_H ($CDCl_3$), 1.16 (t, 6H, N-$CH_2\underline{CH_3}$), 2.87 (m, 2H, N-$C\underline{H_2}CH_3$), 2.98 (m, 2H, N-$C\underline{H_2}CH_3$),

3.02 (m, 1H, CHC<u>H</u>Ph), 3.05 (m, 1H, C<u>H</u>CHPh), 3.25 (s, 3H, C<u>H</u>$_3$), 4.04 (m, 1H, C<u>H</u>), 7.31–7.43 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 14.1 (N-CH_2<u>C</u>H_3), 36.2 (<u>C</u>H_2Ph), 44.5 (N-<u>C</u>H_2CH_3), 51.4 (O<u>C</u>H_3), 65.0 (<u>C</u>H), 126.0–139.1 (<u>C</u>$_6H_4$), 173.0 (<u>C</u>=O).

5.1.3.2. Compound 1b. Yield: 35%. M.p: 89 °C. MW: 343.1964: ESI-MS, m/z: 264.1961 (M^+ without the Br^-). Elem. Anal. Found: C, 55.65; H, 7.80; N, 4.45; Calcd. for $C_{16}H_{26}NO_2Br$: C, 55.82; H, 7.61; N, 4.07. ^{1}H NMR: δ_H ($CDCl_3$), 1.42 (t, 9H, N-CH_2C<u>H</u>$_3$), 3.24 (m, 1H, C<u>H</u>CHPh), 3.38 (m, 1H, C<u>H</u>CHPh), 3.49 (s, 3H, CH_3), 3.66 (m, 6H, N-C<u>H</u>$_2CH_3$), 4.36 (m, 1H, CH), 7.25–7.40 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 11.3 (N-CH_2<u>C</u>H_3), 35.4 (<u>C</u>H_2Ph), 56.4 (O<u>C</u>H_3), 58.0 (N-<u>C</u>H_2CH_3), 75.3 (<u>C</u>H), 130.9–136.1 (<u>C</u>$_6H_4$), 171.0 (<u>C</u>=O).

5.1.3.3. Compound 1c. Yield: 88%. ^{1}H NMR: δ_H ($CDCl_3$), 3.30 (m, 1H, C<u>H</u>CHPh), 3.36 (s, 9H, N-C<u>H</u>$_3$), 3.61 (s, 3H, OCH_3), 3.72 (m, 1H, CHC<u>H</u>Ph), 4.54 (m, 1H, CH), 7.30–7.43 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 32.6 (<u>C</u>H_2Ph), 52.6 (N<u>C</u>H_3), 53.6 (O<u>C</u>H_3), 75.6 (<u>C</u>H), 128.1–132.9 (<u>C</u>$_6H_4$), 167.8 (<u>C</u>=O).

5.1.3.4. Compound 1d. Yield: 38%. ^{1}H NMR: δ_H ($CDCl_3$), 1.28 (t, 9H, N-CH_2C<u>H</u>$_3$), 3.27 (m, 6H, N-C<u>H</u>$_2CH_3$), 7.37–7.65 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 9.6 (N-CH_2<u>C</u>H_3), 30.4 (<u>C</u>H_2Ph), 55.5 (N-<u>C</u>H_2-CH_3), 60.1 (<u>C</u>H), 130.2–138.8 (<u>C</u>$_6H_4$), 168.4 (<u>C</u>=O).

5.1.3.5. Compound 2a. Yield: 68%. ^{1}H NMR: δ_H ($CDCl_3$), 1.01 (t, 6H, N-CH_2C<u>H</u>$_3$), 1.13 (t, 3H, OCH_2<u>C</u>H_3), 2.54 (m, 2H, N-C<u>H</u>$_2CH_3$), 2.76 (m, 2H, N-C<u>H</u>$_2CH_3$), 2.85 (m, 1H, CHC<u>H</u>Ph), 2.90 (m, 1H, C<u>H</u>CHPh), 3.59 (t, 1H, C<u>H</u>), 4.01 (m, 2H, O-C<u>H</u>$_2CH_3$), 7.31–7.43 (m, 5H, Ph).

5.1.3.6. ^{13}C NMR: δ_C ($CDCl_3$), 13.8 (N-CH_2<u>C</u>H_3), 14.1(O<u>C</u>H_2CH_3), 35.3 (<u>C</u>H_2Ph), 44.5 (N-<u>C</u>H_2CH_3), 60.0 (O<u>C</u>H_2CH_3), 65.1 (<u>C</u>H), 126.2–138.8 (<u>C</u>$_6H_4$), 172.6 (<u>C</u>=O).

5.1.3.6. Compound 2b. Yield: 28%. M.p: 90 °C. Elem. Anal. Found: C, 56.23; H, 8.51; N, 4.90; Calcd. for $C_{17}H_{28}NO_2Br$: C, 56.98; H, 7.88; N, 3.91. ^{1}H NMR: δ_H ($CDCl_3$), 0.96 (t, 3H, OCH_2C<u>H</u>$_3$), 1.44 (t, 9H, N-CH_2-C<u>H</u>$_3$), 3.28 (m, 1H, C<u>H</u>CHPh), 3.38 (m, 1H, CHC<u>H</u>Ph), 3.67 (m, 6H, N-C<u>H</u>$_2CH_3$), 4.03 (m, 2H, OC<u>H</u>$_2CH_3$), 4.34 (m, 1H, CH), 7.26–7.41 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 8.20 (N-CH_2<u>C</u>H_3), 13.2 (OCH_2<u>C</u>H_3), 32.9 (<u>C</u>H_2Ph), 55.1 (N-<u>C</u>H_2CH_3), 63.8 (O<u>C</u>H_2CH_3), 72.3 (<u>C</u>H), 128.1–133.6 (<u>C</u>$_6H_4$), 167.7 (<u>C</u>=O).

5.1.3.7. Compound 3a. Yield: 76%. ^{1}H NMR: δ_H ($CDCl_3$), 0.85 (t, 3H, OCH_2CH_2C<u>H</u>$_3$), 1.00 (t, 6H, N-CH_2C<u>H</u>$_3$), 1.57 (m, 2H, OCH_2C<u>H</u>$_2CH_3$), 2.52 (m, 2H, N-C<u>H</u>$_2CH_3$), 2.76 (m, 2H, N-C<u>H</u>$_2CH_3$), 2.87 (m, 1H, CHC<u>H</u>Ph), 3.05 (m, 1H, C<u>H</u>CHPh), 3.60 (m, 1H, C<u>H</u>), 4.01 (t, 2H, O-C<u>H</u>$_2$ CH_2CH_3), 7.31–7.43 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 13.8 (N-CH_2<u>C</u>H_3), 14.1(O<u>C</u>H_2CH_3), 35.3 (<u>C</u>H_2Ph), 44.5 (N-<u>C</u>H_2CH_3), 60.0 (O<u>C</u>H_2CH_3), 65.1 (<u>C</u>H), 126.2–138.8 (<u>C</u>$_6H_4$), 172.6 (<u>C</u>=O).

5.1.3.8. Compound 3b. Yield: 6%. M.p: 92 °C. ^{1}H NMR: δ_H ($CDCl_3$), 0.49 (t, 3H, OCH_2CH_2C<u>H</u>$_3$), 1.26 (t, 9H, N-CH_2C<u>H</u>$_3$), 1.35 (m, 2H, OCH_2C<u>H</u>$_2CH_3$), 3.13 (m, 1H, C<u>H</u>CHPh), 3.25 (m, 1H, CHC<u>H</u>Ph), 3.50 (m, 6H, N-C<u>H</u>$_2CH_3$), 3.78 (m, 2H, OC<u>H</u>$_2CH_2CH_3$), 4.22 (m, 1H, CH), 7.10–7.26 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 10.5 (OCH_2CH_2<u>C</u>H_3), 13.9 (N-CH_2<u>C</u>H_3), 22.0 (OCH_2<u>C</u>H_2CH_3), 36.5 (<u>C</u>H_2Ph), 44.6 (N-<u>C</u>H_2CH_3), 65.1 (<u>C</u>H), 65.7 (O<u>C</u>$H_2CH_2CH_3$), 126.2–138.8 (<u>C</u>$_6H_4$), 172.8 (<u>C</u>=O).

