Lipopeptidic Surfactants. II. Acidic and Basic Nα-Lauroyl-L-Arginine Dipeptides from Pure Amino Acids

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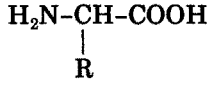
In this paper, the second part of a systematic study whose aim deals with the influence of the terminal amino acid side chain on the properties of Nα-lauroyl arginyl dipeptides is reported. Nα-lauroyl arginyl dipeptides that contain an acidic amino acid (glutamic acid) or a basic amino acid (lysine) as terminal amino acid have been prepared by peptide synthesis methods. These compounds have been synthesized as methyl esters (cationic surfactants) and free α-carboxylic acids (amphoteric surfactants), and their fundamental surfactant properties and antimicrobial activity have been evaluated. The properties of these compounds have been compared to the properties of the cationic monomer derivative methyl ester of Nα-lauroyl arginine and of the amphoteric monomer derivative Nα-lauroyl arginine reported earlier.

The compounds are soluble in water and show surface activity, although in the case of the amphoteric Nα-lauroyl arginyl dipeptide containing glutamic acid these two properties depend on the solution pH. The cationic Nα-lauroyl arginyl dipeptides are antimicrobial agents. However, only the amphoteric compound containing lysine may be considered antimicrobial.

KEY WORDS: Antimicrobial activity, lipo-amino acids, long-chain Nα-acyl dipeptides, surfactants.

Amino acids are the basic structural units of proteins, and they are linked to each other through peptide bonds. Twenty different α-amino acids are commonly present in proteins and their general formula is (R = side chain):



Based on the ionic nature of the side chain the amino acids can be classified as neutral amino acids with a non-ionic side chain (*i.e.*, glycine, phenylalanine, serine, tyrosine), acidic amino acids with a negatively charged side chain (*i.e.*, glutamic acid, aspartic acid) and basic amino acids with a positively charged side chain (*i.e.*, lysine, arginine) (1).

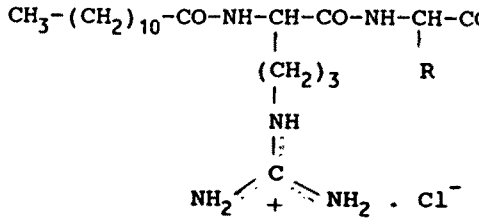
Lipo-amino acid surfactant molecules that contain an amino acid as the hydrophilic part and a long chain as the hydrophobic part constitute an interesting class of biocompatible compounds. Considering the chemical structure of an amino acid, the chain can be introduced through an acyl, ester, amide or alkyl linkage (2,3). Of these lipo-amino acid surfactants, the salts of long-chain Nα-acyl amino acid derivatives are the most widely studied, and they are prepared by acylation of the α-amino group of an α-amino acid with fatty acids (4,5).

Studies on preparation, structure and properties of a series of long-chain Nα-acyl dibasic amino acid derivatives have been published by our research team. Two different types of surfactant compounds were obtained—cationic surfactants by esterification of the α-carboxyl group and amphoteric surfactants with a free α-carboxyl group (6–8).

The cationic surfactants, particularly the methyl and ethyl esters of Nα-lauroyl arginine and Nα-lauroyl lysine, were highly soluble in water and showed good surface activity and high antimicrobial power (7). By contrast, the corresponding amphoteric Nα-lauroyl arginine and Nα-lauroyl lysine were not soluble in water and, consequently, did not show any surface or antimicrobial activity. This insolubility was attributed to the formation of an internal salt between the α-carboxylic group and the basic group of the side chain of the amino acid (8).

To obtain amino acid-based amphoteric surfactants that are soluble in water with antimicrobial activity, we planned a systematic study on Nα-lauroyl arginyl dipeptides prepared by the addition of a second amino acid to the amphoteric Nα-lauroyl arginine compound (LAH) (6). Four amino acids with different side chains were chosen as the terminal amino acid: Glycine and phenylalanine (neutral amino acids), glutamic acid (acidic amino acid) and lysine (basic amino acid). In a recent paper (9) we reported the synthesis and fundamental properties of amphoteric and cationic Nα-lauroyl arginyl dipeptides containing neutral amino acids, such as glycine and phenylalanine. These compounds were highly soluble in water, and they showed good surface activity and high antimicrobial activity, whether they were cationic or amphoteric.

