Optimization of the Synthesis of the Cross-Linked Amino Acid Ornithinoalanine and Nuclear Magnetic Resonance Characterization of Lysinoalanine and Ornithinoalanine

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Lysinoalanine (LAL) and ornithinoalanine (OAL) are unnatural amino acids that can be formed in food submitted to thermal treatment, especially in alkaline conditions. The paper presents an optimization of the synthetic procedure for the preparation of a standard of OAL that could be very useful to study the toxicological and nutritional consequences of the presence of OAL in food. In the meantime, it was possible to develop a method based on nuclear magnetic resonance for the diastereomeric characterization of LAL and OAL without derivatization. Interest in this method is based on the known differences in the nephrotoxicity of the two diastereisomers of LAL.

Keywords: *Lysinoalanine; ornithinoalanine; synthesis; NMR analysis; diastereoisomers*

INTRODUCTION

When food undergoes alkaline processing and/or heating, some reactions can modify the structure of the protein chains. Besides the Maillard reaction, another important interaction is the formation of dehydroalanine residue through elimination of a leaving group from serine, *O*-phosphorylserine, *O*-glycosylserine, and cystine: this reactive intermediate can undergo Michael addition by another nucleophilic amino acid residue (de Koning and van Rooijen, 1982). For example, when lysine is in a suitable position, its ϵ-amino group reacts to give a secondary amine that is normally indicated with the trivial name of lysinoalanine [LAL, N^{ϵ}-(*R,S*-2-amino-2-carboxyethyl)-*S*-lysine]. The principal characteristic of this compound and its analogues is that they are stable in the conditions of acidic protein hydrolysis and are relatively easy to analyze with respect to other compounds.

Some years ago Maga (1984) collected in an excellent review all of the data on LAL known at the moment from the viewpoint of food occurrence, detection, and inhibition of formation.

The analysis in foods can be performed by a number of different methods. A detailed description of the analytical methodologies, especially by high-performance liquid chromatography (HPLC), was reported by Warthesen and Wood-Rethwill (1984): one of the most frequently used is reversed phase HPLC analysis after dansyl derivatization of the three amino groups. This method was recently improved by Moret et al. (1994). Other analytical methods are based on gas chromatography/mass spectrometry (GC/MS) and require derivatization of both the amino and carboxy groups (Hasegawa et al., 1987; Busër and Erbersdobler, 1988; Liardon et al., 1991).

Lactalbumin and caseins are particularly susceptible to LAL formation, and in cheese LAL appears to be a more reliable molecular marker than furosine for the very low concentration of reducing sugars. Very recently Pellegrino et al. (1996) have proposed an HPLC method to determine LAL in natural Mozzarella cheese. Derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl), solid phase extraction, and reverse phase chromatography with fluorescence detection permitted the quantification of LAL, and this parameter was used to distinguish natural Mozzarella cheese from imitations. The contribution of LAL in the cross-linking of proteins has been discussed, but quite recently Henle et al. (1996) have demonstrated that the irreversible oligomerization of casein isolated from UHT-treated skim milk after storage at various temperatures is related only in part to the formation of LAL and similar cross-linked amino acids, the Maillard reaction being a more important source of linking groups. These indications were confirmed by Pellegrino et al. (1998), who have shown clearly that LAL is responsible for intramolecular but not intermolecular cross-linking.

Much research has been devoted to the study of the digestibility of proteins treated with alkali (Savoie et al., 1991) and the toxicological and nutritional consequences of LAL formation in foods (Maga, 1984, and literature cited therein; Friedman et al., 1984; Jonker et al., 1996). LAL provokes lesions in rat kidney cells, causing nephrocitomegaly (Friedman and Pearce, 1989).

