Synthesis of Diethanolamides of N-Lauroyl Dipeptides and Correlation of Their Structures with Surfactant and Antibacterial Properties[1]

S.Y. Mhaskar and **G. Lakshminarayana***
Indian Institute of Chemical Technology, Hyderabad 500 007, India

Diethanolamides (DEA) of proteins possess nonionic skin-compatible, biodegradable, surfactant and antibacterial properties. The DEA of dipeptides are, however, too hydrophilic to be effective surfactants. Hence, DEA of N-lauroyl dipeptides were synthesized and their surfactant and antibacterial properties were correlated with the structures of amino acid moieties. N-Lauroyl condensates of five simple amino acids were coupled with the corresponding amino acid methyl esters and the resulting products were condensed with diethanolamine in the presence of sodium methoxide to yield DEA of N-lauroyl dipeptides. The DEA of N-lauroyl glycine dipeptide showed surfactant properties comparable to lauroyl diethanolamide. The thiol group in the cysteine derivative exerted an unfavorable effect on surfactant properties. The isobutyl side chain in the leucine derivative contributed greatly to the antibacterial activity when compared to the other amino acid derivatives studied.

KEY WORDS: Diethanolamides of N-lauroyl dipeptides, structure *vs.* surfactant and antibacterial properties.

Mono- and diethanolamides of fatty acids, the simplest of polyoxyalkeleneamides, constitute a substantial portion of commercial nonionic surfactants (1,2). The diethanolamides (DEA) of proteins are not only nonionic but also possess skin-compatible, biodegradable, surfactant and antibacterial properties (3,4). To study the structure-property relationships, DEA of dipeptides of simple amino acids (*viz.*, glycine, leucine, phenylalanine, proline and cysteine) were synthesized but they were too hydrophilic to be effective surfactants (Lakshminarayana, G., unpublished work). To increase lipophilicity, an acyl group was incorporated into the dipeptides. For acylation of dipeptides, lauric (dodecanoic) acid was chosen because it generally exhibits optimum properties in surfactants. The synthesis of diethanolamides of N-lauroyldipeptides [$R-NH-CH(R_1)-CO-NH-CH(R_1)-CO-N-(CH_2CH_2OH)_2$ where R = lauroyl, R_1 = H (glycine), isobutyl (leucine), benzyl (phenylalanine), thiomethylene (cysteine) or $-CH_2-CH_2-CH_2-N^{\alpha}$ (proline)] and correlation of the amino acid structures with surfactant and antibacterial properties form the subject matter of this communication. The strategy in the synthesis of these derivatives is to couple synthesized N-lauroyl amino acid with the same amino acid methyl ester and to condense the resulting N-lauroyl dipeptide methyl ester with diethanolamine to yield DEA of N-lauroyl dipeptide. In the case of cysteine the thiol group needs to be protected with a benzyl group.

[1] IICT Communication No. 2830.

*To whom correspondence should be addressed.

MATERIALS

Lauric acid was purchased from BDH Ltd., Poole, England and was 99% pure by gas chromatography (GC). Chromatographically homogeneous amino acids, namely glycine, leucine, phenylalanine, proline and cysteine, were purchased from Hi-Media Ltd., Bombay, India. Silica gel (60–120 mesh) for column chromatography and silica gel G for thin-layer chromatography were obtained from Acme Synthetic Chemicals, Bombay, India. Analytical grade reagents and chemicals (BDH Ltd., Bombay, India, and Indian Drugs and Pharmaceuticals Ltd., Hyderabad, India) were used.

METHODS

Melting points of products were measured in a Ketan melting point apparatus (Shivam Scientific Instruments, Bombay, India). GC was carried out on a Silar 10C column in a Hewlett-Packard 5840 A (Hewlett-Packard Co., Palo Alto, CA) fitted with a hydrogen flame detector and a data processor. The column, injection port and detector were maintained at 195, 250 and 300°C, respectively. Flow rate of carrier gas (N_2) was 30 mL. Infrared (IR) spectra were recorded on a Perkin Elmer 683 spectrometer (Perkin Elmer, Norwalk, CT). Proton nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ on a Varian 60-FT (Varian Associates, Palo Alto, CA). Nitrogen was estimated by the Kjeldahl method as described by Cocks and van Rede (5).

Surfactant properties. Surface tension (ST) was measured with a torsion balance (White Electrical Company Ltd., Worcestershire, England) at constant temperature. Foaming property was determined with a pour foam apparatus as devised by Ross and Miles (6). Emulsifying power was determined according to Subrahmanyam and Achaya (7). The Draves-Clarkson method, as described by Subrahmanyam and Achaya (7), was used for determining the wetting ability. Critical micelle concentration (CMC) was determined by plotting equivalent conductance against concentration of a surfactant solution on a conductometer (Digisun Electronics Ltd., Bombay, India).