5.1.3.9. Compound 4a. Yield: 78%. ^{1}H NMR: δ_H ($CDCl_3$), 1.00 (t, 3H, $OCH_2CH_2CH_2$C<u>H</u>$_3$), 1.03 (t, 6H, N-CH_2C<u>H</u>$_3$), 1.25 (m, 2H, OCH_2CH_2-C<u>H</u>$_2CH_3$), 1.49 (m, 2H, OCH_2C<u>H</u>$_2CH_2CH_3$), 2.56 (m, 2H, N-C<u>H</u>$_2CH_3$), 2.73 (m, 2H, N-C<u>H</u>$_2CH_3$), 2.79 (m, 1H, C<u>H</u>CHPh), 2.87 (m, 1H, CHC<u>H</u>Ph), 3.10 (t, 1H, C<u>H</u>), 3.99 (t, 2H, OC<u>H</u>$_2CH_2CH_2CH_3$), 7.13–7.22 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 13.6 ($OCH_2CH_2CH_2$<u>C</u>H_3), 13.9 (N-CH_2<u>C</u>H_3), 19.1 (O CH_2CH_2<u>C</u>H_2CH_3), 30.7 (OCH_2C<u>H</u>$_2CH_2CH_3$), 36.5 (<u>C</u>H_2Ph), 44.6 (N-<u>C</u>H_2CH_3), 63.9 (<u>C</u>H), 65.1 (OC<u>H</u>$_2CH_2CH_2CH_3$), 126.2–138.8 (<u>C</u>$_6H_4$), 172.8 (<u>C</u>=O).

5.1.3.10. Compound 4c. Yield: 52%. ^{1}H NMR: δ_H ($CDCl_3$), 0.94 (t, 3H, $OCH_2CH_2CH_2$C<u>H</u>$_3$), 1.02 (m, 2H, OCH_2CH_2C<u>H</u>$_2CH_3$), 1.21 (m, 2H, OCH_2C<u>H</u>$_2CH_2CH_3$), 1.27 (m, 6H, N-CH_2C<u>H</u>$_3$), 3.21 (m, 1H, C<u>H</u>CHPh), 3.48 (s, N-C<u>H</u>$_3$), 3.62 (m, 1H, CHC<u>H</u>Ph), 3.67 (m, 2H, N-C<u>H</u>$_2CH_3$), 3.74 (m, 2H, N-C<u>H</u>$_2CH_3$), 3.96 (m, 2H, OCH_2), 4.27 (m, 1H, CH), 7.17–7.25 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 9.2 (N-CH_2<u>C</u>H_3), 13.2 ($OCH_2CH_2CH_2$<u>C</u>H_3), 18.4 (O CH_2CH_2<u>C</u>H_2CH_3), 29.7 (<u>C</u>H_2Ph), 33.3 (OCH_2C<u>H</u>$_2CH_2CH_3$), 46.7 (N-CH_3), 57.3 (N-<u>C</u>H_2CH_3), 67.2 (OC<u>H</u>$_2CH_2CH_2CH_3$), 72.9 (<u>C</u>H), 128.1–132.1 (<u>C</u>$_6H_4$), 166.3 (<u>C</u>=O).

5.1.3.11. Compound 5a. Yield: 76%. ^{1}H NMR: δ_H ($CDCl_3$), 0.89 (t, 3H, O(CH_2)$_9$C<u>H</u>$_3$), 1.03 (t, 6H, N-CH_2C<u>H</u>$_3$), 1.55 (m, 14H, OCH_2CH_2 (C<u>H</u>$_2$)$_7CH_3$), 1.62 (m, 2H, OCH_2C<u>H</u>$_2$(CH_2)$_7CH_3$), 2.70 (m, 2H, NC<u>H</u>$_2CH_3$), 2.76 (m, 2H, NC<u>H</u>$_2CH_3$), 2.81 (m, 1H, C<u>H</u>CHPh), 2.89 (m, 1H, CHC<u>H</u>Ph), 3.06 (m, 1H, CH), 3.59 (m, 2H, OC<u>H</u>$_2$(CH_2)$_8CH_3$), 7.15–7.27 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 13.8 (N-CH_2<u>C</u>H_3), 14.1 (O(CH_2)$_9$<u>C</u>H_3), 22.7–31.9 (OCH_2(<u>C</u>H_2)$_8CH_3$), 36.4 (<u>C</u>H_2Ph), 44.5 (N-<u>C</u>H_2CH_3), 55.7 (O<u>C</u>H_2(CH_2)$_8CH_3$), 65.1 (CH), 126.2–138.8 (<u>C</u>$_6H_4$), 172.8 (<u>C</u>=O).

5.1.3.12. Compound 5b. Yield: 65%. M.p: 129 °C. Elem. Anal. Found: C, 57.30; H, 8.34; N, 3.43; Calcd. for $C_{24}H_{42}NO_2I$: C, 57.25; H, 8.41; N, 2.78. ^{1}H NMR: δ_H ($CDCl_3$), 0.86 (m, 3H, O(CH_2)$_9$C<u>H</u>$_3$), 1.24 (m, 14H, OCH_2CH_2(C<u>H</u>$_2$)$_7CH_3$), 1.48 (m, 6H, N-CH_2C<u>H</u>$_3$), 1.89 (m, 2H, OCH_2C<u>H</u>$_2$(CH_2)$_7CH_3$), 3.22 (m, 1H, C<u>H</u>CHPh), 3.47 (s, 3H, N-CH_3), 3.73 (m, 2H, NC<u>H</u>$_2CH_3$), 3.88 (m, 2H, NC<u>H</u>$_2CH_3$), 3.95 (m, 1H, CHC<u>H</u>Ph), 4.03 (m, 2H, OC<u>H</u>$_2$(CH_2)$_8CH_3$), 4.24 (m, 1H, C<u>H</u>), 7.24–7.30 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 8.9 (N-CH_2<u>C</u>H_3), 14.1 (O(CH_2)$_9$<u>C</u>H_3), 22.7–31.9 (OCH_2(<u>C</u>H_2)$_8CH_3$), 32.8 (<u>C</u>H_2Ph), 46.7 (N-<u>C</u>H_3), 57.3 (N-<u>C</u>H_2-CH_3), 64.1 (O<u>C</u>H_2(CH_2)$_8CH_3$), 72.1 (<u>C</u>H), 128.2–132.1 (<u>C</u>$_6H_4$), 166.6 (<u>C</u>=O).

5.1.3.13. Compound 6a. Yield: 70%. ^{1}H NMR: δ_H ($CDCl_3$), 0.89 (t, 3H, O(CH_2)$_{11}$C<u>H</u>$_3$), 1.03 (t, 6H, N-CH_2C<u>H</u>$_3$), 1.52 (m, 18H, OCH_2CH_2 (C<u>H</u>$_2$)$_9CH_3$), 1.57 (m, 2H, OCH_2C<u>H</u>$_2$(CH_2)$_9CH_3$), 2.71 (m, 2H, NC<u>H</u>$_2CH_3$), 2.79 (m, 2H, NC<u>H</u>$_2CH_3$), 2.82 (m, 1H, C<u>H</u>CHPh), 2.87 (m, 1H, CHC<u>H</u>Ph), 3.09 (m, 1H, CH), 3.94 (m, 2H, OC<u>H</u>$_2$(CH_2)$_{10}CH_3$), 7.15–7.25 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 13.8 (N-CH_2<u>C</u>H_3), 14.1 (O(CH_2)$_{11}$<u>C</u>H_3), 22.7–31.9 (OCH_2(<u>C</u>H_2)$_{10}CH_3$), 36.4 (<u>C</u>H_2Ph), 44.5 (N-<u>C</u>H_2CH_3), 55.7 (O<u>C</u>H_2(CH_2)$_{10}CH_3$), 65.1 (CH), 126.2–138.8 (<u>C</u>$_6H_4$), 172.8 (<u>C</u>=O).

5.1.3.14. Compound 6b. Yield: 43%. M.p: 132 °C. Elem. Anal. Found: C, 55.65; H, 7.80; N, 4.45; Calcd. for $C_{26}H_{46}NO_2I$: C, 55.82; H, 7.61; N, 4.07. ^{1}H NMR: δ_H ($CDCl_3$), 0.88 (m, 3H, O(CH_2)$_{11}$C<u>H</u>$_3$), 1.26 (m, 18H, OCH_2CH_2(C<u>H</u>$_2$)$_9CH_3$), 1.51 (m, 6H, N-CH_2C<u>H</u>$_3$), 1.78 (m, 2H, OCH_2C<u>H</u>$_2$(CH_2)$_9CH_3$), 3.08 (m, 1H, C<u>H</u>CHPh), 3.23 (m, 1H, CHC<u>H</u>Ph), 3.48 (s, 3H, N-CH_3), 3.66 (m, 2H, NC<u>H</u>$_2CH_3$), 3.72 (m, 2H, NC<u>H</u>$_2CH_3$), 3.99 (m, 2H, OC<u>H</u>$_2$(CH_2)$_{10}CH_3$), 4.26 (m, 1H, C<u>H</u>), 7.21–7.34 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 9.2 (N-CH_2<u>C</u>H_3), 14.4 (O(CH_2)$_{11}$<u>C</u>H_3), 23.0–31.2 (OCH_2(<u>C</u>H_2)$_{10}CH_3$), 33.6 (<u>C</u>H_2Ph), 46.8 (N-<u>C</u>H_3), 57.7 (N-<u>C</u>H_2-CH_3), 67.4 (O<u>C</u>H_2(CH_2)$_{10}CH_3$), 72.9 (<u>C</u>H), 128.5–132.3 (<u>C</u>$_6H_4$), 166.8 (<u>C</u>=O).