The present communication is concerned with the synthesis and properties of Nα-lauroyl arginyl dipeptides containing acidic or basic amino acids, such as Nα-lauroyl arginyl glutamic acid and Nα-lauroyl arginyl lysine. These compounds are prepared as methyl ester (cationic surfactants) and free acid (amphoteric surfactants) hydrochloride salts. The general formula is as follows (Scheme 1):



SCHEME 1

Table 1 shows the name, molecular structure and abbreviations of the surfactants synthesized for this study.

Both communications, the previous (9) and the present, constitute a comprehensive study on the influence of the nature of terminal amino acid side chain on solubility, surface activity and antimicrobial properties of Nα-lauroyl arginyl dipeptides with the aim of obtaining amphoteric lipopeptide surfactants with antimicrobial properties. As in the previous communication (9), special emphasis has

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been placed on the amphoteric compounds, which were compared with the water-insoluble monomer Nα-lauroyl arginine (LAH) to show the changes produced by the incorporation of a second amino acid. Cationic Nα-lauroyl arginyl dipeptides also were compared with the methyl ester Nα-lauroyl arginine (LAM) reported earlier (6).

EXPERIMENTAL PROCEDURES

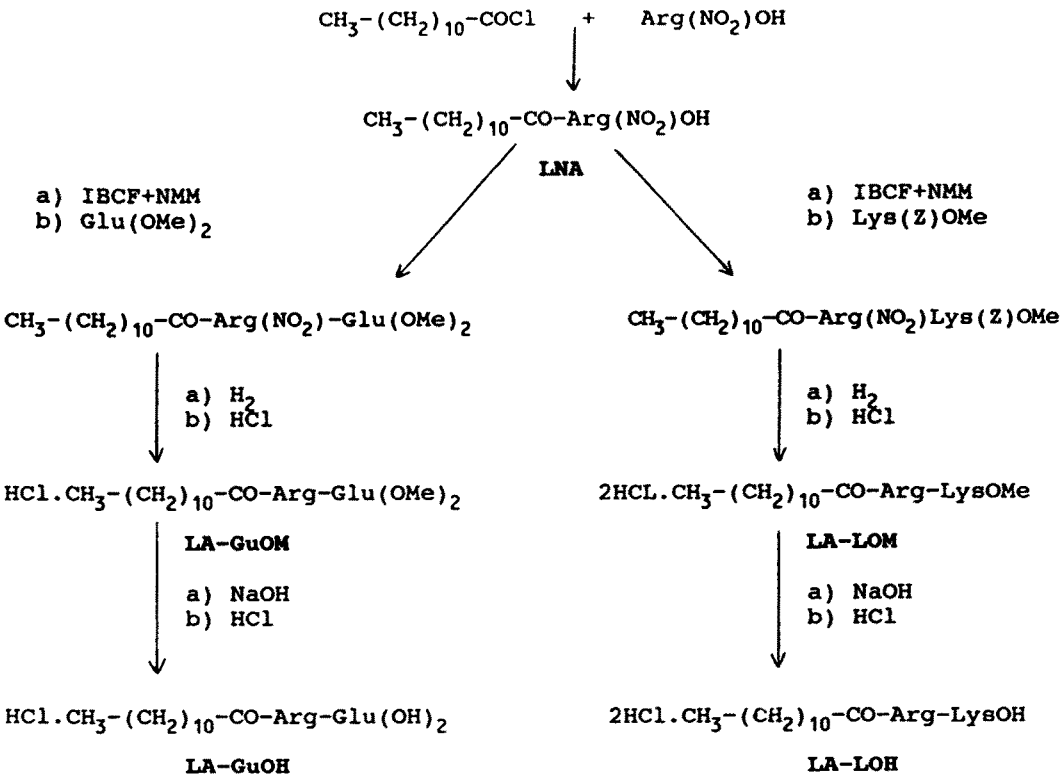
Synthetic method. LA-GuOM, LA-LOM, LA-GuOH and LA-LOH were prepared at laboratory scale in accordance with Scheme 2. Synthetic grade amino acids [L-arginine, L-glutamic acid, L-lysine] and lauroyl chloride were supplied by Merck (Darmstadt, Germany). Nα-lauroyl-L-nitro arginine [LNA], Nε-carbobenzoxy-L-lysine methyl ester [Lys(Z)OMe] and L-glutamic dimethyl ester [Glu $(OMe)_2$] were obtained in our laboratory according to published methods (10–12).