From the point of view of stereochemistry, LAL is a mixture of two diastereoisomers: *S*-lysino-*S*-alanine (*S,S*-LAL) and *S*-lysino-*R*-alanine (*S,R*-LAL), the configuration of lysine being preserved during its formation (Figure 1). They have different affinities to copper(II) and cobalt(II): the greater observed nephrotoxicity of *S,R*-LAL may be related to its higher Cu(II) affinity (Friedman and Pearce, 1989). The stereoisomeric composition of urinary secreted LAL reflects that in ingested proteins (de Weck Gaudard et al., 1988).

On the contrary, the lack of a commercial standard has prevented the development of systematic studies on the formation of ornithinoalanine [OAL, N^{δ}-(*R,S*-2-

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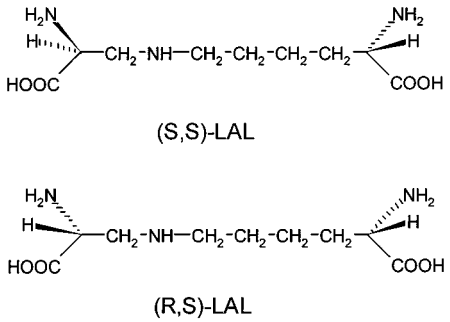
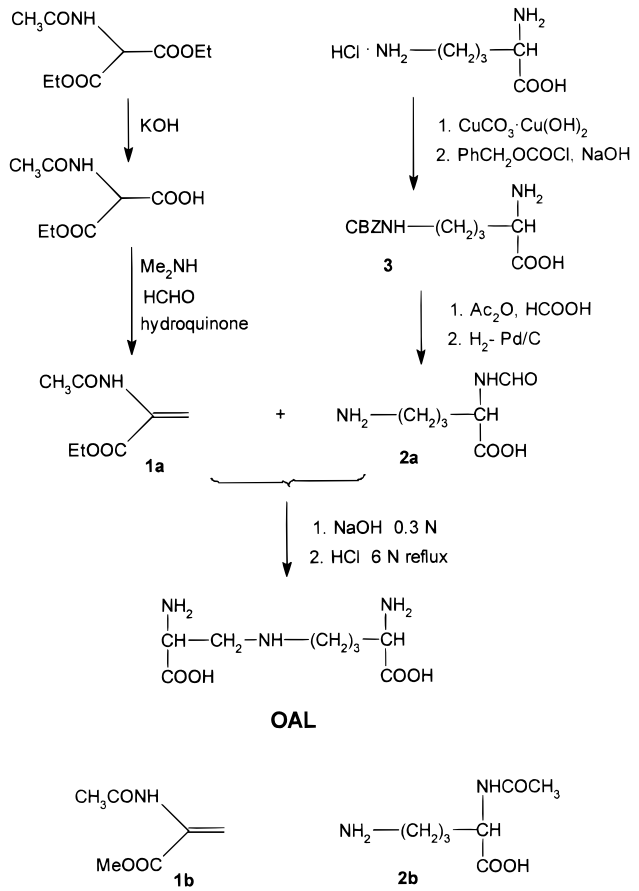


Figure 1. Structure of LAL diastereoisomers.

amino-2-carboxyethyl)-*S*-ornithine]. Ornithine is not normally contained in raw food proteins, but it is formed through the decomposition of arginine, especially in fermented food (Cecchi and Resmini, 1973; Collar et al., 1991). OAL was detected for the first time in food proteins (Ziegler et al., 1967) and then in wool treated with alkali (Mirò et al., 1968). Hasegawa and Iwata (1982) have developed an analytical method for OAL based on GC/MS of the *N*-trifluoroacetyl butyl ester and have observed that it is formed by treatment of lysozyme in a basic medium. As in the case of LAL, it can be easily postulated that two diastereoisomers are formed, but until now they have been neither separated nor characterized. OAL's nephrotoxicity is similar to that of LAL (Friedman et al., 1984).

In this paper we present an optimization of the synthetic procedure for the production of OAL and a method based on nuclear magnetic resonance for the diastereomeric characterization of LAL and OAL.

