Synthesis and Properties of N^{α}-Lauroyl-L-Arginine Dipeptides From Collagen

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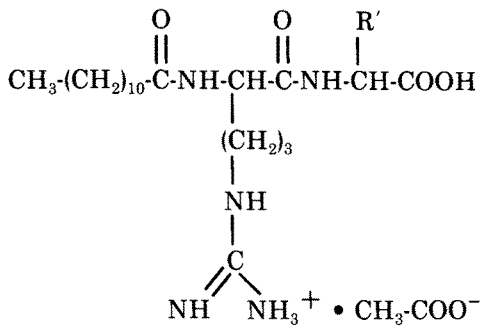
In order to obtain protein-based amphoteric surfactants with antimicrobial properties, N^{α}-lauroyl arginine dipeptides have been prepared by condensation between N^{α}-lauroyl arginine and amino acids which come from a collagen hydrolysate. Some surfactant properties and the minimum inhibitory concentration against Gram positive and Gram negative bacteria have been evaluated. All types of compounds presented a surfactant-like behavior. Furthermore, the compounds described in this paper may be considered as mild preservative or protective substances.

The surfactant and antimicrobial properties of long chain N^{α}-acyl amino acids derived from pure amino acids or protein hydrolysates have been studied by many authors (1, 2).

Previous to this communication and in order to obtain amphoteric surfactant, a series of long chain N^{α}-acyl amino acid derivatives was synthesized in our laboratories. They were prepared from capric, lauric and myristic acids and arginine, lysine, N^{ε}-trimethyl lysine and homologues. The α-carboxyl group was blocked by an alkyl ester or unblocked in the form of a sodium salt or a free acid (3). We found that the amphoteric derivatives (with the α-carboxyl group free) were not soluble in water. On the contrary, the ester derivatives, particularly the methyl or ethyl ester of N^{α}-lauroyl-arginine, were very soluble in water, good surfactants and good antimicrobial agents.

For this reason and in order to obtain protein-based amphoteric surfactants with antimicrobial properties, the present work deals with the synthesis of new kinds of long chain N^{α}-acyl arginine dipeptides obtained by condensation between N^{α}-lauroyl arginine and amino acids from a collagen hydrolysate, and the surfactant and antimicrobial properties of these synthesized dipeptides.

Two types of condensation products were prepared: condensate I and condensate II. The chemical structure of the main component of condensate I corresponds to Formula I, and was N^{α}-lauroyl dipeptides.



Formula I

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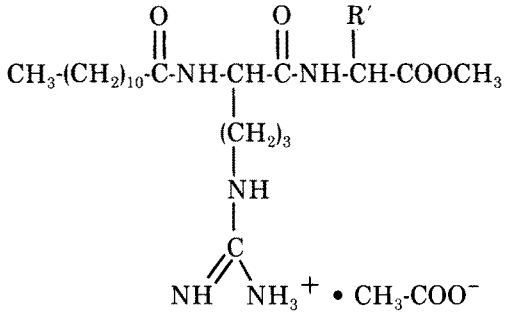
Formula II. In this case the collagen hydrolysate was esterified before the condensation was carried out.



Formula II

R′ residue of amino acid coming from collagen hydrolysate

EXPERIMENTAL PROCEDURES

General reagents were of analytical grade or higher purity. The solvents used were of technical grade. Dimethylformamide (DMF) was dried over a 4 Å molecular sieve for about eight hr under nitrogen. L-Arginine was obtained from Merck (synthetic grade). The protein used was collagen from waste leather trimmings.

The homogeneity of substances was checked by TLC using aluminum plates (Klieselgel-^{G}, Merck, Darmstadt, FRG). The solvent systems were chloroform/methanol/acetic acid (8.5:10.5) and n-butanol/acetic acid/water (4:1:5). Ninhydrin, sakaguchi and Cl_2/o-tolidine were used as developer solutions.

Melting points were measured in a Kofler (Riechert, Vienna, Austria), and they were uncorrected. Optical rotations were measured on a 141 Perkin-Elmer spectropolarimeter. Proton magnetic resonance (1H-NMR) spectra were measured on a Bruker WP 80 S and Spectrometer (80.15 MHz). Amino acid analyses were performed with a Beckman 119 C analyzer.