General reagents and solvents were of analytical grade or higher purity and supplied by Merck. Dimethylformamide (DMF) was dried over a 4-Å molecular sieve for about 8 h in a nitrogen atmosphere. The homogeneity of compounds was checked by thin-layer chromatography on aluminium plates (Kieselgel G, Merck). The solvent systems were (A) chloroform/methanol/acetic acid (8.5:10:5); and (B) chloroform/methanol (7:3). Ninhydrin (13), Sakaguchi (14) and chlorine-toluidine (15) developer solutions were used for qualitative analysis of free amino groups, the guanidyl group of arginine and peptide bonds, respectively.

Optical rotations were measured with a 141 Perkin-Elmer Spectropolarimeter (Norwalk, CT). The pure products were characterized by their elemental analyses, their ^1H nuclear magnetic resonance (NMR) [in a Bruker WP 780 SY, (80 MHz); Bruker, Karlsruhe, Germany] and their amino acid analyses (Biotronik LC-5001, location). Fast-atom bombardment (FAB)-mass spectra of the lipopeptides were determined by a MS9-VG updated system equipped with a VG II-250 unit. The absence of racemization was checked by chiral gas chromatography (GC) (16).

HCl. Nα-lauroyl-Arg-Glu(OMe)$_2$ [**LA-GuOM**]. A solution of 10 g of LNA (10) and an equimolar amount of N-methyl morpholine (NMM) in dry DMF (70 mL) was stirred with cooling at −15°C. Next, 3.3 mL of isobutyl chloroformate (IBCIF) was added dropwise. After the addition, the mixture was stirred for 5 min at a constant temperature of −15°C, and then a cold solution of 5.3 g of Glu $(OMe)_2$ (12) in 50 mL of DMF was added. Stirring was continued for 1 h at −15°C, and then at room temperature for 20–24 h. The resulting mixture was concentrated to dryness under vacuum. The residual paste was washed with water, ice-cold saturated aqueous sodium bicarbonate, water, 0.1N HCl and water. A white solid corresponding to Nα-lauroyl-L-nitro Arg-Glu $(OMe)_2$ was obtained (yield 90%).

The nitro deprotection was carried out as follows: 10 g of Nα-lauroyl nitro Arg-Glu$(OMe)_2$ was dissolved in 150 mL of glacial acetic acid containing 7 mL of 1,4 cyclohexadiene and 10 g of Palladium on activated charcoal (10% Pd). The mixture was hydrogenated with H_2 under normal pressure for 10 h. The catalyst was filtered off, rinsed with acetic acid and water. The filtrates were pooled and concentrated under reduced pressure to



SCHEME 2

dryness. The residue was dissolved in 100 mL of a methanol solution of 0.5N HCl and ether was added until precipitation of an oil corresponding to a crude HCl.Nα-lauroyl-Arg-Glu $(OMe)_2$. Portions of 1 g of the above oil were dissolved in 2 mL methanol and chromatographed on a Merk Silica Gel 60 (400–230 mesh) column (75 × 2.0 cm) by elution with chloroform/methanol mixtures of different polarity. Pure HCl.Nα-lauroyl-Arg-Glu $(OMe)_2$ was obtained in intermediate fractions, which were pooled and lyophylized. In this way a homogeneous hygroscopic powder corresponding to pure LA-GuOM was obtained (yield of the chromatography, 70%). The characteristics of the compound are indicated in Table 1.

HCl. Nα-Lauroyl-Arg-Glu OH [**LA-GuOH**]. To a cold solution of 2.5 g LA-GuOM in 10 mL methanol, 35 mL of 0.5N NaOH was added. The solution was stirred for 3 h at room temperature. HCl (1N) was added until pH 3, and then the desired LA-GuOH was precipitated at 5°C. After filtration the precipitate was washed three times with cold water and dried in a dessicator (yield 50%). The characteristics of this pure hygroscopic amphoteric surfactant are indicated in Table 1.