Scheme 1



EXPERIMENTAL PROCEDURES

Materials. LAL and acetylornithine were purchased from Bachem (Switzerland); 2-acetamidoacrylate methyl ester was from Lancaster.

Nuclear Magnetic Resonance (NMR). ^{1}H NMR and ^{13}C NMR spectra were determined in D_2O using tetramethylsilane as internal standard on a Bruker AMX-300 (300 MHz). High-field ^{13}C NMR and ^{1}H NMR were registered on a Bruker AMX-600 (600 MHz). Chemical shifts were expressed in parts per million (δ). Heteronuclear two-dimensional ^{1}H−^{13}C correlations one-bond, heteronuclear multiple quantum correlation (HMQC; Bax and Morris, 1981), and multiple-bond (HMBC; Bax and Summers, 1986) were carried out in the ^{1}H-detected mode with broad-band decoupling in the ^{13}C domain.

MS. Mass spectra were obtained on a Finnigan TSQ70 equipped with an ICIS data system. FAB analysis was performed using thioglycerol as a matrix.

HPLC Analyses. HPLC analyses were conducted on an HP-1050 quaternary pump fitted with a Rheodyne injector (20 μL loop) and equipped with an HP-1046A programmable fluorescence detector. The system was controlled by an HP ChemStation (DOS Series, Hewlett-Packard). Fluorescence detection was performed with excitation at 266 nm and emission at 310 nm. The analytical column was an Aminoquant (5 μm, 200 × 2.1 mm, Hewlett-Packard), and the flow rate was 0.5 mL/min. The FMOC derivatives of OAL and LAL were prepared, purified on SPE cartridges, and analyzed as indicated by Pellegrino et al. (1996).

***N*$^\delta$-Carbobenzyloxy-L-ornithine (3, Scheme 1).** A solution of *S*-ornithine hydrochloride (6 g, 0.036 mol) in 60 mL of distilled water was added with $CuCO_3$·$Cu(OH)_2$ (8.4 g, 0.038 mol) and refluxed for 1 h. The warm slurry was filtered and the solid washed with distilled water. The solution was evaporated in a vacuum, yielding a blue solid that was dissolved in 2.78 N NaOH (25 mL). At 0 °C, carbobenzyloxy chloride (5.4 mL, 0.038 mol) was slowly added, with the temperature kept between 0 and 3 °C. The solution was then stirred for 2 h at room temperature. The solid formed was filtered, washed with water and acetone, and dissolved in 2 N HCl (300 mL), and then a 0.1 M solution of EDTA disodium salt (600 mL) was added and the solution neutralized with 2 N NaOH (350 mL). The resulting white solid was collected and dried at 40 °C (6.4 g, 67% yield): mp 255 °C; MS-EI, *m*/*z* (%) 267 (3, M^{+1}), 221 (2), 176 (1), 160 (6), 114 (26), 91 (100).

***N*$^\alpha$-Formyl-L-ornithine (2a, Scheme 1).** *N*$^\delta$-Carbobenzyloxy-*S*-ornithine (1.5 g, 5.6 mmol) was dissolved in a mixture of 98% formic acid (9 mL) and acetic anhydride (1 mL), and this solution was stirred at room temperature for 20 h in anhydrous conditions. Distilled water (2.5 mL) was then added, and the solution was concentrated in a vacuum to obtain a clear oily residue, which was dissolved in 25 mL of ethyl acetate and washed with 0.5 N HCl and distilled water. The organic layers were dried over sodium sulfate and evaporated in a vacuum to yield 1.4 g of white solid. Without any purification the product was dissolved in methanol and hydrogenated at room temperature over palladium catalyst (Pd/C 10%). The catalyst was removed by filtration and washed with ammoniacal methanol. The combined filtrate and washings were neutralized with glacial acetic acid and concentrated in a vacuum to a pale yellow oil, 710 mg (80% yield): ^{1}H NMR (300 MHz, D_2O) δ 1.6−1.9 (4 H, m, CH_2CH_2), 3.0 (2 H, t, CH_2NH_2), 4.25 (1 H, t, CH), 8.1 (1 H, s, CHO); MS-FAB (thioglycerol), *m*/*z* (%) 161 (55, M^{+1}), 149 (12), 133 (10), 115 (10), 75 (25).