Antibacterial properties. Cultures of *Staphyloccus aureus* and *Escherichia coli* were grown overnight to a stationary phase at 37°C in a nutrient broth (composition:peptone 0.5%, beef extract 0.3% and NaCl 0.5%). Portions (0.1 mL) of the grown cultures were spread on the plates of nutrient agar (composition: the same as for the broth with additional 1.5% agar). After leaving the plates in an open sterile hood for 15 to 30 min for drying, sterile filter paper discs (5 mm diameter) were placed with sterile forceps. A solution of the sample (10 μL of 1% methanol solution, equivalent to 100 μg/disc) was spotted on the paper discs. The plates were placed in an incubator overnight at 37°C. The diameter of the zone of inhibition was

determined (8). The average value of three determinations was calculated.

Preparation of amino acid methyl ester (AA-OMe). Thionyl chloride (2 mL) was added dropwise to amino acid (2 g) in methanol (1.5 mL) at 0°C. After 10 h of stirring at room temperature, methanol was removed and the product was extracted with chloroform after neutralization with aqueous solution of sodium carbonate. Chloroform was evaporated to yield AA-OMe in 95% yield.

Synthesis of N-lauroyl amino acids (except cysteine). N-Lauroyl amino acids were prepared as per the Schotten-Baumann reaction by adding lauroyl chloride dropwise to an aqueous solution of sodium salt of leucine at pH 10 ± 0.5 (9). All the products except N-lauroyl proline were crystallized from ethyl acetate-hexane (70/30, vol/vol). N-Lauroyl proline was purified on a silica gel column with hexane-ethyl acetate (90:10, vol/vol).

Synthesis of S-bn-N-C_{12}-cysteine. S-Benzyl cysteine was prepared by reacting cysteine (0.055 mole) in 2 N sodium hydroxide (25 mL) and ethanol (60 mL) with benzyl chloride (0.05 mole), precipitating the product by addition of cold 2N HCl and crystallizing from hot water. S-Benzyl cysteine (0.04 mole in 20 mL of 2 N NaOH) was acylated with lauric acid anhydride (0.04 mole) by the carboxylic-carbonic anhydride method while using ethyl chloroformate (0.04 mole) in absolute tetrahydrofuran (75 mL) in the presence of triethylamine (0.04 mole). The product was crystallized from cyclohexane after acidifying with HCl (10).

Synthesis of N-C_{12}-gly-OMe. The methyl ester of N-lauroyl glycine was prepared as described earlier by the $SOCl_2$/methanol method (m.p. 53°C) for comparison with N-C_{12}-gly-gly-OMe.

Synthesis of N-C_{12}-gly-gly-OMe. To a mixture of C_{12}-glycine (0.01 mole) and triethylamine (0.01 mole) in chloroform, cooled to 0°C, ethyl chloroformate (0.01 mole) was added. After 10 min, *p*-nitrophenol (0.01 mole) was added, and the mixture was heated to boiling for 1–2 min. Concentration followed by crystallization from methanol gave N-C_{12}-gly-nitrophenyl ester in 65% yield (11). The ester was taken in a mixture of dioxane (15 mL) and water (15 mL), heated over a steam bath; gly-OMe hydrochloride (0.01 mole) was added and followed by dropwise addition of triethylamine (0.02 mole) over a period of 5 min. The mixture was heated for 5 min more. The product was worked up and purified on a silica gel column to give C_{12}-gly-gly-OMe (m.p. 123°C) in 80% yield.

Preparation of N-C_{12}-leu-leu-OMe and N-C_{12}-phe-phe-OMe. N-Lauroyl amino acid (1 mole) was coupled with its respective amino acid methyl ester (1 mole) by the carboxylic-carbonic anhydride method with ethyl chloroformate (1 mole) in absolute tetrahydrofuran (80 mL) in the presence of triethylamine (1 mole) (12). After 1 h, the solvent was removed. The crude product was extracted with ether and purified on a silica gel column to give N-C_{12}-leu-leu-OMe (liquid) and N-C_{12}-phe-phe-OMe (m.p. 121°C) in 90% yields.

