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Enzymatic synthesis and characterization of N^5-(carboxymethyl)-L-ornithine and N^6-(carboxymethyl)-L-lysine

S. P. F. Miller[1], **J. A. Donkersloot**[2], and **J. Thompson**[2]

[1] Developmental and Metabolic Neurology Branch, National Institute of Neurological Disorders and Stroke, and
[2] Laboratory of Microbial Ecology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, U.S.A.

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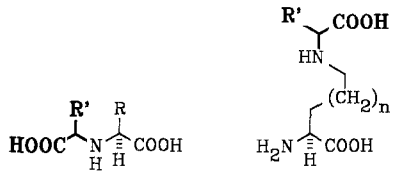
Summary. This report describes the enzyme-catalyzed synthesis, characterization, and chromatographic separation of N^6-(carboxymethyl)-L-lysine and N^5-(carboxymethyl)-L-ornithine. The two N^{ω}-(carboxyalkyl)amino acids are formed via a reductive condensation between glyoxylate and the ε- or δ-amino groups of lysine and ornithine, respectively. Both reactions are catalyzed by the NADPH-dependent enzyme, N^5-(carboxyethyl)ornithine synthase [EC 1.5.1.24], found in some strains of the lactic acid bacterium *Lactococcus lactis* subsp. *lactis*.

Keywords: Amino acids – N-(Carboxyalkyl)amino acids – N^6-(Carboxymethyl)-lysine – N^5-(carboxymethyl)ornithine – *Lactococcus lactis*

Introduction

N-(Carboxyalkyl)amino acids (see Fig. 1) may be regarded either as two amino acids linked by a common imino nitrogen, or alternatively, may be viewed as secondary amines. These compounds are frequently formed via a reductive condensation between the ketone carbonyl of an α-keto acid and the $-NH_2$ group (α or ω) of an amino acid. Enzymatic biosyntheses require NAD(P)H as the reductant, whereas $NaBH_4$ and $NaBH_3CN$ have been used for this purpose in chemical syntheses (for reviews, Miyazawa, 1980; Tempé, 1983; Thompson and Donkersloot, 1992; Thompson and Miller, 1991). N-(Carboxyalkyl)amino acids have been found in hydrolysates of proteins, and they have also been isolated as reduction products of "intermediates" in some enzyme-catalyzed reactions



A B

Fig. 1. General structures for: **A**, N^{α}- and **B**, N^{ω}-(carboxyalkyl) amino acids. Bold type indicates the carboxyalkyl moiety. The amino acid usually has L configuration, while the carboxyalkyl substructure may exhibit D or L stereochemistry

(Chin and Wold, 1975; Gundlach et al., 1959; Kobes and Dekker, 1971; Matsutani et al., 1979; Vlahos and Dekker, 1986). Members of this unusual family of amino acids have also been found in phylogenetically diverse species including bacteria, yeasts, plant tumors and muscle tissue of certain marine invertebrates (Gäde and Grieshaber, 1986; Tempé and Goldmann, 1982; Thompson and Donkersloot, 1992; Thompson and Miller, 1991).

N^{ω}-(Carboxyalkyl)amino acids found in humans include the lysine derivatives N^6-(1,3-L-dicarboxypropyl)-L-lysine (saccharopine), and N^6-(carboxymethyl)-L-lysine [N^6-(CM)lysine]. The latter compound was discovered by Wadman et al. (1975) in the urine of sick children. Later, Liardon and coworkers 1987; and Büser et al., 1987, detected N^6-(CM)lysine in the urine of rats maintained on a dietary regimen of alkali-treated whey proteins.