Preparation of OAL with 2-Acetamidoacrylate Ethyl Ester (1a) as Michael Acceptor. A 0.6 N solution of *N*$^\alpha$-formyl-*S*-ornithine (540 mg, 3.4 mmol) in distilled water (5.7 mL) was prepared, and 0.6 N NaOH was added (5.7 mL). After the addition at 0 °C of 2-acetamidoacrylate ethyl ester (640 mg, 4.01 mmol), the solution was stirred at room temperature for ~19 h. The solvent was removed in a vacuum, and the residue was hydrolyzed with 6 N HCl (3 mL) at reflux for 6 h. The crude OAL was purified by flash chromatography on silica gel with $MeOH/CHCl_3/NH_3/H_2O$ = 22:20:5:5 as eluent. OAL

Table 1. Reaction of N^{α}-Formylornithine (2a) or N^{α}-Acetylornithine (2b) with 2-Acetamidoacrylate Ethyl Ester (1a) or Methyl Ester (1b) (See Scheme 1)

entry	nucleophile	acrylate (equiv)	solution	temp (°C)	reaction time (h)	yield OAL (%)	yield compd **4** (%)
1	**2a**	**1a** (1.2)	0.3 N NaOH	rt[a]	19	18	nd[b]
2	**2b**	**1b** (2)	0.2 M $K_2B_4O_7$ (pH 11)	reflux	14	nd	12
3	**2b**	**1b** (2)	0.3 N NaOH	80	12	nd	20
4	**2a**	**1b** (1.2)	0.3 N NaOH	80	3	15	nd
5	**2a**	**1b** (1.2)	0.3 N NaOH	80	9	nd	10
6	**2a**	**1a** (1.2)	0.3 N NaOH	80	6	nd	8
7	**2a**	**1b** (1.2)	0.5 N NaOH	rt	96	8	tr[c]
8	**2a**	**1b** (1.2)	0.3 N NaOH	rt	144	15	nd
9	**2b**	**1b** (1.2)	0.3 N NaOH	rt	72	28	nd

[a] rt, room temperature. [b] nd, not detected. [c] tr, traces.

was recovered as a clear brown oil that was dried by addition and evaporation of several portions of ethanol, giving a yellowish solid, 132 mg (18% yield): mp = 190 °C; MS-FAB (glycerol), *m/z* (%) 219 (4), 207 (12), 202 (29), 166 (10), 127 (6), 115 (33), 110 (100), 75 (5).

Preparation of OAL with 2-Acetamidoacrylate Methyl Ester (1b) as Michael Acceptor. The condensation procedure was similar to the one described for 2-acetamidoacrylate ethyl ester but the reaction time was ~3 days. During this time, it is important to check the pH, which must be kept above 9 by adding 0.6 N NaOH when necessary. The yield is 28%.

Formation of Product 4. A 0.6 N solution of N^{α}-acetyl-*S*-ornithine in distilled water was prepared, and 0.6 N NaOH was added. After the addition at 0 °C of 2-acetamidoacrylate ethyl ester or methyl ester (1.1 equiv), the solution was heated at 80 °C or refluxed for various times (see Table 1). The solvent was removed in a vacuum, and the residue was hydrolyzed with 6 N HCl at reflux for 6 h. Compound **4** was purified by flash chromatography on silica gel with $MeOH/CHCl_3/NH_3/H_2O$ = 22:20:5:5 as eluent.

