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The isolation of N^{ϵ}-formyl-L-lysine from the reaction between formaldehyde and L-lysine and its identification by OPLC and NMR spectroscopy

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Abstract: From the reaction between L-lysine and formaldehyde, N^{ϵ}-formyl-L-lysine was isolated by means of ion-exchange column chromatography. The identification of N^{ϵ}-formyl-L-lysine was carried out by ion-exchange overpressured-layer chromatography (OPLC) and ^{1}H NMR and ^{13}C NMR spectroscopies. The m.p. and mixed m.p. values, the retention characteristics and the chemical shifts of the isolated product were identical with those of an authentic sample of N^{ϵ}-formyl-L-lysine.

Keywords: *Spontaneous formylation of L-lysine with formaldehyde; N^{ϵ}-formyl-L-lysine; overpressured-layer chromatography (OPLC); ^{1}H NMR spectroscopy; ^{13}C NMR spectroscopy.*

Introduction

In recent years it has been found [1, 2] that free and bound forms of the N^{ϵ}-methylated derivatives [N^{ϵ}-monomethyl-L-lysine (MML), N^{ϵ}-dimethyl-L-lysine (DML), N^{ϵ}-trimethyl-L-lysine (TML)] and the N^{ϵ}-formylated derivative [N^{ϵ}-formyl-L-lysine (FL)] of L-lysine are present in biological systems.

TML and other N^{ϵ}-methylated lysines have a proliferation promoting effect on several normal and neoplastic cell systems [2, 3]. The biological function of FL is little known but FL molecules are present in peptides from bee venom [4] and other formylated amino acids occur in chemotactic peptides [5].

Paik and Kim [6] showed the presence of an enzyme (ϵ-alkyllysinase) in rat kidney. This enzyme dealkylates MML and DML to produce equimolar amounts of L-lysine and formaldehyde. In various biological systems formaldehyde and other products can be formed from nitrosamines by the effect of a demethylase enzyme [7]; similarly

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formaldehyde can be formed in the presence of aromatic hydrocarbons [8] or as a result of viral infections [9–11]. It appears that there are demethylase enzyme systems in many organs for the demethylation of various endogeneous and exogeneous *N*-methylated substances.

In earlier work Tome *et al.* [12, 13] studied by ^{13}C NMR the reaction of formaldehyde with amino acids having a functional side-chain; in particular they studied the reaction with lysine. They found that formaldehyde reacts with the ϵ-amino group of L-lysine to form hydroxymethyl derivatives. Using ^{15}N NMR spectroscopy Naulet *et al.* [14] have confirmed the formation of N^{ϵ}-hydroxymethyl-L-lysine in the reaction between L-lysine and formaldehyde.

In contrast, it has been found in the authors' laboratories that the conversion of L-lysine into the corresponding stable N^{ϵ}-methylated derivatives (MML, DML, TML) can take place spontaneously by treatment with formaldehyde without any reductive catalyst [15, 16]. Results of preliminary investigations suggest that FL is also formed [15, 16].

These controversial results and the unknown biological functions of FL prompted the present work to isolate this derivative in pure form from the reaction between L-lysine and formaldehyde and to identify the isolated derivative by means of modern analytical methods.

Experimental

Reagents and chemicals

L-Lysine (free base), L-lysine HCl and N^{ϵ}-monomethyl-L-lysine HCl (MML) were obtained from Sigma Chemical Co. (USA). N^{ϵ}-Dimethyl-DL-lysine HCl (DML) and N^{ϵ}-trimethyl-DL-lysine 2 HCl (TML) were prepared by total synthesis [17]. N^{ϵ}-Formyl-L-lysine was synthesized by the methods of Okawa and Hase [18] and Hofmann *et al.* [19].

Varion KS® strong cation-exchange resin powder for column chromatography was obtained from Nitrokémia (Balatonfüzfö, Hungary).

Precoated plates of Ionpres™ 6 special ion-exchange resin for HPTLC and OPLC separations were obtained from LABOR MIM (Budapest, Hungary).

Ninhydrin spray solution was made by dissolving 0.2 g of ninhydrin and 0.05 g of $CuSO_4$ in 80 ml of CH_3OH and 20 ml of glacial acetic acid.

All chemicals were of analytical grade and were used without purification.