N^6-(CM)lysine has attracted the attention of medical researchers by its association with the aging process of human skin collagen (Dunn et al., 1991), and in the pathology and progressive complications of diabetes (Baynes, 1991; Gould et al., 1989). Studies of the Maillard (browning) reaction (Ahmed et al., 1986, 1988; Dyer et al., 1991) have shown that N^6-(CM)lysine is an oxidation product of N^6-fructose lysine which, in turn is formed via the non-enzymatic glycosylation (glycation) of ε-NH_2 groups of lysyl residues in proteins with reducing sugars, (e.g. glucose). In a significant contribution, Krook et al. (1993) have reported the first enzyme-catalyzed carboxyalkylation of a specific lysyl residue in a protein, by their finding of N^6-(carboxyethyl)lysine [N^6-(CE)lysine] in $NADP^+$-linked prostaglandin dehydrogenase/carbonyl reductase.

N^6-(CE)lysine and its lower homolog, N^5-(CE)ornithine, occur in the cytoplasm of certain bacterial strains belonging to the species *Lactococcus lactis* subsp. *lactis* (Miller and Thompson, 1987; Thompson and Miller, 1988). The two compounds are synthesized by the NADPH-dependent enzyme N^5-(carboxyethyl) ornithine synthase (EC 1.5.1.24) in reactions involving a reductive condensation between pyruvic acid and the side-chain amino groups of lysine and ornithine, respectively (Thompson, 1989). We have now discovered that glyoxylate may also serve as a substrate for the enzyme, and this has allowed the facile synthesis of N^6-(CM)lysine and the novel compound, N^5-(CM) ornithine. This communication documents the physicochemical characteristics of the two N^{ω}-(CM)amino acids, and procedures are described for their separation and detection by ion-exchange and thin-layer chromatography.

Materials and methods

Organism and culture conditions

N^5-(Carboxyethyl)ornithine synthase (EC 1.5.1.24) was initially purified from *Lactococcus lactis* K1 (Thompson, 1989). The gene (*ceo*) encoding this tetrameric enzyme (subunit M_r-35,323) has recently been cloned and sequenced (Donkersloot and Thompson, J Biol Chem: submitted). For the present study a plasmid containing *ceo* on a 7-kb *EcoRI-XhoI* fragment (in vector pBluescript SK-) has been used . This plasmid (p493) was transformed into *Escherichia coli* TG1 which also contained the plasmid pGP1-2 (Tabor and Richardson, 1985). One of the transformants, CEO521, was used to obtain high expression of N^5-(CE)ornithine synthase by the procedure of Tabor and Richardson (1985). Strain CEO521 was grown on a shaker (220 rpm) at 30°C in one liter of medium supplemented with ampicillin (50 μg/ml) and kanamycin (40 μg/ml). At an absorbance (at 600 nm) of 1.0, the temperature of the culture was raised to 42°C for 30 minutes. Thereafter, the temperature was lowered to 37°C and 10 ml of rifampicin solution (10 mg/ml in methanol) was added to the culture. Two hours after addition of rifampicin, the cells were harvested by centrifugation. The cells were washed twice by resuspension and centrifugation from 100 ml of phosphate-buffered saline (pH 7.2), and the resultant cell pellet was stored at −20°C.

Preparation of cell extract

Frozen cells of *E. coli* CEO521 (approx. 7 g wet weight) were thawed by resuspension in 12 ml of 25 mM potassium phosphate buffer (pH 7.0) at room temperature. The organisms were disrupted (at 0°C) by three 1-min periods of sonic oscillation using a Branson Model 350 sonifier operating at 75% of maximum power. Intact cells and cell debris were removed by centrifugation (25,000 × g at 5°C for 30 min), and the supernatant fluid was further clarified by ultracentrifugation (180,000 × g at 5°C for 2 h). This preparation was used for the enzymatic syntheses of the N^{ω}-(carboxyalkyl) amino acids.