5.1.3.15. Compound 7a. Yield: 78%. ^{1}H NMR: δ_H ($CDCl_3$), 0.86 (t, 3H, O(CH_2)$_{13}$C<u>H</u>$_3$), 1.03 (t, 6H, N-CH_2C<u>H</u>$_3$), 1.49 (m, 22H, OCH_2CH_2

($C\underline{H}_2)_{11}CH_3$), 1.54 (m, 2H, $OCH_2C\underline{H}_2(CH_2)_{11}CH_3$), 2.54 (m, 2H, $NC\underline{H}_2CH_3$), 2.76 (m, 2H, $NC\underline{H}_2CH_3$), 2.82 (m, 1H, C<u>H</u>CHPh), 2.87 (m, 1H, CHC<u>H</u>Ph), 3.09 (m, 1H, CH), 3.94 (m, 2H, $OC\underline{H}_2(CH_2)_{12}CH_3$), 7.16–7.25 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 13.8 (N-$CH_2\underline{C}H_3$), 14.1 ($O(CH_2)_{13}\underline{C}H_3$), 22.7–31.9 ($OCH_2(\underline{C}H_2)_{12}CH_3$), 36.4 ($\underline{C}H_2$Ph), 44.6 (N-$\underline{C}H_2CH_3$), 63.0 ($O\underline{C}H_2(CH_2)_{12}CH_3$), 65.1 (CH), 126.2–138.8 ($\underline{C}_6H_4$), 172.8 (<u>C</u>=O).

5.1.3.16. Compound 7b. Yield: 42%. M.p: 152 °C. MW 559.38: ESI-MS, m/z: 432.38 (M^+ without the I^-) Elem. Anal. Found: C, 60.37; H, 8.65; N, 2.97; Calcd. for $C_{28}H_{50}NO_2I$: C, 60.10; H, 9.01; N, 2.50. ^{1}H NMR: δ_H ($CDCl_3$), 0.86 (m, 3H, $O(CH_2)_{13}C\underline{H}_3$), 1.24 (m, 22H, $OCH_2CH_2(C\underline{H}_2)_{11}CH_3$), 1.52 (m, 6H, N-$CH_2C\underline{H}_3$), 1.75 (m, 2H, OCH_2-$\underline{C}H_2(CH_2)_{11}CH_3$), 2.77(m, 1H, CHC<u>H</u>Ph), 3.15 (m, 1H, C<u>H</u>CHPh), 3.46 (s, 3H, N-CH_3), 3.70 (m, 2H, $NC\underline{H}_2CH_3$), 3.84 (m, 2H, $NC\underline{H}_2CH_3$), 3.96 (m, 2H, $OC\underline{H}_2(CH_2)_{12}CH_3$), 4.26 (m, 1H, C<u>H</u>), 7.25–7.27 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 9.1 (N-$CH_2\underline{C}H_3$), 14.2 ($O(CH_2)_{13}\underline{C}H_3$), 22.7–32.0 ($OCH_2(\underline{C}H_2)_{12}CH_3$), 33.3 ($\underline{C}H_2$Ph), 46.7 (N-$\underline{C}H_3$), 57.2 (N-$\underline{C}H_2$-$CH_3$), 67.3 ($O\underline{C}H_2(CH_2)_{12}CH_3$), 72.7 (<u>C</u>H), 128.3–132.1 ($\underline{C}_6H_4$), 166.7 (<u>C</u>=O).

5.1.3.17. Compound 8a. Yield: 73%. ^{1}H NMR: δ_H ($CDCl_3$), 0.99 (t, 3H, $O(CH_2)_{15}C\underline{H}_3$), 1.23 (t, 6H, N-$CH_2C\underline{H}_3$), 1.48 (m, 26H, OCH_2CH_2 ($C\underline{H}_2)_{13}CH_3$), 1.55 (m, 2H, $OCH_2C\underline{H}_2(CH_2)_{13}CH_3$), 2.56 (m, 2H, $NC\underline{H}_2CH_3$), 2.73 (m, 2H, $NC\underline{H}_2CH_3$), 2.78 (m, 1H, C<u>H</u>CHPh), 2.86 (m, 1H, CHC<u>H</u>Ph), 3.06 (m, 1H, C<u>H</u>), 3.94 (m, 2H, $OC\underline{H}_2(CH_2)_{14}CH_3$), 7.14–7.26 (m, 5H, Ph).

5.1.3.18. Compound 8b. Yield: 65%. M.p: 164 °C. Elem. Anal. Found: C, 60.65; H, 9.46; N, 2.29; Calcd. for $C_{30}H_{54}NO_2I$: C, 61.31; H, 9.26; N, 2.38. ^{1}H NMR: δ_H ($CDCl_3$), 0.83 (m, 3H, $O(CH_2)_{15}C\underline{H}_3$), 1.24 (m, 26H, $OCH_2CH_2(C\underline{H}_2)_{13}CH_3$), 1.53 (m, 6H, N-$CH_2C\underline{H}_3$), 1.75 (m, 2H, $OCH_2CH_2(CH_2)_{13}CH_3$), 2.77 (m, 1H, CHC<u>H</u>Ph), 3.16 (m, 1H, C<u>H</u>CHPh), 3.49 (s, 3H, N-CH_3), 3.67 (m, 2H, $NC\underline{H}_2CH_3$), 3.73 (m, 2H, $NC\underline{H}_2CH_3$), 3.95 (m, 2H, $OC\underline{H}_2(CH_2)_{14}CH_3$), 4.26 (m, 1H, C<u>H</u>), 7.24–7.28 (m, 5H, Ph).

^{13}C NMR: δ_C ($CDCl_3$), 9.1 (N-$CH_2\underline{C}H_3$), 14.2 ($O(CH_2)_{15}\underline{C}H_3$), 22.7–32.0 ($OCH_2(\underline{C}H_2)_{14}CH_3$), 33.3 ($\underline{C}H_2$Ph), 46.7 (N-$\underline{C}H_3$), 57.2 (N-$\underline{C}H_2$-$CH_3$), 67.3 ($O\underline{C}H_2(CH_2)_{14}CH_3$), 72.7 (<u>C</u>H), 128.3–132.1 ($\underline{C}_6H_4$), 166.7 (<u>C</u>=O).

5.2. Antimicrobial activity

A growth inhibition assay was performed using the Kirby-Bauer disc diffusion method [26] using Muellor Hinton agar and Muellor Hinton broth (full strength) for bacterial culture. Minimum Inhibitory Concentrations (MICs) of the synthesized compounds were determined by a dilution assay. The stock solutions of the compounds were prepared in distilled water/TBS (0.05 M, pH 7.4) and were serially diluted with concentration of the L-Phenylalanine QUATS ranging from 1000 μg/mL to 1 μg/mL. 10 μL of these sample solutions were pipetted onto the discs and allowed to incubate at 37 °C for 24 h. The MICs were determined by measuring the diameter of the zone of inhibition in mm. CTAB which is commonly use as an antiseptic was used as a positive control using the same concentration of the tested compounds. The lowest concentration that prevented colony formation was defined as the MIC.

5.3. Hemolytic activity

Erythrocytes were suspended in saline buffer/TBS (0.05 M, pH 7.4). 0.5 mL of a series of surfactant solutions with concentration ranging from 1 mg/mL to 0.01 mg/mL were placed in centrifuge tube containing saline buffer/TBS. An aliquot of 50 μL of erythrocyte suspension were added to each tube and were incubated for 30 min at room temperature. Following incubation, the tubes were centrifuged (5 min, 3000 rpm). The hemolysis degree was determined by comparing the absorbance (540 nm) of the supernatant with that of the control samples which are totally hydrolysed with distilled water.