2HCl. Nα-Lauroyl Arg-Lys OMe [**LA-LOM**]. This compound was prepared from 10 g LNA and 7.4 g Lys(Z)OMe (11) by the mixed anhydride method under the same experimental conditions as described for LA-GuOM. After the coupling reaction, 10 g Nα-lauroyl-nitro-Arg-Lys(Z)OMe were dissolved in 150 mL glacial acetic acid containing 50 mL formic acid and 10 g Palladium on activated charcoal (10% Pd). The mixture was hydrogenated with H_2 and a similar procedure as described for LA-GuOM was carried out. An oil corresponding to crude 2HCl Nα-lauroyl-Arg-Lys OMe was obtained (yield 90%). This oil was purified by column chromatography as above. The yield of the column chromatography was about 70%. The characteristics of the pure compound are shown in Table 1.

2HCl. Nα-Lauroyl-Arg-Lys OH [**LA-LOH**]. Saponification of LA-LOM was carried out under the same experimental conditions as for LA-GuOH. The yield of this reaction was 65%. The characteristics of pure LA-LOH are indicated in Table 1.

Physicochemical properties and antimicrobial activity. Surface tension (δ), critical micellar concentration (CMC), and area per molecule (A_m) were determined by standard methods described previously (9). In the present study, the solutions were allowed to equilibrate from 4–10 h at 25°C until stabilization. Constant ionic strength was not maintained. Minimum inhibitory concentration (MIC) data were measured under identical conditions and against the same microorganisms as described earlier (9).

RESULTS AND DISCUSSION

The synthesized compounds and their characteristic properties are listed in Table 2. The synthesis of cationic compounds was accomplished by coupling Nα-lauroyl-L-nitro arginine and the appropriate methyl ester derivative of glutamic acid and lysine through the liquid-phase methodology. Temporary protection of the guanidino group of arginine and the Nε amino group of lysine was carried out by using the nitro group (NO_2) and the carbobenzoxy group (Z), respectively. The coupling reaction was achieved in a yield of 90% by the mixed anhydride method (17) with isobutyl chloroformate at −15°C. The protection of arginine and lysine side chains was effective, and no undesirable by-products were obtained.

The deprotection of the NO_2 and Nε-Z groups was carried out by catalytic hydrogenation in the presence of a proton donor, which gave a high yield. This combined deprotection was simultaneous for the Nα-lauroyl-nitro Arg-Lys(Z)OMe compound. Several strategies have been described to achieve this deprotection reaction (18). In an earlier attempt to achieve this reaction, hydrogenation over Palladium in glacial acetic acid of Nα-lauroyl-nitro Arg-Glu $(OMe)_2$ and Nα-lauroyl nitro Arg-Lys(Z)OMe removed the nitro and the carbobenzoxy groups at a yield of 40%, with many by-products.

The presence of a proton donor, 1,4-cyclohexadiene (19) in the hydrogenation medium of Nα-lauroyl-nitro-Arg-Glu$(OMe)_2$ and formic acid (20) in the medium of Nα-lauroyl-nitro Arg-Lys(Z)OMe, increased the deprotection reaction to 70% and 90%, respectively. The deprotection of the nitro group of Nα-lauroyl-nitro Arg-Glu$(OMe)_2$ was not tested in the presence of formic acid. Because the crude hydrochloride salts of methyl ester Nα-lauroyl arginine dipeptides could not be crystallized in the usual manner, the purification of these compounds was carried out by column chromatography. Although there was a retention of 30% of the product in the silica gel during the chromatography, the effectiveness of this technique was demonstrated by the analysis of the final compounds (see Table 1).