Synthesis of N^{ω}-(CM) amino acids

Two reaction mixtures, each of 10-ml were prepared containing: 0.1 M potassium phosphate buffer (pH 7.0); 5 mM sodium glyoxylate; 5 mM NADPH; 0.5 ml cell extract; and either 5 mM L-ornithine or 5 mM L-lysine. After 3 h of incubation at room temperature (approx. 22°C), the two mixtures were frozen in dry ice and lyophilized. Each residue was reconstituted with 2.5 ml of distilled water, and proteinaceous and other high molecular weight compounds were removed by gel filtration through PD-10 columns. Fractions containing low molecular weight compounds were pooled, lyophilized and the pale yellow residues were dissolved in 0.5 ml of water. The two solutions were applied to separate sheets of Whatman 3 MM chromatography paper, and components were separated by descending chromatography (24 h) in a solvent containing *n*-butanol/acetic acid/water (v/v 12:3:5). Ornithine and the putative N^5-(CM) derivative migrated about 4 cm, and lysine and putative N^6-(CM)lysine about 5 cm, from the origins. The appropriate areas were cut from the chromatograms, and the amino acids were eluted with water and the solutions were lyophilized. Thereafter, the (CM) derivatives were separated from the parent amino acids (ornithine and lysine) by preparative cellulose 300 MN thin-layer and Dowex 50 (H^+-form) ion-exchange procedures (Miller and Thompson, 1987; Thompson et al., 1986; Thompson and Miller, 1988). Approximately 1 mg of each CM derivative was obtained which represents a 10% yield.

Amino acid chromatography

Amino acids were separated on a Beckman Instrument Inc, system 6300 high performance amino acid analyzer equipped with a Li^+-high performance column for physiological methods (Beckman Model 338051 cation-exchange resin; column dimensions 4mm ID × 10

cm length). Standard amino acids and the (CM) derivatives of L-ornithine and L-lysine, were resolved by procedures supplied by the manufacturers.

Citrate-based Li^+-A, B and C high performance buffers were used, and compounds were detected by post-column reaction with ninhydrin. Chromatographic analyses were conducted at the Protein Structure Laboratory, University of California at Davis.

Spectral data and instrumentation

Proton and ^{13}C-NMR data were collected on a Varian XL-300 spectrometer. The bis-trifluoroacetyl-butyl-ester (bis-TAB) derivatives of the amino acids were prepared via the dimethyl esters (Thompson et al., 1986). Gas chromatography/electron impact mass spectrometry (GC/EI-MS) was performed on a VG-7070F quadrupole instrument equipped with a wide-bore methylsiloxane capillary column (0.53 mm ID, 10 m length). The temperature was raised 20°C per minute. Ammonia chemical ionization mass spectrometry (CI/MS) was performed on an Extrel 400-3 quadrupole instrument.

Results

Biosynthesis and characterization of N^{ω}-(CM)amino acids

The *L. lactis* enzyme, N^5-(CE)ornithine synthase is unable to use α-ketobutyrate as an alternative substrate to pyruvate (Thompson, 1989). However, extracts from the *E. coli* CEO493 clone readily catalyzed the NADPH-dependent reductive condensation between glyoxylate and the ε- and δ-NH_2 groups of lysine and ornithine, to yield N^6-(CM)lysine and N^5-(CM)ornithine respectively (Fig. 2). The K_m for glyoxylate (3.8 mM) is approximately twenty-five fold higher than the K_m for pyruvate, ~0.15 mM, (Thompson, 1989).

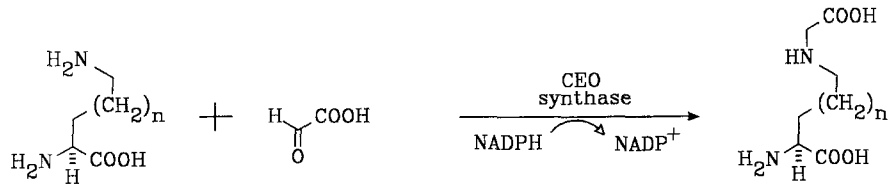


Fig. 2. Enzymatic synthesis of N^5-(CM)ornithine ($n = 1$) and N^6-(CM)lysine ($n = 2$) by reductive alkylation of the appropriate amino acid with glyoxylate. Both reactions are catalyzed by NADPH-dependent N^5-(carboxyethyl) ornithine synthase [EC 1.5.1.24]

Approximately 1 mg each of N^6-(CM)lysine and N^5-(CM)ornithine was obtained by the procedures described in Materials and methods. The following ^{1}H and ^{13}C-NMR spectral data were obtained:

N^6-(Carboxymethyl)-L-lysine

^{1}H-NMR: 9.8 mM in D_2O; (0.2% CH_3OH = 3.3 ppm); 300 MHz 3.68 (1H, t, J = 5.9 Hz, $CHCO_2$); 3.56 (2H, s, CH_2CO_2); 3.03 (2H, t, J = 7.8 Hz, CH_2N); 1.84 (2H, bq, J = 6–7 Hz, $CHCH_2$); 1.71 (2H, p, J = 7.7 Hz, CH_2CH_2N); 1.44 (2H, 7 lines, J = 8.1 Hz, $CHCH_2CH_2$).

^{13}C-NMR: 9.8 mM in D_2O; (0.2% CH_3OH = 49 ppm); 75 MHz
174.72, C-1; 171.34, C-8; 54.67, C-2; 49.25, C-7*; 47.11, C-6*; 30.04, C-5; 25.18, C-3; 21.62, C-4.

CI-MS on the bis-TAB derivative of N^6-(CM)-L-Lysine:
527 (26%) $(M + 1) \cdot NH_4^+$, 526 (100%) $M \cdot NH_4^+$, 509 (12%) $M \cdot H^+$, 484 (15%), 435 (16%), 413 (9%), 412 (26%).

N^5-(Carboxymethyl)-L-ornithine

^{1}H-NMR: 6.8 mM in D_2O; (2% CH_3OH = 3.3 ppm); 300 MHz
3.70 (1H, t, J = 5.1 Hz, $CHCO_2$); 3.56 (2H, s, CH_2CO_2); 3.05 (2H, t, J = 7.2 Hz, CH_2N); 1.9 (2H, m, $CHCH_2$*); 1.8 (2H, m, CH_2CH_2N*).

^{13}C-NMR: 6.8 mM in D_2O; (2% CH_3OH = 49 ppm); 75 MHz
174.52, C-1; 171.39, C-7; 54.30, C-2; 49.38, C-6*; 46.91, C-5*; 27.80, C-3; 21.83, C-4.

CI-MS on the bis-TAB derivative of N^5-(CM)-L-Ornithine:
513 (25%) $(M + 1) \cdot NH_4^+$, 512 (100%) $M \cdot NH_4^+$, 495 (23%) $M \cdot H^+$, 470 (4%), 421 (16%), 399 (5%), 398 (26%)

The spectra for N^6-(CM)lysine and N^5-(CM)ornithine were very similar to those obtained for the N^{ω}-(CE) derivatives of ornithine and lysine which have previously been isolated from *L. lactis* (Miller and Thompson, 1987; Thompson et al., 1986; Thompson and Miller, 1988). The major differences were the replacement of the $CHCH_3$ resonances in the N^{ω}-(CE) derivatives by a CH_2 resonance in the N^{ω}-(CM) analogs. The two N^{ω}-(CM) compounds were converted to their bis-TAB derivatives for analysis by mass spectrometry. The ammonia chemical-ionization MS data showed the molecular weights predicted for the bis-TAB derivatives of both N^6(CM)lysine and N^5(CM)ornithine. By GC/MS, the lysine derivative eluted at 178°C (Fig. 3, inset), and the electron impact mass spectra (Fig. 3) revealed a molecular ion at 508 and a fragmentation pattern that is analogous to the published spectra of bis-TAB-N^6-(CE)lysine (Thompson and Miller, 1988). The fragmentation pattern in Fig. 3 can be interpreted as follows. The parent ion can lose butanol (M/Z = 434) or the elements of butyl radical and CO_2 (407). The ion at 407 can sequentially lose butylene (351), water (333), CO (305) and CF_3CONH_2 (192). This interpretation is supported by the observation of metastable ions in the mass spectrum of bis-TAB-N^6-(CE)lysine (Thompson and Miller, 1988), which was collected without data digitization. The ions at 280, $M^{+\cdot}$-[$CF_3CONHCH_2CO_2Bu + H^{\cdot}$], and 180, $M^{+\cdot}$-[$CF_3CONHCH_2Bu + {}^{\cdot}CO_2Bu$], arise by loss of N^6 with its substituents, and occur at the same M/Z values as the corresponding ions from the N^6-(CE)lysine derivative. The N^5-(CM)ornithine derivative elutes as a single peak at 154°C (Fig. 4, inset) and displays a consistent fragmentation pattern in which the previously mentioned ions are further reduced by 14 mass units. The NMR and MS data clearly

*Assignments may be reversed.