5.4. Phospholipid binding studies

5.4.1. 1-Anilino-8-naphthalene sulfonate (ANS)

Fluorometric titrations of solutions containing 5×10^{-4} M QUATS (6b, 7b, 8b) and phospholipid (1×10^{-3} M) with stock solutions of ANS were carried out. Phospholipid vesicles were prepared using the method described previously [20]. The excitation and emission wavelength were 380 nm and 480 nm respectively. The slope of the linear plot was taken to be the emission coefficient of the ternary ANS complex (A_t). For the quantitative determination of the binding of ANS to lipid in the presence of the QUATS, a solution containing 2×10^{-5} M lipid and 5×10^{-4} M QUAT was titrated with stock solution of ANS and the fluorescence of each titration increment was measured. The concentrations of the bound ANS, X_b, in these solutions were calculated according to the Eq. (1). The free and bound concentrations of ANS corresponding to each total ANS concentration along the titration curve were calculated.

$$X_b = (F_b - F_0)/(A_t - A_0) \tag{1}$$

F_b and F_0 are the fluorescence intensities for solutions with and without lipid, while A_t and A_0 are the emission coefficients for the lipid-bound and free ANS respectively. From the value of X_b, the concentrations of free and bound ANS were calculated for each solution and the data were then treated using the Scatchard equation (Eq. (2)). The binding constant and the binding capacity between ANS and DPPC vesicles were determined using this equation, assuming equal binding strength for all binding sites.

$$\frac{v}{[P]} = nK = vK \tag{2}$$

where v is the number of moles of bound probe per mol of lipid; [P] is the free concentration of the probe, ANS; n is the maximum value of v which indicates the binding capacity of the lipid for ANS; and K is the binding constant. Previous studies carried out on the binding of ANS to DPPC, the emission coefficients of free ANS, A_0 and that of lipid bound ANS, A_b were found to be 1×10^7 and 5×10^8 respectively [34]. The binding capacity of the lipid, n and the binding constant, K were found to be 0.0016 and 2×10^6 M^{-1} respectively

5.4.2. DPH (1,6-diphenyl-1,3,5-hexatriene)

For the competitive binding studies, mixtures containing DPPC (2×10^{-5} M), and DPH were titrated against varying concentrations of the QUATS, and the quenching of the fluorescence were recorded as a function of QUATS concentrations. The binding of the QUATS to DPPC were determined using Eq. (3). This equation was derived from the competitive binding mechanism assuming that the QUATS competes for n_1 of the n probe binding sites with an intrinsic binding strength of K_1.

$$\frac{(n - v_1)v_1}{(n - v^* - v_1)[D]} = n_1K_1 - v_1K_1 \tag{3}$$

where v^* is the ratio of bound-probe per total lipid concentration in the presence of the QUATS; v_1 is the ratio of bound QUATS per total lipid concentration; n_1 is the maximum value of v_1 which indicates the probe binding sites which may be replaced by QUATS; [D] is the free QUATS concentration; K_1 is the binding constant for the QUATS-lipid interaction; and v_1 was obtained from Eq. (4), using n and K values from Eq. (2).

$$v_1 = n - v^* - \frac{v^*}{[P]^*K} \tag{4}$$

[P]* represents the free concentration of the probe in the presence of the QUATS. [D] was taken to be the difference between the total concentration of the QUATS and the product of v_1 and total lipid concentration.

6. Results and discussion

6.1. Synthesis of quaternary ammonium compounds derived from L-Phenylalanine esters

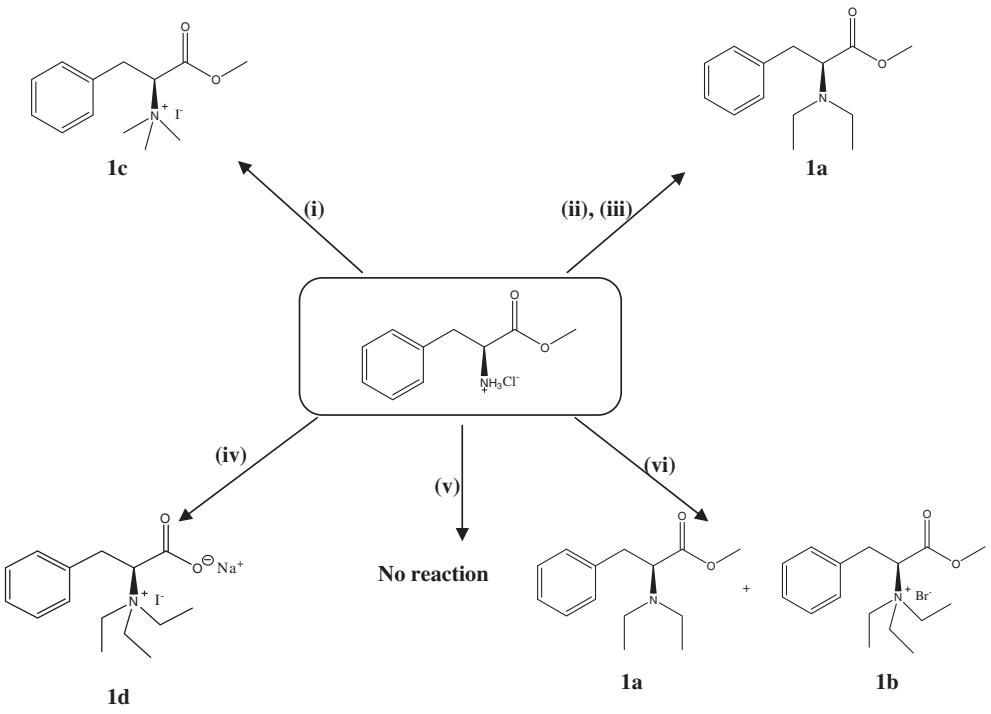
L-Phenylalanine QUATS were synthesised by quaternising the amino group of L-Phenylalanine esters with alkyl halides. The method was standardised using L-Phenylalanine methyl ester as precursor. A number of methods for quaternising primary and secondary amines involving the use of alkylating agents in the presence of strong inorganic bases have been reported [27,28]. Most of these reactions often involve prolonged reaction times and heat which are conducive to undesirable side reactions [29]. Several attempts were made in order to quaternise L-Phenylalanine methyl ester using ethyl groups to yield compound 1b (Scheme 1).

Reaction of L-Phenylalanine methyl ester with MeI in the presence of $NaHCO_3$ yielded the *N,N,N*-trimethyl phenylalanine methyl ester 1c derivative in 88% yield. However, when the same reaction was repeated using ethyl iodide, only the dialkylated product 1a was formed rather than the *N,N,N*-triethyl derivative. Heating the reaction to 90 °C in a sealed tube for 18 h yielded only the dialkylated derivative 1a. When EtI/NaOH was employed, the amino group was successfully quaternised but the ester moiety hydrolysed to the carboxylate salt 1d. Triethylamine (TEA), a milder base was then employed. However, as it was envisaged that the formation of the quaternised base rendered the isolation of the desired quaternary ammonium compound difficult [30], the phenylalanine ester hydrochloride was first made to react with TEA, to produce the free amine which was then reacted with EtI. However, the desired product was not formed and this may be due to the absence of base in the reaction medium during quaternisation. The reaction of a primary amine with an alkyl halide involves the liberation of a hydrohalic acid which combines with amines to form a mixture of amine hydrohalide salts [31]. Consequently, very low concentration of the free amine remained for subsequent alkylation.

Using EtBr in acetonitrile at 90 °C and K_2CO_3 as base in a sealed tube gave a mixture of the desired product 1b together with the dialkylated product 1a as the major product. The structure of compound 1b was confirmed by spectral data and X-ray crystallography. From the ^1H NMR spectrum the presence of the three ethyl groups in 1b were confirmed by the triplet (9 H) at 1.42 and a multiplet of 6 H at 3.66 ppm. The structure of 1a was confirmed by spectral data. The N-ethyl groups appeared as a triplet at 1.16 ppm corresponding to two CH_3 protons and 2 multiplets at 2.87–2.98 ppm N-CH_2 moieties.

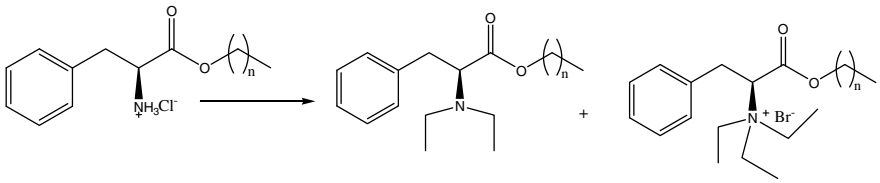
Different phenylalanine esters with alkyl chain lengths varying from C_1 to C_4 were quaternised using EtBr/K_2CO_3 in a sealed tube at 90 °C (Scheme 2).

An increase of the alkyl ester chain length from methyl to propyl ester caused a decrease in the yield of the quaternary ammonium compounds and an increase in the diethyl derivative. In the case of the butyl ester, the diethyl derivative 4a was formed as the only product. An increase in the alkyl chain length of the esters rendered quaternisation more difficult, possibly due to steric hindrance. The bulky ethyl groups rendered quaternisation unfavourable with phenylalanine esters of chain length >3. Reaction of 4a with excess bromoethane did not give the desired triethyl derivative. When compound 4a was reacted with excess MeI in a sealed tube, the quaternary ammonium compound 4c was formed as an



(i) $NaHCO_3$/MeI, MeOH, RT, **(ii)** $NaHCO_3$/EtI, EtOH, RT, **(iii)** $NaHCO_3$/ EtBr, 90°C, sealed tube, **(iv)** NaOH/EtI, 90°C, sealed tube, **(v)** TEA/ EtI, 90°C, sealed tube, **(vi)** K_2CO_3/EtBr, 90°C, sealed tube.