PREPARATION OF COLLAGEN HYDROLYSATES

As free amino acids (R=H). Waste leather trimmings were hydrolyzed with 6N HCl at 105 C for 24 hr (4). The hydrolysate was used without further purification. The amino acid composition of the hydrolysate is indicated in Table 1.

As methyl ester amino acids ($R=CH_3$). Dry protein hydrolysate (1 g) was suspended in 50 ml of absolute methanol, and 0.41 ml of concentrated HCl was added. The reaction mixture was stirred at room temperature for four days (5). About 70% esterification was obtained (Table 1).

TABLE 1

Amino Acid Composition (μmol/g) of Collagen Hydrolysate, Esterified Collagen Hydrolysate, Condensate I and Condensate II

Amino acid	Collagen hydrolysate	Esterified collagen hydrolysate	Condensate I	Condensate II
Histidine	30	11	-	-
Lysine	130	36	22	-
Arginine	271	65	817	875
Asparagine	284	32	39	58
Threonine	66	41	10	12
Serine	204	81	25	31
Glutamine	417	38	66	81
Proline	680	165	111	147
Glycine	1762	472	276	339
Alanine	704	185	130	113
Valine	66	0	12	17
Methionine	34	9	10	-
Isoleucine	87	54	15	15
Leucine	171	53	31	27
Tyrosine	30	32	3	3
Phenylalanine	70	23	13	12
Hydroxyproline	566	138	115	146
Hydroxylysine	32	9	20	-

PREPARATION OF N^{α}-LAUROYL-L-NITROARGININE

To an ice-cooled solution of one g (4.6 mm) of L-nitroarginine (6) and 9.14 ml of 0.5 N NaOH, 1.09 ml (4.6 mm) of lauroyl chloride and 4.6 ml of 1N NaOH were added simultaneously, maintaining the pH between 9 and 10. The mixture was stirred for several hr at 0 C and a white solid was obtained. Then, the dry solid was washed successively with diethyl ether, HCl solution and water, and finally dried over P_2O_5 in vacuo. The N^{α}-lauroyl-L-nitroarginine thus obtained was recrystallized from a mixture of ethanol and water. Yield, 41.53%; melting point, 177-179 C; $[\alpha]^{D}$ = 3.6 ± 0.5 (c=1, MeOH); anal. calcd for $C_{18}H_{35}N_5O_5$: C 53.86, H 8.73, N 17.46; found: C 54.23, H 8.78, N 17.19.

PREPARATION OF N^{α}-LAUROYL-L-ARGININE DIPEPTIDES AS FREE ACID OR AS METHYL ESTER DERIVATIVES

N^{α}-Lauroyl-L-nitroarginine (0.8 g) was dissolved in dry DMF (40 ml) and then 0.22 ml of N-methylmorpholine was added. The mixture was cooled to -15 C and an equimolar amount of isobutylchloroformate was added slowly. The resulting mixture was then stirred for 90 seconds with cooling. A suspension of 0.300 g of collagen hydrolysate (either as free amino acid or methyl ester amino acids) plus 0.25 ml of N-methylmorpholine and 40 ml of DMF was then added. Stirring was continued for one hr at -15 C and for four hr at room temperature. The mixture was evaporated to dryness in vacuo and the residue dissolved in ethyl acetate. The solution was washed several times with water, dried over sodium sulphate and evaporated to dryness. The N^{α}-lauroyl-L-nitroarginine dipeptides, either as free acid or methyl ester derivatives, were thus obtained as an oil which did not crystallize from several mixtures of solvents. This oil did not contain either free amino acids or N^{α}lauroyl-nitroarginine. The N^{α}-lauroyl-L-arginine dipeptides as acetate salts (condensate I and II) were obtained by hydrogenating, over 0.200 g of Pd/C, a solution containing 0.571 g of N^{α}-lauroyl-L-nitroargine dipeptides, 15 ml of methanol and 1.5 ml of glacial acetic acid for 8 hr. The desired products I or II were obtained in oil form from a mixture of methanol diethyl ether. The reaction products were not purified. The amino acid compositions of both condensates are indicated in Table 1.