Instrumentation

A Chrompres 10 pressurized ultramicro chamber with an S 13 Micropump (Labor MIM, Budapest, Hungary), a Nanomat applicator (Camag Co., Muttenz, Switzerland), a Hamilton syringe, a Shimadzu CS-920 microcomputer-controlled zig-zag scanner (Shimadzu Co., Japan) and a JEOL-FX-100 NMR spectrometer were used.

Methods

High-performance thin-layer chromatography (HPTLC) [20]. HPTLC separation was performed on precoated plates of Ionpres™ 6 without impregnated edges in a normal Desaga chamber. Standard solutions were prepared by dissolving the N^{ϵ}-methylated lysines and N^{ϵ}-formyl-L-lysine in 10% (v/v) isopropyl alcohol. Sample application was accomplished with a Nanomat applicator or a Hamilton syringe. The solvent was a 0.9% (m/v) sodium chloride solution. After development, the plates were air-dried and then

sprayed uniformly with ninhydrin reagent. After drying under a stream of cold air, the plates were heated in an oven for 8–9 min at 100°C; violet spots were detected on a yellow background. *In situ* quantitative evaluation of these spots was accomplished by a microcomputer-controlled high-speed zig-zag scanner at 570 nm.

Overpressured-layer chromatography (OPLC) [21]. OPLC separation was performed on precoated plates of Ionpres^{TM} 6 with impregnated edges (in an inert plastic dispersion). The solvent, sampling, detection and quantitative evaluation were the same as for the HPTLC separation. The solvent was admitted into the pressurized chamber with the micropump at different flow rates. The external pressure on the membrane was 1.2 MPa [22, 23].

NMR. The ^{1}H and ^{13}C NMR spectra were recorded in the PFT mode at 99.6 and 25.0 MHz, respectively, with an internal deuterium lock at ambient temperature. The ^{1}H and ^{13}C chemical shifts were determined on the δ scale using a solution of 2,2-dimethyl-2-silapentane-5-sulphonate (DSS; $\delta_{DSS} = 0$) in D_2O as the internal standard. The parameters for the acquisition of the spectra were: 6002 Hz frequency range; data points 16K; acquisition time 1.364 s; t_{pulse} 3 μs (25° flip angle); repetition time 1.5 s; number of transients 28,207 (isolated FL) and 1752 (authentic FL), respectively.

Isolation of FL from the reaction mixture by ion-exchange column chromatography. The reaction mixture of 1.0 g of L-lysine (free base) and 5 ml of HCHO (36%, m/v) in 50 ml of water at approximately pH 9 was kept at room temperature for 12 h and applied on a column (100 × 3 cm i.d.) packed with the NH_4^+-form of Varion KS strong cation exchange resin. The column was first washed with distilled water (450 ml) for the removal of HCHO and then with further distilled water (500 ml) for the elution of FL. Lysine and its N^{ε}-methylated derivatives were bound on the column. The column fractions were analyzed by ion-exchange HPTLC and OPLC. After reaction with ninhydrin the fractions containing FL were collected and evaporated to dryness under reduced pressure and the product was recrystallized from ethanol. The chromatographically pure compound (approximately 60 mg) was identical with authentic FL in all respects: m.p. (authentic FL) [19] 214–215°C (decomp.); m.p. (isolated FL) 215–216°C (decomp.); mixed m.p. 214–216°C. Elemental analysis. Calculated for $C_7H_{14}O_3N_2$: C, 48.3; H, 8.1; N, 16.1%. Found: C, 48.35; H, 8.15; N, 16.4%.

Results and Discussion

During the potentiometric titration of L-lysine with formaldehyde, the pH of the aqueous system decreased in accordance with what would be expected on the basis of the Sörensen formol titration [24]. After neutralization there was no further change in the pH of the aqueous system with further increase in formaldehyde concentration.

If, however, the reaction mixture was examined by HPTLC and OPLC ninhydrin-positive spots could be observed after the first minute at the same positions as the spots of reference samples of FL, Lys, MML, DML and TML. The amounts of these derivatives increased for a few min but approximately one-third only of the L-lysine was modified after a few days.