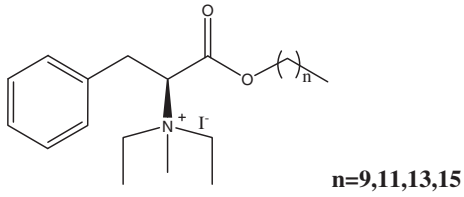
Scheme 1. Synthesis attempts of *N,N,N*-triethyl phenylalanine methyl ester.



1a-4a; n=0-3 1b-4b; n=0-3

n	Compound number	% Yield	Compound Number	% Yield
0	1b	35	1a	63
1	2b	28	2a	68
2	3b	6	3a	76
3	4b	0	4a	78

Scheme 2. Quaternisation attempts of esters with varying chain length.



n=9,11,13,15

Fig. 3. General structure of *N*,*N*-Diethyl,*N*-methyl L-Phenylalanine esters.

oil in moderate yield. Higher homologues of the L-Phenylalanine QUAT derivatives ($n = C_{10}$–C_{16}) were prepared by alkylating the amino group with ethyl and methyl groups. The products were obtained in moderate yield (Fig. 3).

6.2. X-ray analysis of compound 1b

The compound 1b crystallised in the monoclinic space group $P2_1$ with two independent cations and the associated bromide anions in the asymmetric unit, $Z = 4$. The compound crystallised as the dihydrate (one water molecule per cation, two per asymmetric unit) (Fig. 4).

Selected bond lengths and bond angles are summarised below in Table 2.

The data in Table 2 illustrate the similarity of the two cations in the asymmetric unit. The most notable difference between the two structures is the C7–C8–C9–C14 torsion angles which measure 76.8(2)° and 70.0(2)° for molecules A and B, respectively. Although there is a moderate difference between the C7–N1–$C_{methylene}$ bond angles of molecules A and B, both illustrate the sp^3 hybridisation of the quaternary nitrogen atoms. A Mogul structural search [32] shows that the bond lengths and bond angles of compound compare favourably to those of related structures in the Cambridge Structural Database [33].

The three-dimensional structure of 1b is interesting in that the cations pack such that there are channels running through the lattice (Fig. 5a). The channels are co-linear with the *b*-axis. In the present structure these channels are occupied by the bromide counter ions and the solvent molecules. These bromide ions and water molecules are linked through extensive hydrogen bonding; refer to Fig. 5b and Table 3. The water molecules act as the hydrogen bond donors and the bromide ions as the hydrogen bond

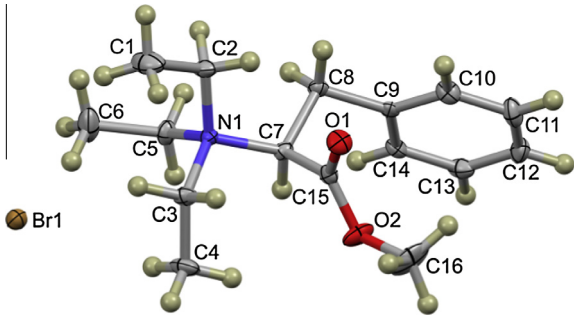


Fig. 4. Fully labelled thermal ellipsoid plot of 1b shown at 50% probability. Since the geometrical parameters of molecules A and B differ only marginally, molecule A is shown as being representative of both molecules. Water molecules have been omitted for clarity. Hydrogen atoms have been rendered as spheres of arbitrary radius.

acceptors in all cases. Although hydrogen bond length does not necessarily correlate linearly to bond strength due to packing constraints in the lattice [34], these hydrogen bonds are all significantly shorter than the sum of the van der Waals radii of the interacting atoms. This coupled with the fact that the hydrogen bond angles do not deviate significantly from the optimum bond angle suggests that these are likely to be moderately strong interactions. There are few significant interactions linking the cations into an extended supramolecular structure. The most notable is a weak C–H···π interaction between the methine C–H and the carbonyl oxygen atom of an adjacent molecule.

6.3. CMC (Critical micelle concentration)

The CMCs of the C_{10}–C_{16} of the L-Phenylalanine QUATS were determined in aqueous solution by measuring the conductivity values of different concentrations of surfactant solutions (Table 4).

The CMC was estimated from the breakpoint in the conductivity versus surfactant concentration plots. As expected, the CMC decreases with increasing alkyl chain as a consequence of a higher hydrophobic content of the molecule which involves more interactions between the chains, favouring micellar formation (Fig. 6).

Considering the Kleven's equation for homologous straight chain ionic surfactants in aqueous medium [35], the relation

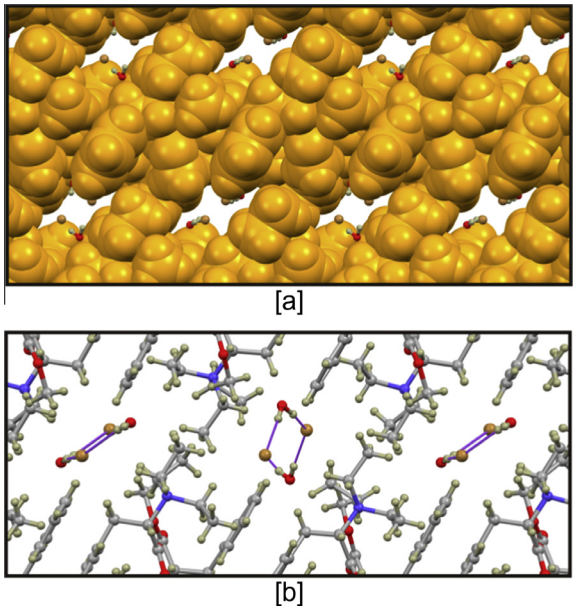


Fig. 5. (a) Partial space-filling model illustrating the channels in the lattice of 1b, viewed down the *b*-axis. The cations have been rendered as space filling models, the bromide ions and water molecules as ball and stick models; (b) Hydrogen bonding (illustrated as broken purple tubes) between the bromide ions and water molecules within the channels.

Table 3
Summary of hydrogen bond lengths (Å) and bond angles (°) of 1b.

Hydrogen Bond	D–H	H···A	D···A	D–H···A
O1s–H1s···Br2	0.75(2)	2.67(2)	3.41(2)	168(2)
O1s–H2s···Br2	0.79(3)	2.60(3)	3.38(2)	171(3)
O2s–H3s···Br1	0.88(4)	2.49(4)	3.31(2)	156(3)
O2s–H4s···Br1	0.78(4)	2.63(4)	3.40(2)	170(3)

Table 4
CMC of the homologous series of L-Phenylalanine QUATS.

Compound	Chain length	CMC value (μM)
5b	10	455
6b	12	74.1
7b	14	5.66
8b	16	1.96

between log CMC (μM) and the number of carbon atoms n in the hydrophobic chain of the homologous series of L-Phenylalanine QUATS at 25 °C, is given in Eq. (5)

$$\log \mathrm{CMC} = 6.7325 - 0.4107n \quad (5)$$

The CMC of the QUATS were found to be much lower compared to those previously reported for the hydrochloride salts of L-Phenylalanine ester series. This is in accordance with previous studies whereby it was observed that surfactants with a more hydrophobic head group present lower CMC values [36]. Ionic surfactants have higher CMC values compared to non-ionic ones due to repulsion between their ionic groups. However, the head group repulsion is counteracted when there is a hydrophobic attraction between the head groups [37,38]. In this case the additional alkyl groups attached to the nitrogen moiety of phenylalanine screens the electrostatic head-group repulsion and strongly facilitates the surfactants self assembly, giving rise to a much lower CMC value compared to the hydrochloride salts of phenylalanine esters.

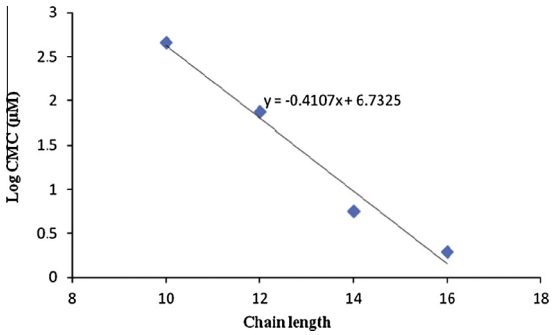


Fig. 6. CMC versus chain length of L-Phenylalanine QUATS.

Table 5
MIC values for compounds 5b, 6b, 7b and 8b and CTAB.