PHYSICOCHEMICAL AND OTHER PROPERTIES

Surface tension. The surface tension values were measured at room temperature by the Du Noüy method (7), with a Lauda Tensiometer.

Critical micellar concentration. The critical micellar concentration (CMC) was determined by means of surface tension isotherms at room temperature and by the conductivity method (3).

Antimicrobial activity. The minimum inhibitory concentration (MIC) was determined by a dilution test in Müeller-Hinton agar medium, against Gram positive and Gram negative bacteria and one yeast.

The dilution test (8) was performed to determine the minimal concentration of an antimicrobial agent required to inhibit microbial growth. Serial dilutions of the antimicrobial agent were inoculated with the organism and incubated. The MIC is the lowest concentration without visible growth.

Preparation of medium. Solutions of antimicrobial agent in a concentration range of 0.5-256 μg/ml were prepared. One volume of each dilution of antimicrobial agent was added to nine volumes of Müeller agar and put in different plates. The pH of this medium was in the range of 7.2-7.4. Control plates containing no antimicrobial agent also were prepared.

Preparation of inoculum. Portions of four or five discrete colonies representative of the microorganisms

to be tested were inoculated into four or five ml of a saline solution (0.1% NaCl) and adjusted to the turbidity of the barium sulfate standard. A 1:20 dilution was then prepared in saline solution for inoculation on the agar containing antimicrobial agents.

Inoculation of medium. The agar surface of the plates containing the dilutions of antimicrobial agents and the control plate were spot inoculated with a loop calibrated to deliver one to two μl. Inoculated agar plates were then incubated at 37 C for 24 hr.

Testing results. The MIC was determined by comparing microbial growth in the presence of antimicrobial agents with the controls. The MIC represents the lowest concentration of antimicrobial agent at which complete inhibition of the test organism occurred; a very fine, barely visible haze or a single colony was disregarded.

Origin of the test organisms. Staphylococcus aureus ATCC6538P, *Staphylococcus epidermidis* ATCC1228, *Streptococcus faecalis* ATCC10536, *Klebsiella pneumoniae* ATCC13883, *Citrobacter freundii* ATCC11606, *Corynebacterium agropyri* CM, *Serratia marcescens* ATCCF8412, *Micrococcus luteus* ATCC9341, *Bacillus subtilis* ATCC6633, *Bacillus megaterium* ATCC1023, *Pseudomonas aeruginosa* ATCC27853, *Bacillus cereus* ATCC11778, *Salmonella derby* ATCC4698, *Micrococcus auranticus* ATCC11731, *Acinetobacter calcoaceticus* CM and *Candida albicans* ATCC10231 (CM Cátedra de Microbiologia. Fac. Farmacia de Barcelona).

RESULTS AND DISCUSSION

Synthesis. The introduction of the strongly electronegative nitro function depresses the basic nature of the guanidino group, thus facilitating the incorporation of arginine into peptides. Moreover, this group can easily be removed by catalytic hydrogenation (9, 10). Of the reported methods to prepare arginine dipeptides (11), the mixed anhydride method described in this paper was the most quick, clean and effective.

The yield of the reaction of both condensates I and II was similar (about 65%). They were obtained in oil form as acetate or hydrochloride salt and without purification. By amino acid analysis, TLC and NMR it was shown that these compounds did not contain either free amino acid, N^{α}-lauroyl-nitroarginine or isobutyloxycarbonyl derivative (urethane).

Condensates I and II were hydrolyzed and their amino acid composition determined in a Beckman 119 C analyzer. Table 1 shows that in both cases the N^{α}-lauroyl-arginine dipeptides of proline, glycine and hydroxyproline were the principal dipeptides in the mixtures.

The average molecular weights of N^{α}-lauroyl-arginine dipeptides were determined from the amino acid composition. In condensate I this was 538 g/mol, and in condensate II this was 545 g/mol.

Properties. Table 2 shows the water solubility values of both condensates at different concentration and pH values.

TABLE 2.