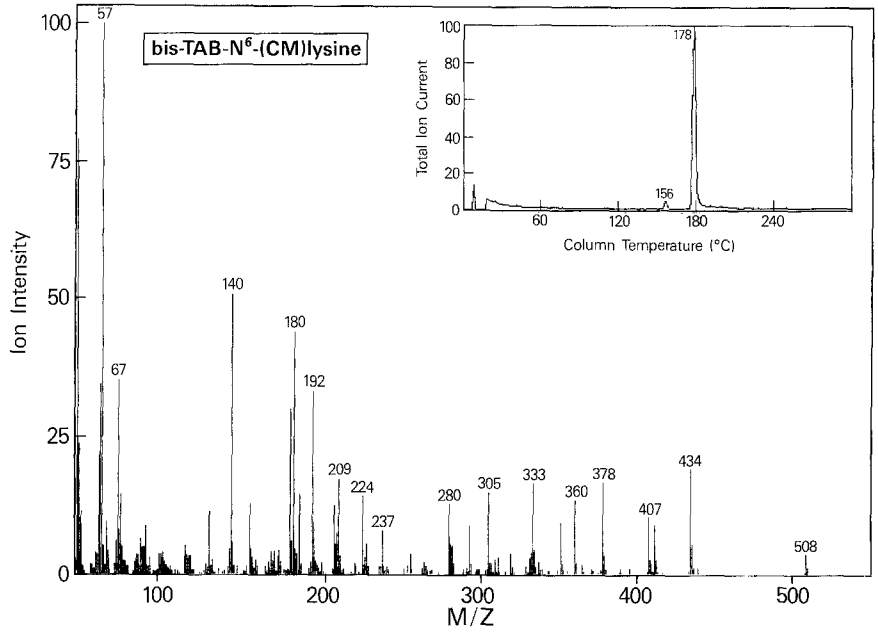


Fig. 3. Analysis of the bis-TAB derivative of N^6-(CM)lysine by GC/EI-MS. The elution profile of the gas chromatograph is shown as a plot of the reconstructed total ion current versus column temperature (inset). The electron-impact mass spectra is plotted as the mass to charge ratio (M/Z) versus the relative ion intensity