Microorganisms		MIC (μM)				
		5b	6b	7b	8b	CTAB
Gram-positive	*S. aureus* (ATCC 29213)	94	6.6	3.6	8.5	8.2
	S. aureus (ATCC 25923)	30	7.5	3.6	12	8.2
	S. epidermidis (ATCC 12228)	20	5.7	4.5	12	2.7
	B. cereus (ATCC 10876)	131	9	5.4	17	27
	B. cereus (ATCC 11778)	150	15	5.4	17	27
Gram-negative	*K. pneumoniae* (ATCC 13883)	139	19	89	170	27
	S. typhimurium (ATCC 14028)	198	94	178	425	13700
	E. coli (ATCC 22922)	99	19	178	340	2200
	P. aeruginosa (ATCC 27853)	397	94	268	425	1370

6.4. Antibacterial activity

The antibacterial activity of the synthesised L-Phenylalanine QUATS was determined by their minimum inhibitory concentration (MIC) values. The results are summarised in Table 5.

The antibacterial effects were clearly strain-dependant. The compounds (5b-8b) showed better activity against Gram-positive than Gram-negative strains. An increase in activity against Gram-positive bacteria was observed with increasing chain length which then tails off, exhibiting a cut-off effect at C_{14}. Lukac et al. reported the antibacterial activity of a series of quaternary ammonium compounds derived from phenylalanine, namely *N*-alkyl-*N*,*N*-dimethyl-(1-hydroxy-3-phenylpropyl)-2-ammonium bromides [19]. The phenylalanine QUATS (5b–8b) displayed relatively better antibacterial activity against *S. aureus* compared to the phenylalanine derivatives reported by Lukac et al. However, in the case of *E. coli*, the phenylalanine QUATS were found to be less active. The phenylalanine QUATS displayed a cut-off point at C_{14} and C_{12} with respect to Gram-positive and Gram-negative bacteria respectively, whereas the phenylalanine quaternary derivatives reported by Lukac et al. showed a cut-off effect at C_{14} for both Gram-negative and Gram-positive strains.

The increase in antibacterial activity with respect to chain length might be attributed to an increase in hydrophobic interactions between the surfactant chain and those of the neighbouring lipid molecule of the bacterial membrane, which in turn pulls the surfactant molecule further into the membrane. These factors including the interaction of the quaternary ammonium moiety of the surfactant and the polar head of lipid tend to destabilise the membrane causing lysis. However, a further increase in chain length (>C_{14}) causes a decrease in antibacterial activity, a phenomenon referred to as the cut-off effect. Janoff and Pringle associated this effect with a decrease in solubility of the surfactant with chain length [39,40]. Lobecce et al. and Sarapuk et al. stated the possibility of a sewing of the lipid bilayer caused by the incorporation of

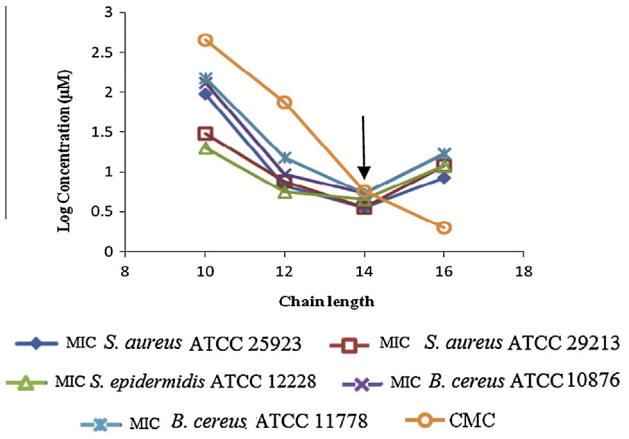


Fig. 7a. Correlation between CMC and MIC with respect to Gram-positive bacteria.

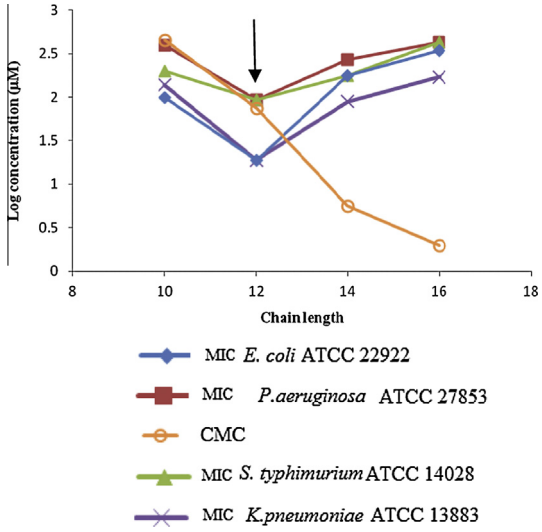


Fig. 7b. Correlation between CMC and MIC with respect to Gram-negative bacteria.

the surfactant molecule, stabilising it and making it more resistant to lysis [41,42].

The presence of the quaternary ammonium moiety was found to enhance the antibacterial activity of the L-Phenylalanine QUATS as compared to the hydrochloride salt due to the additional hydrophobic interactions between the alkyl groups attached to the nitrogen atom with the lipid of the bacterial membrane, and hence aiding to disruption. The cut-off effect was observed at a higher chain length (C_{14}) for the L-Phenylalanine QUATS compared to the hydrochloride salt of L-Phenylalanine esters (C_{12}) which we have reported earlier [20]. The increase in hydrophobic interaction caused by the alkyl groups attached to the nitrogen might be destabilising the membrane much more than the NH_3^+, hence leading to a greater free volume in the membrane. The free volume can therefore accommodate a longer chain length (C_{14}).

The compounds exhibited a strain-dependant action in which the Gram-negative species were more resistant than the Gram-positive bacteria. This was also observed with many other surfactants and is attributed to the presence of the cell wall lipopolysaccharide (LPS) and the outer membrane, two structures both absent in Gram-positive bacteria which could prevent the active compounds to reach the cytoplasmic membrane of the Gram-negative species [43].

The MIC of the quaternary ammonium surfactants were correlated with their CMCs in order to relate their activities with their micellar forming abilities (Figs. 7a and 7b).

Comparing the CMC and the MIC, it was found that these 2 parameters intersect each other at the cutoff point, as previously observed in the case of L-Phenylalanine ester hydrochloride series. In the case of Gram-positive bacteria, the MIC of the C_{10}–C_{14} was found to be below the CMC, while that of the C_{16} derivatives was above the CMC. The CMC line also intersects the MIC at the cut-off point in the case of Gram-negative bacteria. This could be explained by the fact that the antibacterial activities of these surfactants are primarily due to their presence as monomers and not to that of their micellar form, which was consistent to what we have reported previously [20].

6.5. Phospholipid binding assay

The phospholipid molecules present in bacterial membranes seem to be the target for quaternary ammonium surfactants. Phosphatidyl choline (PC) is the major membrane-forming phospholipid in cells. Although many prokaryotes lack PC, it can be found in significant amounts in membranes of rather diverse bacteria [44]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is considered to be a simple and well characterized model which has several advantages over more complex model systems [45]. DPPC has been used as model membrane to provide valuable information for understanding the mechanism of action of surfactant molecules as antibacterial agents [15,45,46]. Therefore, to provide more insight on the mechanism of the synthesised L-Phenylalanine QUATS on antibacterial activity, the binding studies of phospholipid (DPPC) with the C_{12}, C_{14} and C_{16} QUAT derivatives were carried out using the fluorescence probe technique. These QUATS (6b–8b) were chosen in order to relate their binding ability with phospholipid and their antibacterial activity. 1-Anilino-8-naphthalenesulfonate (ANS) and 1,6-diphenyl-1,3-5-hexatriene (DPH) were used as fluorescent probes.

ANS shows enhanced fluorescence intensity at 480 nm (excited at 380 nm) when bound to DPPC. ANS bind to phospholipid molecules in such a way that the anionic sulfonate group is orientated towards the hydrophilic head group of the lipid and the addition of cationic species capable to bind with the phospholipid molecule was found to bring about more fluorescence enhancement of the lipid-bound ANS [20].

The fluorescence of the DPPC bound ANS was further increased by the addition of the synthesized QUATS, suggesting the formation of QUATS–lipid–ANS complex in the ternary system.

From previous studies, the binding capacity of DPPC for ANS, n and the binding constant, K were found to be 0.0016 and $2 \times 10^6\ M^{-1}$ respectively. The value of A_b was found to be 5×10^8. Addition of the QUATS (6b–8b) to the ANS–DPPC system enhanced the fluorescence intensity of DPPC bound ANS in the order of 7b > 6b > 8b (Fig. 8).

The binding constants (K) and the binding capacity (n) in the presence of the QUATS were obtained from Eq. (2). A plot that illustrates the binding of ANS to DPPC in the presence of 7b is shown in Fig. 9.

Table 6 summarises the binding constant and binding capacity of the ANS–DPPC complex in the presence of the QUATS.