The pH had an important influence on the composition of the reaction mixture. The amount of FL-like substance was small in the reaction mixture when the pH decreased

after the mixing of the diluted formaldehyde solution and L-lysine (formol titration); in addition, other unknown substances on the chromatogram were present under the spot of the FL-like substance. If the pH of the reaction mixture was adjusted to the basic range (e.g. pH 9) only one substance was present on the chromatogram at the position of FL.

Figure 1 demonstrates that this OPLC system is also suitable for determining the composition of the reaction mixture using a microcomputer controlled zig-zag scanner. The amounts of FL and other components in the reaction mixture varied continuously because formaldehyde is a very reactive molecule. Thus Fig. 1 only represents an OPLC chromatogram under certain conditions.

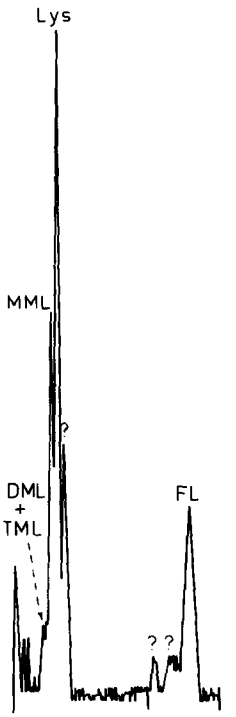


Figure 1
Quantitative evaluation of the OPLC chromatogram for the reaction mixture (pH 9) after 12 h by the Shimadzu CS-920 zig-zag high speed scanner at 525 nm. Sorbent Ionpres^{TM}6; eluent, 0.1 M NaCl; external pressure on membrane, 1.2 MPa; development distance, 160 mm; x, 12 mm; rate, 20 mm/min.

It is known that a constant solvent front velocity (u) can be employed in OPLC and that there is an optimum solvent front velocity where the average plate height (H) [25] is smallest. In TLC and HPTLC this value is much higher.

^{1}H NMR and ^{13}C NMR chemical shifts (Tables 1 and 2) brought confirmatory evidence that the structure of the FL-like substance isolated from the reaction mixture was identical with that of the authentic sample of FL.

According to the results the formaldehyde is capable not only of methylating but also of formylating the ϵ-NH_2 group of L-lysine. It is very interesting that the formation of FL in this reaction is limited (4–6%); the amount formed is much less than the stoichiometric amount.

In the reaction between formaldehyde and L-lysine an addition compound (**I**) is produced; this very labile, unseparable compound (N^{ϵ}-hydroxymethyl-L-lysine) may be stabilized by resonance to an iminium cation (**II**) (no Schiff base) or the reaction may be

Table 1
^{1}H NMR chemical shifts (D_2O; DSS), ppm

	A*	B†	C‡
(a)	1.58	1.50	1.52 m, 4H, 2xCH_2
(b)	1.85	1.83	1.83 m, 2H, CH_2 C3
(c)	3.25	3.24	3.24 m, 2H, CH_2–N
(d)	3.75	3.73	3.72 t, J = 6Hz, 1H, –CH–
(e)	8.05	8.03	8.01 s, 1H, N–CHO

*A = FL synthesized by the method of Okawa and Hase [18].
†B = FL synthesized by the method of Hofmann *et al.* [19].
‡C = FL isolated from the reaction between L-lysine and formaldehyde.

Table 2
^{13}C NMR chemical shifts* (D_2O; DSS), ppm

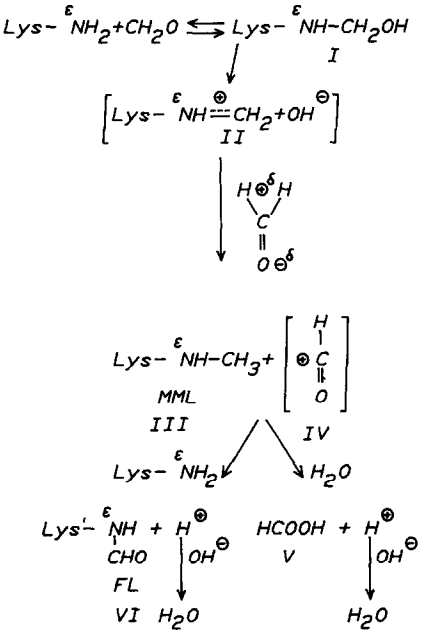
	Lysine HCl	A†	B‡	C§
–COOH	176.8	177.2s	177.1	177.4
α	57.0	57.2d	57.1	57.3
β	32.3	32.7t	32.5	32.7
γ	24.0	24.4t	24.2	24.3
δ	28.8	30.7t	30.4	30.6
ε	41.6	40.2t	40.0	40.2
–N–CHO	—	166.7d	166.5	166.5