Preparation of N-C_{12}-pro-pro-OMe and N-C_{12}-S-bn-cys-S-bn-cys-OMe. The N-lauroyl amino acid (1 mole) in dry ethyl acetate (75 mL) and amino acid methyl ester (1 mole) in dry ethyl acetate (75 mL) were mixed and dicyclohexylcarbodiimide (1 mole) was added to the mixture (13). In case of cysteine, the methyl ester used was S-bn-cys-OMe. After 10 h, the precipitate dicyclohexyl urea was filtered to get N-lauroyl dipeptide methyl ester, which, after removal of solvent, was purified on a silica gel column to give N-C_{12}-pro-pro-OMe (liquid) and N-C_{12}-S-bn-cys-S-bn-cys-OMe (m.p. 75°C).

Preparation of diethanolamide (DEA) from N-lauroyl dipeptide methyl ester. To a solution of diethanolamine (10 mmole) in anhydrous methanol (5 mL) under inert atmosphere, sodium methoxide was added in catalytic quantity. After heating for 15 min at 60°C, N-lauroyl dipeptide methyl ester (10 mmole) in methanol (5 mL) was added slowly and refluxed for 5 min. Methanol was removed under vacuum, and the reaction mixture was stirred for 1 h at 110°C (14). The pure DEA was separated on a silica gel column. In the case of DEA of cysteine, the benzyl protecting groups were removed with Na -liquid NH_3 in absolute tetrahydrofuran as solvent. The crude product was separated on a silica gel column to give pure product N-C_{12}-cys-cys-DEA in 40% yield. For reference, lauroyl diethanolamide was also prepared from methyl laurate.

RESULTS AND DISCUSSION

Synthesis of N-lauroyl dipeptide diethanolamides. N-Lauroyl amino acids were obtained by the acid chloride method in 70–80% yield. The thin-layer chromatography (TLC), NMR and mass spectral analyses showed that these compounds did not contain either free amino acid or fatty acid. The thiol group of cysteine was protected with a benzyl group and the overall yield of N-C_{12}-S-bn-cys was 70%. The active ester method used for synthesis of glycine dipeptide gave 90% yield. The carboxylic-carbonic anhydride method used for synthesis of leucine as well as phenylalanine dipeptide offered good yield (80%). For synthesis of dipeptides of proline and cysteine, the use of expensive dicyclohexylcarbodiimide was necessary because all the other methods gave low yield, probably because of a hindered molecular system. The yield of the coupling reaction of diethanolamine with dipeptide methyl ester decreased as the structural hindrance of dipeptide methyl ester increased from glycine (90% yield) to proline (30% yield). The unreacted dipeptide methyl ester can be recovered. The presence of sodium methoxide catalyst reduced both the reaction time and temperature and also gave light-colored products. The thiol group regeneration by benzyl group removal gave 40% of the final product.

Spectral properties. The IR spectra of all the DEA derivatives showed characteristic alkyl stretching at 2920 cm^{-1} and bending at 1460 cm^{-1}, secondary amide stretching at 1650 cm^{-1} and hydroxyl stretching at 3500 cm^{-1}. In case of proline (cyclic amino acid with tertiary amide), amide carbonyl stretching was seen at 1620 cm^{-1} and the thiol group of cysteine was seen at 2500 cm^{-1}.

The proton NMR spectra of all the N-lauroyl dipeptide esters showed a singlet of methyl ester protons at 3.7 δ (ppm). All the diethanolamide condensates showed some common peaks as follows: 5.5–7.0 (*d*, 1H, 2×[–NH–CO]), 4.0–5.2 (*m*, 2H, 2×[–C^{α}H–]), 3.1–3.9 (*m*, 8H, N–CH_2, CH_2–OH), 2.1 (*t*, 2H, –CH_2–CO), 1.3 (*s*, 19H, –CH_2–) and 0.9 (*t*, 3H, –CH_3). The ^1H NMR data of the amino acid side-chain (R) are given in Table 1. Protons relating to amide linkage were not observed for the proline derivative because it is a tertiary amide.

TABLE 1

^{1}H NMR Data (δ ppm) of Side-Chain R of Diethanolamide of N-Lauroyl Dipeptide

Amino acid moiety	β-CH_2	Characteristic peaks
Glycine	—	—
Leucine	1.3	0.96 (*d*, 6H, 2 x-CH_3)
Phenylalanine	2.1	7.24 (*s*, 5H, aromatic)
Proline	—	3.6 (*t*, 2H, N-CH_2) 2.2 (*m*, 4H, cyclic-CH_2)
Cysteine	3.2	1.2 (*br*, 1H, -SH)

Surfactant properties. The CMC of the DEA of N-lauroyl dipeptides decreased with increase in the hydrophobic nature of the amino acids from glycine (0.13%) to phenylalanine (0.06%) (Table 2). The pyrrolidine ring in proline (0.06%) had the same effect as the benzyl group of phenylalanine in reducing CMC, but the presence of two thiol groups in the cysteine derivative (0.13%) had the effect of increasing CMC. The CMC of C_{12}-gly-DEA (0.2%) was higher than that of C_{12}-gly-gly-DEA (0.13%), exhibiting the unfavorable effect of reducing the number of amide linkages from three to two. All the derivatives showed higher CMC values compared to the standard, lauroyl DEA, because of the reduction of hydrophobicity due to the dipeptide moieties.