TABLE 1

Synthesized Surfactants and Their Abbreviations

	R	R′	Abbreviation
Amphoteric compounds			
Nα-lauroyl-L-arginyl-L-glutamic chloride	$-(CH_2)_2-COOH$	-H	LA-GuOH
Nα-lauroyl-L-arginyl-L-lysine dichloride	$-(CH_2)_4\overset{\oplus}{N}H_3$	-H	LA-LOH
Cationic compounds			
Methyl ester of Nα-lauroyl-L-arginyl-L-glutamic chloride	$-(CH_2)_2-COOCH_3$	$-CH_3$	LA-GuOM
Methyl ester of Nα-lauroyl-L-arginyl-L-lysine dichloride	$-(CH_2)_4\overset{\oplus}{N}H_3$	$-CH_3$	LA-LOM

TABLE 2

Characteristic Properties of the Synthesized Compounds

Compound	m.p. (°C)	Rf	$[\alpha]^{25}$ c = 1% MeOH	Elemental Analysis Calc.	Elemental Analysis Found	FAB-MS m/e $[M^+H]^+$	AA analysis molar ratio
LA-GuOH	Hygroscopic	0.80[a]	−2.54	C 52.97 H 8.44 N 13.43	52.42 8.20 12.80	486	Glu/Arg: 1.004
LA-LOH	Hygroscopic	0.54[a]	−1.42	C 51.70 H 8.90 N 15.08	51.54 8.40 14.30	485	Lys/Arg: 0.97
LAGuOM	Hygroscopic	0.88[a] 0.53[b]	−1.59	C 54.59 H 8.73 N 12.74	54.06 9.40 12.59	514	Glu/Arg: 0.96
LA-LOM	Hygroscopic	0.47[a] 0.25[b]	−1.85	C 52.63 H 9.10 N 14.73	52.70 10.00 14.20	499	Lys/Arg: 1.08

[a]Chloroform/methanol/acetic acid (8.5:10:5). [b]Methanol/chloroform (3:7).

The purity of the obtained compounds was confirmed by amino acid analysis and FAB-mass spectrometry. The amino acid analysis of LA-GuOM and LA-LOM gave a purity of 98 ± 0.5%, and their FAB-mass spectra showed only one mass peak corresponding to the molecular ion. The amphoteric compounds LA-GuOH and LA-LOH were obtained as hydrochloride salts by subsequent saponification of the cationic salts. Although this reaction was complete, the yield of the reaction decreased due to the washes of the final product with cold water. Racemization was not detected by GC, and the analysis of these compounds was acceptable. Likewise, the ^1H NMR spectra of all compounds were in accordance with their proposed structures.

Table 3 shows the water solubility up to 25 wt%, tested at different pH values at room temperature. In the pH range of 3.0–9.0, all compounds display good water solubility except LA-GuOH, which is soluble at pH ≤ 3.0 or pH ≥ 9.0. The insolubility of LA-GuOH in this pH range could be attributed to the capability of this molecule to form an intramolecular guanidine-carboxylate interaction between the side chains of arginine and glutamic acid, thus producing a water-insoluble complex in which only carboxylate anions remain free.

According to the spectroscopy studies of Lancelot *et al.* (21) for several dipeptide derivatives containing arginine and glutamic or aspartic acid, the following structural model could be proposed to explain the insolubility of LA-GuOH at this pH range (see Fig. 1). In this model the positive charge of the arginine side chain could be neutralized by the γ-carboxylate anion of glutamate, whose two oxygen atoms could form hydrogen bonds with two NH groups of arginine. This interaction could disappear at pH ≤ 3.0 or pH ≥ 9.0, where the γ-carboxylate anion is protonated or the positive charge strength of guanidinium is decreased.

TABLE 3

Water Solubility of Synthesized Compounds at 25% wt and Room Temperature

	pH 3.0	pH 4.0	pH 6.0	pH 8.0	pH 9.0
LA-GuOH	S[a]	I[b]	I	I	S
LA-LOH	S	S	S	S	S
LA-GuOM	S	S	S	S	S
LA-LOM	S	S	S	S	S

[a]Soluble. [b]Insoluble.

The conformational studies of Lancelot *et al.* (21) for several dipeptide models indicated that the Arg-Glu sequence is the most appropriate for such an interaction, in which the γ-carboxylic group of glutamic acid and the guanidinium group of arginine could have a conformation where the planar guanidinium group and the carboxilic group are coplanar. However, no intramolecular interaction between the guanidinium group of arginine and the terminal α-carboxylic group in the dipeptide models was observed. Therefore, on the basis of these models, the water solubility of the amphoteric compound LA-LOH and the amphoteric Nα-lauroyl arginyl dipeptides containing glycine or phenylalanine as second amino acid (9) could be explained.