Solubility in Water at Room Temperature at Different pH Values

		pH Values	
	Conc. (w/v)	2-7	7-8
Condensate I	0.5 %	S[a]	I[b]
	0.4 %	S	I
	0.2 %	S	I
	0.03 %	S	S
	0.01 %	S	S
Condensate II	0.5 %	S	S
	0.4 %	S	S
	0.2 %	S	S
	0.03 %	S	S
	0.01 %	S	S

[a]Soluble.
[b]Insoluble.

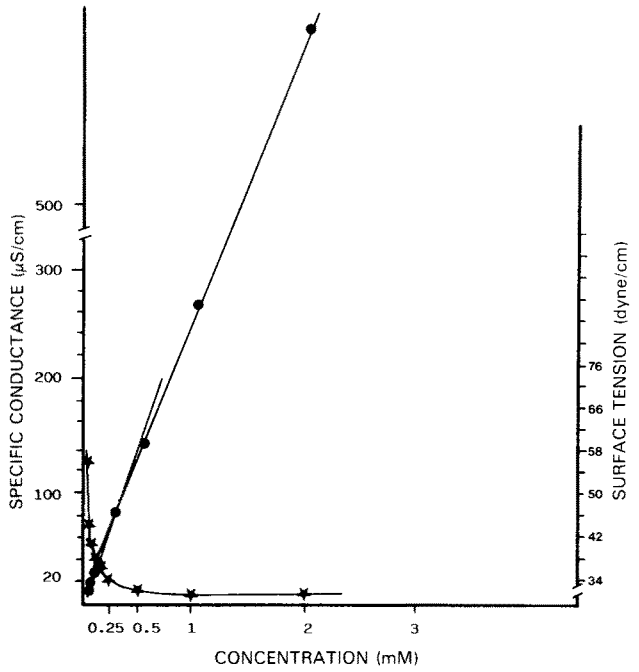


FIG. 1. Surface tension (★) and specific conductance (•) vs concentration plot for Condensate I at room temperature.

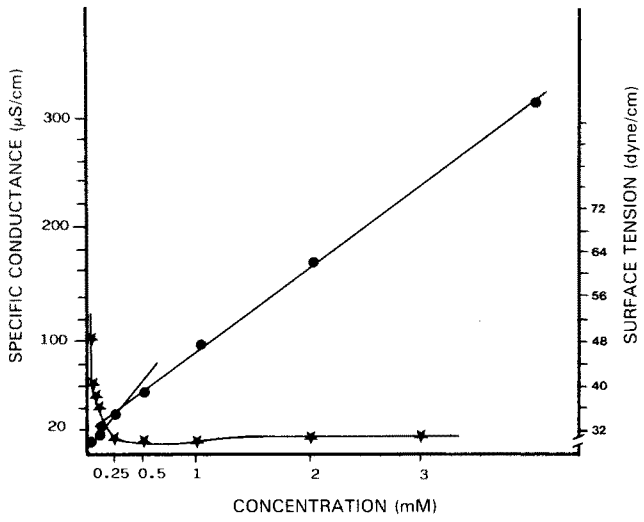


FIG. 2. Surface tension (★) and specific conductance (•) vs concentration plot for Condensate II at room temperature.

TABLE 3

Minimum Inhibitory Concentration (μg/ml) of Condensate and the References L-LAM[a] and L-LAE[b]

	Gram negative													Gram positive				
Compounds	*Candida albicans*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Streptococcus faecalis*	*Corynebacterium agropyri*	*Sarcina lutea*	*Bacillus subtilis*	*Bacillus megaterium*	*Bacillus cereus*	*Micrococcus luteus*	*Micrococcus auranticus*	*Escherichia coli*	*Klesbiella pneumoniae*	*Citrobacter freundii*	*Serratia marcescens*	*Pseudomonas aeruginosa*	*Salmonella derby*	*Acinetobacter calcoaceticus*
Condensate I	256	>256	64	256	256	256	128	256	256	256	128	>256	256	>256	>256	>256	>256	256
Condensate II	128	64	32	128	16	32	32	32	128	32	32	256	64	>256	>256	256	128	32
L-LAM	8	8	4	16	2	8	8	16	16	8	8	16	16	32	128	64	32	16
L-LAE	16	8	2	8	2	8	8	8	16	4	4	16	16	32	128	64	32	8

[a] N^{α}-Lauroyl-L-arginine methyl ester as hydrochloride salt (3).
[b] N^{α}-Lauroyl-L-arginine ethyl ester as hydrochloride salt (3).