*The assignation was made according to the literature data for lysine base [29]. Multiplicity of the signals in the off-resonance decoupled spectra: s, singlet; d, doublet; t, triplet.
†A = FL synthesized according to Okawa and Hase [18].
‡B = FL synthesized according to Hofmann *et al.* [19].
§C = FL isolated from reaction between L-lysine and formaldehyde.

reversed to form the initial compound. The next step is the formation of the ε-NH-methyl group (N^{ϵ}-monomethyl-L-lysine, MML) (**III**) by the reduction of the iminium cation group with a further formaldehyde molecule since formaldehyde is itself a hydride anion donor ($H^{\ominus}$) (e.g. instead of $NaBH_4$) in the reduction mechanism. In this reaction step the formyl cation (**IV**) is formed from formaldehyde. This formyl cation can react with water or the ε-NH_2 group of another L-lysine molecule. Since water is always in excess in this reaction mixture, the amount of formic acid (**V**) is always higher than that of FL (**VI**). This explains the small amount of FL formed in the reaction between formaldehyde and L-lysine.

The amount of FL depends on the pH of the reaction mixture. A basic pH (e.g. pH 9, borate buffer) is more favourable for the formation of FL than is an acidic or a neutral pH. This shows that in a basic medium there are many more free ε-NH_2 groups which can react with the formyl cation group. This is in agreement with the described mechanism.

This reaction mechanism differs from that of the classical Eschweiler–Clarke reaction [26, 27]; in that reaction the aliphatic primary amines or amino acids are methylated by formaldehyde in the presence of an excess of 90% (m/v) formic acid (1 mol of amine or



amino acid, 2.2 mol of formaldehyde and 5 mol of 90% formic acid), the reaction mixture being heated under reflux about 100°C for 2–4 h. The reaction is as follows:

$$R\text{–}NH_2 + 2\ CH_2O + 2\ HCOOH \rightarrow R\text{–}N\ CH_3\ _2 + 2\ H_2O + 2\ CO_2$$

In the Eschweiler–Clarke reaction the hydride anion donor is formic acid; thus the liberation of CO_2 is stoichiometric and FL cannot be formed. By contrast, in the reaction that occurs in the present work, formaldehyde is itself the hydride anion donor and the formyl cation is formed; the formylation reaction can then take place spontaneously at room temperature without any formic acid.

Lindeke *et al.* [28] recently re-evaluated the Eschweiler–Clarke reaction with specific deuteromethylation. They found that the iminium cation is reduced by $H\text{–}COO^-$ or $D\text{–}COO^-$. The hydride anion donor is the formic acid anion or $D\text{–}COO^-$; CO_2 is liberated but the formyl cation is not formed, in contrast to the reaction in the present work.

It should be noted that formaldehyde in aqueous solution exists mainly as the hydrate, $CH_2(OH)_2$, but in the reaction mixture there is always a small amount of the active form, CH_2O, which can react with L-lysine.

In the authors' opinion the formation of FL in the reaction between formaldehyde and L-lysine is very important in biological systems since endogeneous releasable formaldehyde is known to occur at low concentrations in different tissues and fluids. Thus in normal human blood 0.4–0.6 μg/ml and in urine 2.8–4.0 μg/ml of formaldehyde have been detected with [^{14}C]-dimedone reagent (T. Szarvas, E. Szatlóczky, J. Volford, L. Trézl, E. Tyihák and I. Rusznák, unpublished observations).

It appears that the formylation reaction of formaldehyde with free and bound L-lysine may be important mainly in the protection reactions of the biological systems.

It is obvious that the formylated and methylated derivatives of L-lysine were present in the reaction mixtures made by Tome *et al.* [12, 13] and Naulet *et al.* [14], too. They could not observe these stable compounds in their reaction mixtures because they investigated the reaction mixture itself without separation. Of course N^{ε}-hydroxymethyl-L-lysine cannot be detected on the chromatogram because this very labile compound decomposes during the chromatographic procedure.