The ST of 0.5% aqueous solutions of the DEA derivatives increased with increase in molecular weight of the alkyl side chain of the amino acid in the dipeptides, from glycine (31.5 dynes/cm) to phenylalanine (34.5 dynes/cm) and also to a small extent with reduction in amide linkages, *e.g.*, from C_{12}-gly-gly-DEA (31.5 dynes/cm) to C_{12}-gly-DEA (32.0 dynes/cm). The pyrolidine ring of proline (36.0 dynes/cm) showed an unfavorable effect on ST-lowering ability of its diethanolamide derivative. The thiol group in the cysteine derivative contributed to the lowering of ST to 33.5 dynes/cm, though not to the same extent as the isobutyl group in the leucine derivative (32.0 dynes/cm). The lauroyl DEA lowered ST more than any other derivative. The trend of ST-lowering ability of the dipeptide derivatives was similar for 0.1% aqueous solutions. There was little or no change in ST, as expected, with change in surfactant concentration from 0.1% to 0.5% where the CMC was less than 0.1% (*e.g.*, phenylalanine and proline derivatives).

The C_{12}-gly-gly-DEA produced maximum foam of 170 mm height compared to 70 mm produced by lauroyl DEA. Foaming power decreased as the hydrophobic nature of the amino acid side-chain increased from glycine (170 mm) to phenylalanine (25 mm) and also with the substitution of glycine dipeptide (170 mm) to glycine (50 mm). The proline and cysteine derivatives foamed well to a height of 135 mm and 145 mm, respectively, showing the favorable effect of pyrrolidine and thiol groups on foaming ability.

Wettability, tested as Draves sinking time in s, was best in the proline derivative (1 s), showing clearly the excellent contribution of cyclic structure to wetting compared to any other structure of the tested derivatives and lauroyl DEA (3 s). The wetting time was 6 s for C_{12}-gly-gly-DEA. The wetting ability decreased rapidly as the molecular weight of the alkyl chain of amino acids in the dipeptide increased (phenylalanine, 125 s). Reduction of the number of amide linkages led to reduction in wetting ability (C_{12}-gly-DEA, 65 s). The hydrophilic thiol group in the cysteine derivative showed a deterrent effect on wetting (35 s).

Emulsification power was highest for C_{12}-gly-gly-DEA (27 min) among all the derivatives, which could only be matched by the standard lauroyl DEA. The emulsifying ability of the derivatives decreased as the hydrophobicity of the alkyl side-chain in the amino acid increased (phenylalanine, 1.75 min) and as the number of amide linkages decreased (C_{12}-gly-DEA, 2.75 min). The cyclic pyrrolidine structure (18.5 min) enhanced emulsification, but the presence of the hydrophilic thiol group in cysteine (5.5 min) destabilized the emulsion.

In considering the surfactant properties in totality, it was found that C_{12}-gly-gly-DEA showed optimum surfactant properties that could only be matched by lauroyl DEA. The increase in bulkiness of the alkyl side-chain of amino acids in the dipeptide (*i.e.*, hydrophobicity) or reduction in the number of amide linkages exhibited negative effects on the surfactant properties. The cyclic structure in proline contributed to wetting, foaming and

TABLE 2

Physicochemical and Surfactant Properties of Diethanolamides of N-Lauroyl Dipeptides[a]

Acylated dipeptide moiety	m.p.[b] (°C)	CMC (wt %)	Surface tension 0.5 wt % (dynes/cm)	Surface tension 0.1 wt % (dynes/cm)	Foam ht Initial (mm)	Foam ht After 5 min (mm)	Wetting time (s)	Aqueous phase separation from emulsion 10 mL (min)	Aqueous phase separation from emulsion 20 mL (min)
C_{12}-gly-gly	116	0.13	31.5	32.5	170	155	6	27.00	30.00
C_{12}-leu-leu	liq.	0.1	32.0	34.0	65	50	9	8.75	19.50
C_{12}-phe-phe	109	0.06	39.0	40.0	25	15	125	1.75	2.25
C_{12}-pro-pro	liq.	0.06	36.0	36.0	135	120	1	18.50	30.00
C_{12}-cys-cys	liq.	0.13	33.5	35.5	145	125	35	5.50	10.50
C_{12}-gly	70	0.2	32.0	39.5	50	40	65	2.75	5.25
C_{12}[c]	—	0.03	30.0	30.5	70	40	3	26.00	30.00

[a]Determined on 0.5% aqueous solutions at 28°C.