To determine the influence of the terminal amino acid side chain on the physicochemical properties of these Nα-lauroyl arginyl dipeptides, we measured the CMC, surface tension and area per molecule for the amphoteric compounds (LA-GuOH, LA-LOH) and for the cationic compound (LA-GuOM, LA-LOM) at 25°C. The aqueous solutions for the LA-GuOH and LA-GuOM determinations were prepared at pH 3.0 by the addition of dilute HCl. Table 4 shows the physicochemical data for the synthesized compounds. For the sake of comparison, physicochemical data for the amphoteric Nα-lauroyl arginine (LAH) and for the cationic methyl ester of Nα-lauroyl-arginine (LAM) described previously are also indicated.

From the results of Table 4 we may conclude that both the amphoteric Nα-lauroyl-arginyl dipeptides and the cationic methyl ester Nα-lauroyl arginyl dipeptides behave as surfactants. The introduction of a second basic amino acid to the amphoteric Nα-lauroyl arginine dramatically changes its surface activity and produces a water-soluble amphoteric surfactant. At a suitable pH the amphoteric

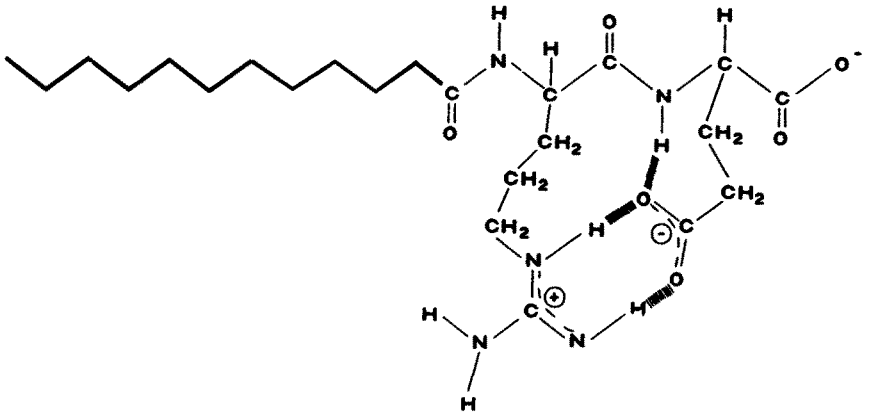


FIG. 1. Proposed conformation for LA-GuOH.

TABLE 4

Critical Micellar Concentration and Surface Properties of Synthesized Compounds

	CMC (mmolar)	Surface tension at CMC (mNm^1)	Area per molecule ($nm^2 \times 10^2$)
Amphoteric compound			
LA-GuOH	0.25	34	56
LA-LOH	1.2	30	49
LAH[a]	—[b]	—[b]	—[b]
Cationic compound			
LA-GuOM	0.70	34	65
LA-LOM	1.5	35	51
LAM[a]	5.8	32	60

[a]Reference 6. [b]Insoluble in water.

LA-GuOH also is soluble in water and shows surface activity. Regarding the cationic derivatives, it should be noted that by increasing the hydrophylic part of the LAM by one more amino acid (acidic or basic), an important reduction of the CMC is produced. Both in the amphoteric and cationic surfactants the compound containing glutamic acid as terminal amino acid exhibits smaller CMC values.

All compounds produced practically the same decrease in the surface tension of water, and only small differences in the A_m of the amphoteric compounds are observed. However, by comparing the A_m values of the cationic surfactants, more significant differences can be observed, such as LA-GuOM being higher than LA-LOM and LAM. Nα-lauroyl-arginyl dipeptides containing glycine and phenylalanine, particularly the latter, presented A_m values above 70 $nm^2 \times 10^2$ (9).