In general, the binding constant in the presence of the QUATS were greater than that of the binary ANS–DPPC system, suggesting that these surfactant–lipid binding induces a secondary binding between the probe and the lipid. The binding constant, K is higher than that previously observed with the phenylalanine ester hydrochloride derivatives showing that the QUATS have higher ionic attraction for the phospholipid molecule. The C_{14} derivative 7b showed the highest binding constant and the highest binding

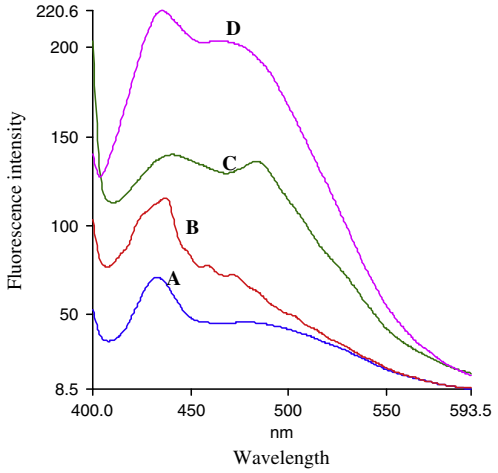


Fig. 8. Fluorescence intensity spectra solutions containing 5×10^{-8} M ANS and 1×10^{-3} M DPPC; A: no QUATS, B: 5×10^{-4} M of 8b, C: 5×10^{-4} M of 6b, D: 5×10^{-4} M of 7b.

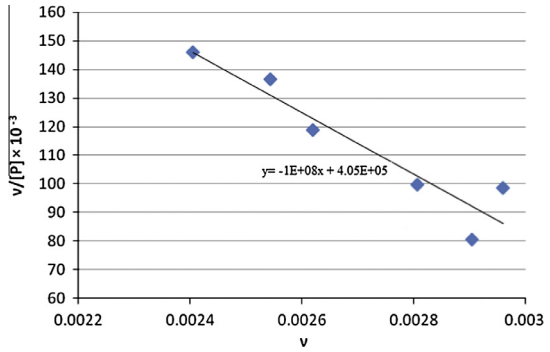


Fig. 9. Scatchard plot (Eq. (1)) for the binding of ANS to DPPC (2×10^{-5} M) at 25 °C in the presence of 7b; ν is the number of moles of bound ANS per mol of lipid; [P] is the free concentration of ANS.

Table 6
Effect of the different L-Phenylalanine QUATS on the binding of binary ANS-DPPC complex.

Compound	Chain length	A_t/A_b	K (M^{-1})	n
6b	12	4	8.0×10^7	0.001
7b	14	4	1.0×10^8	0.004
8b	16	4	3.0×10^7	0.002
CTAB	16	4	6.0×10^7	0.001
Streptomycin	–	4	9.0×10^6	0.002
Control[a]	–	–	2.0×10^6	0.002

[a] Binary system consisting of ANS and DPPC in the absence of the QUATS.

capacity. The binding of ANS to DPPC were also determined in the presence of a cationic surfactant CTAB, and a clinical antibiotic streptomycin, with no surfactant-like structure. CTAB was found to enhance the binding between ANS and DPPC showing that CTAB also binds to the phospholipid via electrostatic interaction. However, no significant binding enhancement between ANS to DPPC was observed in the presence of streptomycin, showing that this antibiotic has a low tendency to bind to negatively charged phospholipids.

DPH was used as probe to determine the hydrophobic interaction between the QUATS and the phospholipid. DPPC bound DPH shows enhanced fluorescence intensity at 440 nm (excited at 368 nm) and compounds capable to bind with the phospholipid

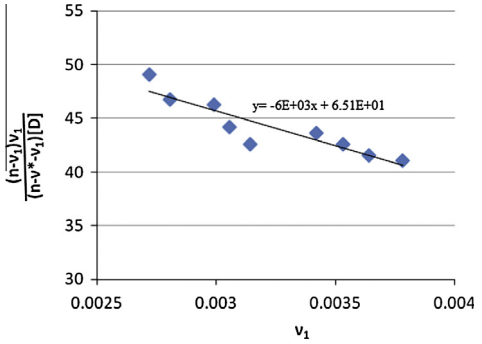


Fig. 10. Binding of 7b to DPPC measured by using DPH as probe (Eq. (4)).

through hydrophobic interactions are able to quench the fluorescence of the lipid–DPH solution. From previous studies on the binding of DPH to DPPC, the value of the binding constant K, and the binding capacity n from Eq. (2) were calculated to be $3 \times 10^6\ M^{-1}$ and 0.0323 respectively, which indicates a strong hydrophobic interaction between the probe and the lipid [20].

The L-Phenylalanine QUATS 6b–8b were able to quench the fluorescence of DPPC–DPH solution indicating a binding displacement of DPH by these surfactants which binds in close proximity to the DPH binding sites. The binding constant K_1 and binding capacity n_1 were obtained from Eq. (3). A plot that illustrates the competitive binding between 7b and DPH to DPPC (Eq. (4)) is shown in Fig. 10.

The binding constant and binding capacity is much lower in the presence of the surfactants 6b–8b compared to the binary DPH–DPPC (Table 7).

The surfactants were able to quench the fluorescence of the binary DPH–DPPC showing that these surfactants interact with the phospholipid via hydrophobic interactions in the order 7b > 6b > 8b (Fig. 11).

The C_{14} derivative 7b showed the lowest binding constant and binding capacity showing that this particular QUAT derivative was able to interact more with the lipid, and therefore was able to quench the fluorescence of DPH bound lipid much more than the other QUATS. The binding of DPH to DPPC was also determined in the presence of CTAB and streptomycin. The results showed that K_1 value decreases in the presence of CTAB, indicating that this surfactant was able to bind with DPPC by competing with DPH binding site, in the same manner to that of the phenylalanine QUATS. However, the phenylalanine QUATS were able to compete with DPPC much more than CTAB, due to a higher clog$P$ value (clog$P$ CTAB: 2.68; clog$P$ QUATS > 4). This implies that the phenyl groups in these amino acid surfactants render the QUATS more lipophilic and contribute to a certain extent to the hydrophobic interactions between the surfactant and the lipid. However, streptomycin did not show any significant binding displacement of the DPH-DPPC complex, showing that this antibiotic does not bind to DPPC via

Table 7
Effect of the different L-Phenylalanine QUATS on the binding of binary DPH-DPPC complex.

Compound	Chain length	K_1 (M^{-1})	n_1
6b	12	3.6×10^4	0.023
7b	14	6.5×10^3	0.010
8b	16	4.2×10^5	0.013
CTAB	16	1.5×10^5	0.014
Streptomycin	16	3.0×10^6	0.015
[a]control	–	3.0×10^6	0.032

[a] Binary system consisting of DPH and DPPC in the absence of the QUATS.

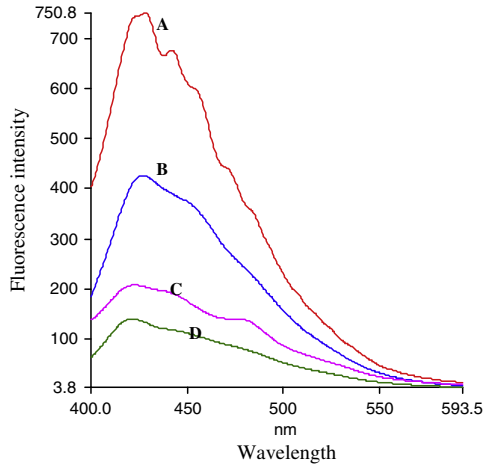


Fig. 11. Fluorescence spectra of solutions containing 5×10^{-8} M DPH and 2×10^{-5} M DPPC in the presence and absence of QUATS. A: no QUATS, B: 5×10^{-5} M of 8b, C: 5×10^{-5} M of 6b and D: 5×10^{-5} M of 7b.

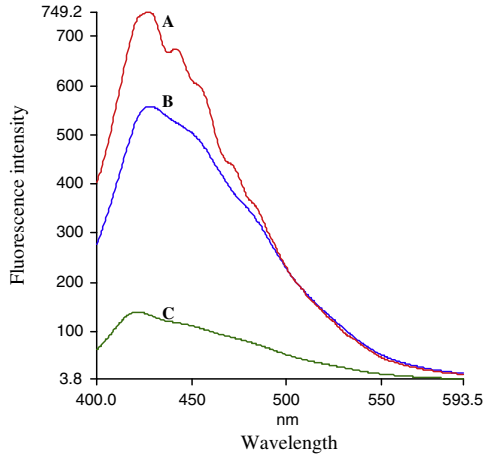


Fig. 12. Fluorescence spectra of solutions containing 5×10^{-8} M DPH and 2×10^{-5} M DPPC in the presence and absence of QUATS. A: no QUATS, B: 5×10^{-5} M of C_{14} L-Phenylalanine ester hydrochloride, C: 5×10^{-5} M of C_{14} L-Phenylalanine QUATS.

hydrophobic interactions. It was observed that the L-Phenylalanine QUATS were able to quench the fluorescence of DPPC-DPH solution much more than the L-Phenylalanine ester hydrochloride (Fig. 12) indicating a greater hydrophobic interactions between the QUATS and the phospholipids.