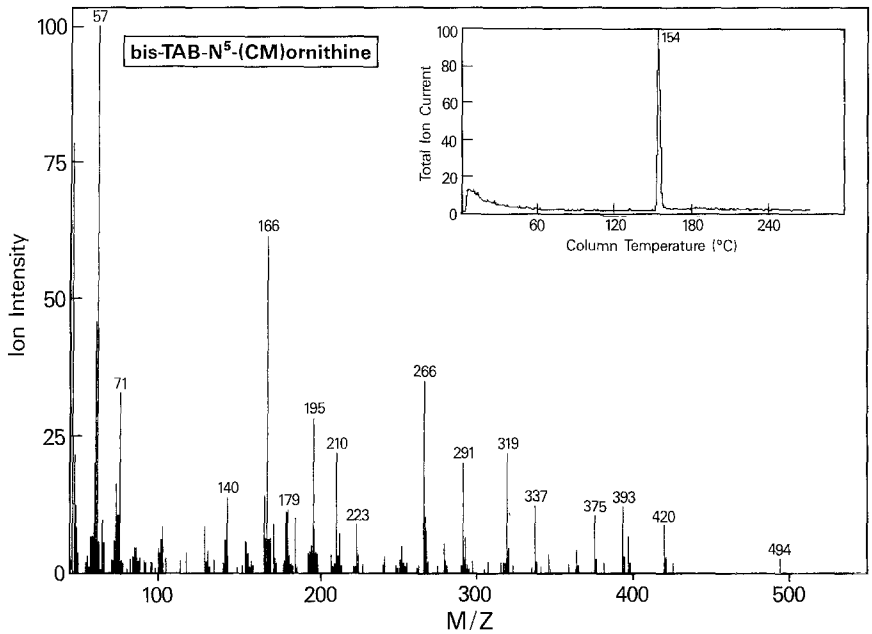


Fig. 4. Analysis of the bis-TAB derivative of N^5-(CM)ornithine by GC/EI-MS. Other parameters as described in the legend to Fig. 3

establish the structures of the enzymatically synthesized products to be N^5-(CM)ornithine and N^6-(CM)lysine.

Chromatographic separation and identification of N^{ω}-(CM) amino acids

The two N^{ω}-(CM) amino acids were readily separable by ion-exchange chromatography and both compounds migrated with the neutral amino acids during HPLC (Fig. 5). N^5-(CM)ornithine eluted (48.6 min) just before isoleucine (49.28 min), whereas N^6-(CM)lysine (50.67 min) eluted shortly after leucine (50.14 min).

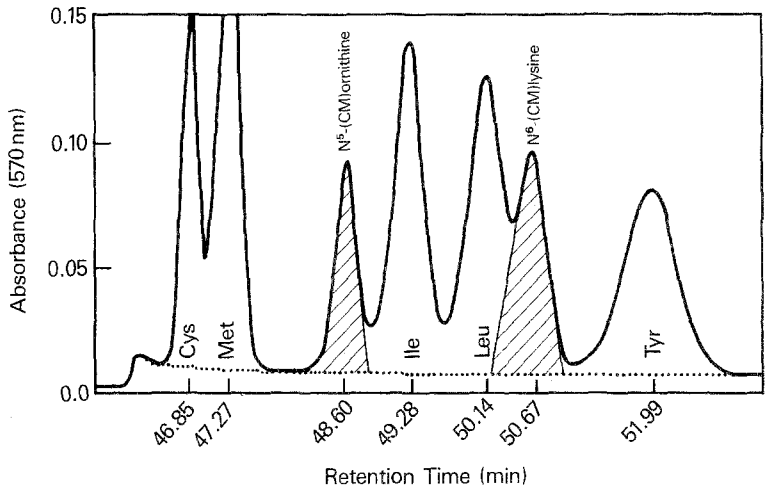


Fig. 5. Separation and migration characteristics of N^5-(CM)ornithine and N^6-(CM)lysine by HPLC. Details of instrumentation and experimental procedures are presented in "Materials and methods"

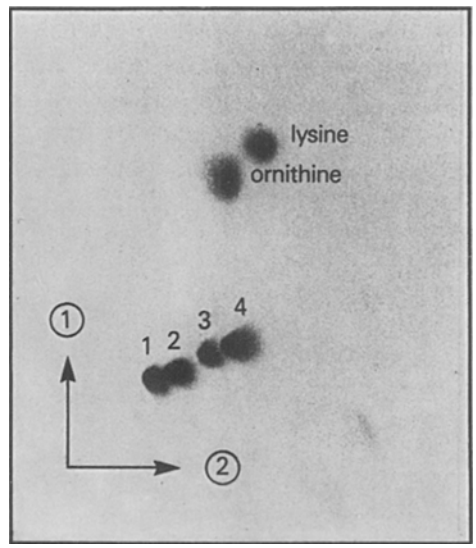


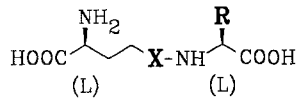
Fig. 6. Separation of N^{ω}-(carboxyalkyl) derivatives from the parent amino acids (ornithine and lysine) by cellulose 300MN- TLC. Numbered arrows indicate the order and directions of solvent flow: *Solvent 1*, *n*-butanol/acetone/NH_4OH/H_2O (20:20:10:4, vol/vol); *Solvent 2*, isopropanol/formic acid/H_2O (20:1:5, vol/vol). The mixture contained 10 nmoles of each compound, and 0.5% (w/v) ninhydrin in acetone was used for detection. The derivatives are: *1*, N^5-(CM)ornithine; *2*, N^6-(CM)lysine; *3*, N^5-(CE)ornithine and *4*, N^6-(CE)lysine

The components of a mixture containing ornithine, lysine, and the two (CM) and (CE) derivatives, were readily separated by two dimensional cellulose-TLC (Fig. 6).