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References

[1] W. K. Paik and S. Kim, *Protein Methylation*. John Wiley, New York (1980).
[2] E. Tyihák, B. Szende and K. Lapis, *Life Sci.* **20**, 385–392 (1977).
[3] B. Szende, E. Tyihák, L. Kopper and K. Lapis, *Neoplasma (Bratisl.)* **17**, 433–434 (1970).
[4] S. Doonan, A. J. Garman, J. M. Hanson, A. G. Loudon and Ch. A. Vernon, *J. Chem. Soc. Perkin Trans* **1978**, 1157–1160.
[5] V. M. Mackin and E. L. Becker, *Int. J. Immunopharmac.* **5**, 365–377 (1983).
[6] W. K. Paik and S. Kim, *Arch. Biochem. Biophys.* **165**, 369–378 (1974).
[7] C. Turberville and V. M. Craddock, *Biochem. J.* **124**, 725–739 (1971).
[8] J. C. Arcos, A. H. Conney and Ng. Ph. Buu-Hoi, *J. Biol. Chem.* **236**, 1291–1293 (1961).
[9] E. Tyihák, J. Balla, R. Gáborjányi and E. Balázs, *Acta Phytopathol. Acad. Sci. Hung.* **13**, 29–31 (1978).
[10] T. Szarvas, É. János, R. Gáborjányi and E. Tyihák, *Acta Phytopathol. Acad. Sci. Hung.* **17**, 7–10 (1982).
[11] J. Burgyán, T. Szarvas and E. Tyihák, *Acta Phytopathol. Acad. Sci. Hung.* **17**, 11–15 (1982).
[12] D. Tomé and N. Naulet, *Int. J. Peptide Protein Res.* **17**, 501–507 (1981).
[13] D. Tomé, N. Naulet and G. J. Martin, *J. Chem. Phys.* **79**, 361–363 (1982).
[14] N. Naulet, D. Tomé and G. J. Martin, *Org. Magn. Reson.* **21**, 564–566 (1983).
[15] E. Tyihák, L. Trézl and I. Rusznák, *Pharmazie* **35**, 18–20 (1980).
[16] L. Trézl, I. Rusznák, E. Tyihák, T. Szarvas and B. Szende, *Biochem. J.* **214**, 289–292 (1983).
[17] J. Puskás and E. Tyihák, *Period. Polytechn. Chem. Eng.* **13**, 261–265 (1969).
[18] K. Okawa and S. Hase, *Bull. Chem. Soc. Jn* **36**, 754 (1963).
[19] K. Hofmann, E. Stutz, G. Spühler, H. Yajima and E. T. Schwartz, *J. Am. Chem. Soc.* **82**, 3727–3732 (1960).
[20] R. E. Kaiser (Ed.), *Einführung in die Hochleistungs-Dünnschicht-Chromatographie*. Institut für Chromatographie, Bad Dürkheim (1976).
[21] E. Tyihák, E. Mincsovics and H. Kalász, *J. Chromatogr.* **174**, 75–81 (1979).
[22] E. Mincsovics, E. Tyihák and H. Kalász, *J. Chromatogr.* **191**, 293–300 (1980).
[23] E. Tyihák, E. Mincsovics and H. Kalász, *J. Chromatogr.* **211**, 45–51 (1981).
[24] S. P. L. Sörensen, *Biochem. Z.* **2**, 45–101 (1908).
[25] G. Guiochon and A. Siouffi, *J. Chromatogr. Sci.* **16**, 470 (1978).
[26] W. Eschweiler, *Chem. Ber.* **38**, 880 (1905).
[27] H. T. Clarke, H. B. Gillespie and S. Z. Weisshaus, *J. Am. Chem. Soc.* **55**, 4571–4587 (1933).
[28] B. Lindeke, E. Anderson and D. J. Jenden, *Biomed. Mass Spectrom.* **3**, 257–259 (1976).
[29] E. Pretsch, Th. Clerc, J. Seibl and W. Simon, *Tabellen zur Strukturaufklärung organischer Verbindungen mit Spektroskopischen Methoden*, p. C 210. Springer, Berlin (1981).

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