6.6. Hemolytic activity

Hemolysis by surfactant molecules is a process of great fundamental and practical importance [47]. Hemolytic activity of surfactants can also be used as a mean for their toxicological assessment [48]. Evaluation of the concentration that induces the hemolysis of 50% of the erythrocytes was determined. The L-Phenylalanine QUATS series showed moderate hemolytic activity with an increase in activity with increasing chain length of the hydrophobic tail (Table 8).

This behaviour is in line with those described for typical cationic surfactant [49]. The hemolytic activity is lower than those found for CTAB. Comparing the CMC with the hemolytic activity, the HC_{50} was found to be higher than the respective CMC value of the QUATS, except for 5b, whereby the HC_{50} value was much below its CMC value.

Table 8
Hemolytic activity of the different L-Phenylalanine QUATS.

Compound	HC_{50} (μM)
5b	120
6b	75
7b	35
8b	17
CTAB	5.5

Because the human red blood cell lacks internal organelles, the only way for the surfactant molecule to interact with it is via the cell membrane [50]. However, the lysis process is still unclear despite the numerous advances made in understanding the mechanism. The most accepted hypothesis is that surfactants are intercalated into the lipid bilayer of the cell membrane of the red blood cells, such that the hydrophobic chain lies within the hydrophobic core region of the bilayer while the hydrophilic head is located at the polar region of the membrane. Surfactant intercalation into membrane leads to changes in the membrane permeability that concludes with cell lysis [51]. Hemolysis also depends on the adsorption of the surfactant to components of the membrane surface which is influenced by electrostatic interaction [17].

For these L-Phenylalanine QUATS, the HC_{50} was found to be above the CMC, except for 5b, showing that hemolysis is induced above the micelle concentration rather than at monomer concentration. Different studies were performed to demonstrate a positive correlation between HC_{50} and CMC of surfactant; however, no clear conclusions were reported in the literature [52].

The selectivity of the QUATS for bacterial cell over mammalian cell was calculated by the ratio of their HC_{50} to their MIC values with respect to all the bacterial strains tested. The results are summarised in Table 9.

In general, the results showed a good selectivity of the compounds for bacterial cells. A relatively better selectivity was observed for Gram-positive bacteria. In this case, the MIC values of the L-Phenylalanine QUATS were much lower compared to their HC_{50} values. The antibacterial activities of these QUATS were found to increase at monomer concentration whereas the hemolytic activity occurs mostly at micellar concentration. In this way, these surfactants can act as antibacterials, yet non toxic compounds at a concentration lower than their respective CMCs, hence providing a good selectivity for bacterial cells.

6.7. Comparing the CMC, antibacterial and hemolytic activity of the L-Phenylalanine QUATS in the same medium

Maisuria et al. studied the antibacterial activity of di- and tricarboxyl dendritic amphiphiles in brain–heart infusion broth using the broth dilution method [53]. To assess the potential safety of

Table 9
Selectivities of L-Phenylalanine QUATS for bacterial cells over mammalian cells.

Microorganisms		Selectivity HC_{50}/MIC			
		5b	6b	7b	8b
Gram-positives	*S. aureus* (ATCC 29213)	1.3	11	10	2
	S. aureus (ATCC 25923)	4	10	10	1.4
	S. epidermidis (ATCC 12228)	4	13	8	1.4
	B. cereus (ATCC 10876)	0.9	8	6.7	1
	B. cereus (ATCC 11778)	0.8	5	6.7	1
Gram-negatives	*K. pneumoniae* (ATCC 13883)	0.9	4	0.4	0.1
	S. typhimurium (ATCC 14028)	0.3	0.8	0.1	0.04
	E. coli (ATCC 22922)	0.6	0.8	0.2	0.04
	P. aeruginosa (ATCC 27853)	1.2	4	0.2	0.05

Table 10
CMC, antibacterial and hemolytic activity of phenylalanine QUATS in TBS.

Compound	CMC (μM)	MIC (μM)		HC_{50} (μM)
		S. aureus (ATCC 25923)	*S. epidermidis* (ATCC 12228)	
5b	307	99	20	152
6b	28.3	19	15	92
7b	3.7	3.6	3.6	71
8b	1.0	17	170	66
CTAB	720	28	28	9.2

using these dendritic amphiphiles as drugs, measurements of micellar and hemolytic properties were conducted in the same medium (full-strength BHIB) that was used for antibacterial activity. In this study, the micellar, hemolytic and antibacterial data reported were intercorrelated to provide a relationship between micellization and biological properties. In view of providing a fair assessment of the relationship between CMC, MIC and HC_{50}, a common medium was chosen to measure all three parameters. As hemolysis could not be measured in distilled water due to lysis of red blood cells, measurements of all the three properties were made in TBS solution. TBS was chosen in order to emulate physiological conditions with a slightly alkaline pH (7.4) and an isotonic NaCl concentration (150 mM or 0.9% NaCl). The disc diffusion method was used for antibacterial evaluation of the phenylalanine QUATS where solutions of varying concentration of the compound dissolved in TBS. Table 10 summarises the CMC, MIC (with respect to two selected Gram-positive bacteria) and hemolytic activity of the phenylalanine QUATS.

The change in the ionic strength of TBS slightly changed the values of the different properties measured. The CMC of the phenylalanine QUATS in TBS were found to be lower than those measured in deionised water which is due to the effect of the dissolved NaCl that facilitates micellar formation. Similar effect was observed for CTAB (CMC in water: 1050 μM). The HC_{50} values of the QUATS in TBS were larger than those observed in saline solution, indicating that these compounds have a lower tendency to lyse red blood cells under these conditions. The MIC values of the QUATS dissolved in TBS with respect to *S. aureus* (ATCC 25923) and *S. epiderrmidis* (ATCC 12228) were found to be generally higher than those observed for solutions of the QUATS dissolved in water. This might be attributed to a lower solubility of these compounds in TBS than in water. However, the MIC values of 7b in both water and TBS were comparable. The relationship between the CMC, MIC and HC_{50} in TBS were comparable to the results obtained when these parameters were determined in different media, which showed that in most cases, hemolytic activities were governed by their micellar forms, while the monomers were responsible for their antibacterial effects (Fig. 13).

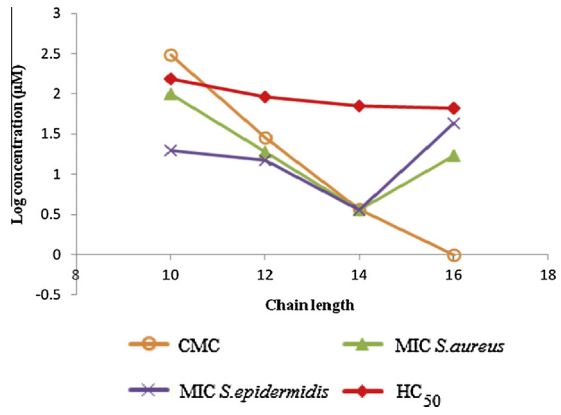


Fig. 13. Comparison of CMC, antibacterial and hemolytic activities in TBS.

7. Conclusion

The synthesised L-Phenylalanine QUATS were found to display good antibacterial activities, which were found to increase up to a certain chain length, above and below which activity decreases, exhibiting a cut-off effect. For Gram-positive bacteria, the cut-off point was observed at C_{14} while in the case of Gram-negative bacteria, the cut-off point was observed at a lower chain length (C_{12}). This difference between the two types of bacterial strains may be accounted by the difference in their cell wall structures. The L-Phenylalanine QUATS displayed relatively better antibacterial property with a higher cut-off point compared to the ester hydrochlorides. The study showed the monomers of the QUAT derivatives were responsible for their antibacterial activities while their hemolytic activities were primarily governed by their micellar form. The use of TBS as common medium showed similar interrelationship between micellisation, antibacterial and hemolytic activities. The binding studies carried out with phospholipid demonstrate that the QUATS bind with DPPC via both electrostatic and hydrophobic interactions. The study shows that these interactions have a direct effect on the antibacterial activities of the esters. Greater hydrophobic interactions were found between the QUATS and the phospholipid compared to the hydrochloride salt of phenylalanine, showing that the presence of the quaternary ammonium moiety accounts for further interactions between the surfactant and the lipid, giving rise to a better antibacterial activity. This study has given insights regarding the mode of action of the QUATS as antibacterial agents, which includes their interaction with phospholipid molecules in the bacterial cell membrane, giving rise to cell lysis.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2015.01.001.

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