Discussion

To our knowledge this is the first reported enzymatic synthesis of the N^{ω}-(CM) derivatives of lysine and ornithine. The biosynthesis of these unusual compounds is mediated via an NADPH-dependent reductive condensation reaction catalyzed by N^5-(CE)ornithine synthase. This enzyme has been found in certain Group N lactococci (Donkersloot and Thompson, 1990; Thompson et al., 1990) used as "starter" organisms in various dairy fermentations. These strains frequently contain high intracellular concentrations (5–20 mM) of the N^{ω}-(carboxyethyl) derivatives of ornithine and lysine, which are formed via the reductive condensation between pyruvic acid and either ornithine or lysine. The biochemical function(s) of the N^{ω}-(CE)amino acids are presently unknown. To date neither of the carboxymethyl compounds described in this communication has been detected in lactococcal cells. This may be due to insufficient concentrations of glyoxylate within the cells or more likely, to the inability of these organisms to generate this compound.

Chemical methods are usually employed for the preparation of N^6-(CM)-lysine and other N-(carboxyalkyl)amino acids (Thompson and Donkersloot, 1992). However enzyme-catalyzed syntheses (when available) provide an attractive alternative to the former procedures since they are efficient, and simple to perform. Furthermore, N,N-dialkylation does not occur and the enzyme-catalyzed reactions are both regio- and stereochemically specific. Whereas the amounts of the two (CM) derivatives obtained are rather small ($\sim$ 1 mg), scale-up should be relatively straightforward. For instance, about 3 mg of pure N^5-(CE)ornithine synthase can readily be obtained from 1 g (wet weight) of recombinant *E. coli* cells (Donkersloot and Thompson, submitted) and about 5 mg of product can be synthesized per mg of enzyme per min. To limit costs, NADPH could be regenerated with a glucose 6-phosphate: glucose 6-phosphate dehydrogenase coupled reaction.



X	R	Compound
CO	H	γ-L-glutamyl-glycine
CH_2	H	N^5-(carboxymethyl)-L-ornithine
CH_2CH_2	H	N^6-(carboxymethyl)-L-lysine
CO	CH_3	γ-L-glutamyl-L-alanine
CH_2	CH_3	N^5-(1-L-carboxyethyl)-L-ornithine
CH_2CH_2	CH_3	N^6-(1-L-carboxyethyl)-L-lysine

Fig. 7. Structural relationships between N^{ω}-(carboxyalkyl)amino acids and some γ-glutamyl peptides

The N^{ω}-(CM) and (CE) derivatives of ornithine and lysine are structurally similar to some γ-glutamyl peptides, including γ-L-glutamylglycine and γ-L-glutamyl-L-alanine (Fig. 7). The latter compounds are substrates for γ-glutamyl transpeptidase(s) (Anderson and Meister, 1986) and γ-glutamylcyclotransferase (Taniguchi and Meister, 1978). It will be of interest to determine whether N^{ω}-(carboxyalkyl)amino acids (which contain $—CH_2$ or $—CH_2.CH_2$ in lieu of the —CO— moiety) serve as non-metabolizable inhibitors of γ-glutamyl transpeptidase and related enzymes (Griffith and Meister, 1977). Conversely, some γ-glutamyl peptides may also inhibit N^5-(carboxyethyl)ornithine synthase.

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Authors' address: Dr. J. Thompson, National Institute of Dental Research, Building 30, Room 528, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, U.S.A.

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