The antimicrobial activity of all synthesized compounds was established by estimating their corresponding minimum inhibitory concentration (MIC) values (in μg/mL) at pH 7.0 against Gram-positive and Gram-negative bacteria. The MIC values for the compounds are given in Table 5. Aqueous solutions of the antimicrobial agents in a concentration range of 0.5–256 μ/mL were prepared for the MIC determination of all compounds except for the LA-GuOH, where 0.1 N HCl solution prepared in methanol was used as solvent. The same solvent was used for the MIC determination of the amphoteric compound Nα-lauroyl arginine (LAH) (6).

Table 5 shows that the cationic surfactants LA-GuOM and LA-LOM showed antimicrobial activity, although the effectiveness of inhibiting the growth of bacteria decreases in the order of LAM > LA-LOM > LA-GuOM. The MIC results of LA-LOM show that the resulting compound is less active than LAM, despite having a more cationic character. The antimicrobial activity of the methyl ester of Nα-lauroyl-arginyl dipeptides containing glycine and phenylalanine (9) was much higher than that of LA-LOM, their activity being similar to that of LAM. This activity could be attributed to the ability of the guanidinium group to interact with polyanionic components of the cell surface, due to their cationic condition. A weakness of the cell wall would occur, allowing the surfactant to pass through the membrane, thus preventing microbial growth. However, the amphoteric compounds reported here were practically inactive, particularly the compound containing glutamic acid. The inactivity of the LA-GuOH could be attributed to a reduction in the interaction between the guanidinium basic group of arginine with specific anionic sites in the cell membrane because of the formation of intramolecular guanidinium carboxylate interactions, as in the case of the LAH molecule. By contrast, the antimicrobial activity increased dramatically in the amphoteric Nα-lauroyl-arginyl dipeptides containing glycine or phenylalanine (9).

The following conclusions may be drawn from the present study: i) Pure Nα-lauroyl arginyl dipeptides, such as methyl esters (cationic surfactants) or free carboxylic acids (amphoteric surfactants) containing acidic or basic amino acids give good yields by the application of classical methods for peptide synthesis. ii) The addition of a basic amino acid (such as lysine) to the water-insoluble amphoteric derivative Nα-lauroyl-arginine changed its water solubility. Nevertheless, the addition of an acidic amino acid, such as glutamic acid, to Nα-lauroyl-arginine produced an amphoteric compound that was insoluble in water in the pH range 3.0–9.0. iii) The acidic or basic nature of the second amino acid had no important effect

TABLE 5

Minimum Inhibitory Concentration (μg/mL)

Bacteria	Amphoteric compounds			Cationic compounds		
	LAH[a]	LA-GuOH	LA-LOH	LAM[a]	LA-GuOM	LA-LOM
Gram-positive						
Candida albicans (CCM)	R[b]	R	R	32	128	128
Staphylococcus epidermidis (ATCC 12228)	R	R	R	8	R	32
Streptococcus faecalis (ATOC 10541)	R	R	R	16	R	64
Corynebacterium agropyri (CCM)	R	R	32	8	32	32
Bacillus subtilis (ATCC 6623)	R	R	128	8	R	32
Bacillus pumilus (CCM)	—	R	128	—	R	32
Micrococcus lutcus (ATCC 10240)	R	R	128	16	64	32
Micrococcus surantiacus (ATCC 11731)	R	R	128	16	128	32
Gram-negative						
Alcaligenes faecalis (ATCC 8750)	R	R	R	R	R	R
Escherichia coli (ATCC 10536)	R	R	32	16	128	32
Klebsiella pneumoniae (ATCC 13883)	R	R	R	R	R	R
Citrobacter freundii (ATCC 22636)	R	R	R	R	R	R
Serratia marcescens (ATCC 13880)	R	R	R	R	R	R
Pseudomonas seruginosa (ATCC 10145)	R	R	R	128	R	R
Salmonella typhimurium (ATCC 14028)	R	R	R	32	R	R
Bordetella bronchiseptica (ATCC)	R	R	R	16	R	R

[a]Reference 6. [b]Resistant, MIC > 128.

on surfactant behavior, regardless of whether they were cationic or amphoteric surfactants. iv) Amphoteric Nα-Lauroyl arginyl dipeptide surfactants containing glutamic acid or lysine were not antimicrobial agents. However, the cationic Nα-Lauroyl arginyl dipeptides of glutamic acid or lysine were antimicrobial agents, particularly the dipeptide derivative containing lysine.

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