Higher concentrations were not tested. Condensate II was soluble in all pH ranges tested. Condensate I presented turbidity at concentrations greater than 0.2% at alkaline pH values. Probably because of the amphoteric character of I and due to the strongly basic nature of the guanidino group, the alkaline zone could correspond to the isoelectric point of I and therefore to the zone of its minimum solubility (12).

Both types of compounds presented a surfactant-like behavior producing copious foam in aqueous solution. The surface tension decreased until 32 and 30 dyne/cm and the CMC values in water media are shown in Figures 1 and 2, respectively. These were determined by surface tension and conductivimetry. The values from the conductivity method were more exact than from surface tension isotherms.

The antimicrobial activity of both condensation products was established by estimating their corresponding MIC values (in μg/ml), which are listed in Table 3.

Although both compounds exhibit antimicrobial activity, condensate II was much more active against Gram positive and Gram negative bacteria than condensate I. This was related to the higher cationic character of the dipeptides, in this condensate.

A large number of protein-based surfactants derived from protein hydrolysates (casein, keratin, collagen. . .) and fatty acids have been reported as amphoteric surfactants (13-16). Although these products generally can be considered as nonsensitizing and nonirritating, it should be remembered that they may offer an excellent growth medium for biological organisms if they are not properly protected. Because of their antimicrobial activity, although less than the methyl (LAM) or ethyl ester (LAE) of N^{α}-lauroyl-arginine, the compounds described in this paper may be considered as preservative or protective substances (i.e., in cosmetic formulations) even though they are protein-based surfactants.

In order to obtain amphoteric protein-based surfactants which present better antimicrobial properties, research will continue to study the influence of the chain length and the terminal amino acids on the surfactant and antimicrobial properties of this type of compound.

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REFERENCES

1. Yoshida, R., K. Baba, T. Saitó and I. Yoshimura, *J. Jpn. Oil Chem. Soc. 25*:404 (1976).
2. Goldschmiedt, H., and I. Lubowe, *Amer. Cosm. Perf. 87*:35 (1972).
3. Infante, M.R., J. Molinero, P. Erra, M.R. Juliá and J.J. García-Daminguez, *Fette, Seifen, Anstrichm. 87*:309 (1985).
4. Moore, S., and W.H. Stein, *Methods Enzymol. 6*:819 (1963).
5. Fraenkel-Conrat, H., and H.S. Olcott, *J. Biol. Chem. 161*:259 (1945).
6. Hayakawa, T., Y. Fujiwara and J. Noguchi, *Bull. Chem. Soc. Jap. 40*:1205 (1967).
7. Du Noúy, L., *J. Gen. Physiol. 1*:521 (1919).
8. Washington, J.A. II, in *Manual of Clinical Microbiology*, edited by E.H. Lennette, A. Belows, W.J. Hansler Jr. and H.J. Shadomy, American Society of Microbiology, Washington, D.C., 1985, p. 967.
9. Hofmann, K., W.O. Peckham and A. Rheiner, *J. Am. Chem. Soc. 78*:238 (1956).
10. Hayakawa, T., T. Mizmo and J. Noguchi, *Nippon Kagaku Zasshi, J. Chem. Soc. Japan 81*:618 (1960).
11. Gross, E., and J. Meinhofer, in *The Peptides, Vol. I*, Academic Press, New York, NY, 1979, p. 264.
12. Moore, C.D., *J. Soc. Cosmet, Chem. 11*:13 (1960).
13. Narasimhan, K., P.K. Sehgal and J.K. Thomas, *J. Am. Leather. Chem. Assoc. 75*:211 (1980).
14. Stepan Chemical Co., Protein Derivatives Fact File, Form No. 6178-S (1978).
15. Sokol, P., *J. Am. Oil Chem. Soc. 52*:101 (1974).
16. Bluestein, B.R., and C.L. Hilton, in *Amphoteric Surfactants, Surf. Sci. Ser. Vol. 12*, Marcel Dekker, New York, NY, 1982, p. 265.

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