[b]Melting point of diethanolamide of N-lauroyl dipeptide; liq. = liquid at room temperature.

[c]Lauroyl diethanolamide (reference compound).

TABLE 3

Antibacterial Activity[a] of Diethanolamides of N-Lauroyl Dipeptides[b]

Acylated dipeptide moiety	*Staphylococcus aureus* diameter (cm)	*Escherichia coli* diameter (cm)
C_{12}-gly-gly	1.1	0.8
C_{12}-leu-leu	1.5	1.5
C_{12}-phe-phe	0.8	0.7
C_{12}-pro-pro	1.1	—
C_{12}-cys-cys	—	—
C_{12}-gly[c]	1	1.5
Streptomycin (control)	2.2	2.1

[a]Determined as zone of inhibition of bacterial growth on nutrient agar plate.
[b]Tested as 1% solution in methanol.
[c]For comparative studies.

emulsification abilities. The hydrophilic thiol group of cysteine, in general, showed an unfavorable effect on the surfactant properties. The C_{12}-gly-gly-DEA can thus be used as an overall surfactant, C_{12}-pro-pro-DEA as a wetting and emulsifying agent and C_{12}-cys-cys-DEA as a foaming agent.

Antibacterial properties. Antibacterial activity of diethanolamides of N-lauroyl dipeptides was tested on 1% solutions in methanol (Table 3). Of the derivatives studied, the leucine derivative showed maximum inhibition of 1.5 cm against both gram-positive and gram-negative bacteria, whereas the cysteine derivative did not show any inhibition against both types of bacteria. The DEA of glycine and phenylalanine were active against both bacteria, but the cyclic structure of proline contributed to the activity only against gram-positive bacteria (1.1 cm). All the diethanolamide derivatives containing glycine showed inhibition around 1 cm, but the C_{12}-gly-DEA showed higher activity against gram-negative bacteria (zone of inhibition, 1.5 cm). The DEA of N-lauroyl phenylalanine dipeptide exhibited moderate activity (0.7–0.8 cm) against both types of bacteria. The antibacterial studies thus showed that the activity of the isobutyl side-chain in leucine in inhibiting the bacteria was more pronounced than the benzyl group in phenylalanine and the hydrogen atom of glycine, and that the thiol group in cysteine did not impart any antibacterial activity to the derivative.

REFERENCES

1. Griffin, W.C., *J. Soc. Cosmet. Chemists. 5*:249 (1954).
2. Schick, M., *Nonionic Surfactants*, Marcel Dekker, New York, NY, 1967, pp. 1–7.
3. Young, H.H., W. Springs and K.H. Spitzmueller, U.S. Patent 3,230,210 (1966).
4. Tamer, E., O. Schneidere and Quaedulieg, Belgian Patent 642,397 (1966).
5. Cocks, L.V., and C. van Rede, *Laboratory Handbook for Oils and Fats Analysis*, Academic Press, New York, NY, 1966, p. 30.
6. Ross, J., and G.D. Miles, *Oil and Soap 18*:99 (1941).
7. Subrahmanyam, V.V.R., and K.T. Achaya, *J. Chem. and Eng. Data 6 (no. 1)*:38 (1961).
8. Salle, A.J., *Fundamental Principles of Bacteriology*, McGraw-Hill Book Company, Inc., New York, NY, 1961.
9. Jungermann, E., J.F. Geretch and I.J. Krems, *J. Am. Chem. Soc. 78*:172 (1956).
10. Heitmann, P., *Eur. J. Biochem. 3*:346 (1968).
11. Bodanszky, M., *Acta Chim. Acad. Sci. Hung. 10*:335 (1957).
12. Vaughan, J.R., and R.L. Osato, *J. Am. Chem. Soc. 74*:676 (1952).
13. Winitz, M., L. Bloch-Frankenthel, N. Izumiya, S.M. Birnhaum, C.K. Baber and J.P. Greenstein, *Ibid. 78*:2423 (1956).
14. Gast, L.E., W.J. Schneider and J.C. Cowan, *J. Am. Oil Chem. Soc. 43*:418 (1966).

[Received June 1, 1991; accepted January 2, 1992]