The same procedure was followed for the condensation between N^{α}-formyllisine and 2-acetamidoacrylate methyl ester; the product obtained was the N^{δ}-acetyl derivative on the lysino residue of LAL: ^{1}H NMR (300 MHz, D_2O) δ 4.5 (1 H, CH-α ala), 4.0 (1 H, CH-β ala), 3.8 (1 H, CH-β ala), 3.6 (1 H, CH-α lys), 3.4 (2 H, CH_2-ϵ lys), 3.2 (1 H, CH-β lys), 3.0 (1 H, CH-β lys), 2.1 (3 H, $COCH_3$), 1.7 (4 H, CH_2-γ and δ lys).

RESULTS AND DISCUSSION

Synthesis. The strategy generally applied to the synthesis of cross-linked amino acids resembles their formation in food: the approach involves the reaction of the nucleophilic group of an N^{α}-protected amino acid with a protected form of dehydroalanine (DHA). A literature method was applied to the synthesis of LAL (Pintauro et al., 1985): N^{α}-formyllysine (Ledger et al., 1965; Wolf et al., 1952) was treated with 2-acetamidoacrylate ethyl ester **1a**, a suitable form of N-protected dehydroalanine (Hellmann et al., 1958), in the presence of 0.3 N NaOH at room temperature to give crude LAL that was purified by flash chromatography on silica gel using the mixture $MeOH/CHCl_3/NH_3/H_2O$ = 22:20:5:5 as eluent, a method that appeared to be more efficient than ion exchange chromatography (Henle et al., 1993) for LAL purification. Crystallization was avoided because, owing to the different solubilities in water of the two diastereoisomers, it induces a selective enrichment in the less soluble *S,R* isomer (Tas and Kleipool, 1976). The yield of the condensation and hydrolysis calculated on purified LAL was 41%.

The same scheme was adopted for the preparation of OAL (Scheme 1). The synthesis of N^{α}-formylornithine (**2a**) began with the formation of the ornithine copper salt by treatment with $CuCO_3 \cdot Cu(OH)_2$ followed by benzyloxycarbonylation (group Cbz) of its N^{δ} with benzyloxycarbonyl chloride and NaOH. The subsequent treatment with EDTA gave N^{δ}-Cbz-ornithine (**3**) in 66% yield. The formation of N^{α}-formyl-N^{δ}-Cbz-ornithine was accomplished by formylation with formic acid and acetic anhydride, and the N^{δ}-Cbz group was then removed by hydrogenation over 10% Pd/C in MeOH to give **2a** in 80% yield. The condensation of **2a** and **1a** in 0.3 N NaOH at room temperature, followed by the removal of the protective groups with HCl, gave crude OAL, which was purified by flash chromatography on silica gel as indicated for LAL. The condensation/deprotection yield was 18%.

Table 2. NMR Data of Compound 4

position	^{1}H (ppm)	^{13}C one-bond (ppm)	^{13}C long-range (ppm)
CO ala		178.75	
CO orn		176.80	
CO acetyl		169.44	
α ala	4.6	60.31	55.54, 169.44, 178.75
β ala	4.2	55.54/55.50[a]	60.31, 169.44, 178.75
β ala	3.9	55.54/55.50[a]	60.31, 169.44, 178.75
α orn	3.8	56.94/56.83[a]	30.01, 176.80
δ orn	3.5	47.85	30.01, 55.54, 169.44
CH_3	2.3	13.91	169.44
β orn and γ orn	1.7−1.9	30.01/29.85[a] and 25.15/24.83[a]	47.85, 56.94, 176.80

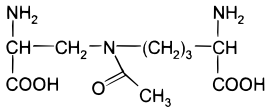
[a] Values refer to the two diastereoisomers.

As very recently lysinomethylalanine was prepared starting from *N*-acetyldehydroaminobutyric acid methyl ester (Walter et al., 1994) and histidinoalanine from DHA methyl ester **1b** (Henle et al., 1993), a commercial derivative, we decided to try to use **1b** (Scheme 1) for the preparation of OAL. However, as at room temperature the reaction was very slow, it was decided to heat the reaction mixture at reflux, but in these conditions an unexpected compound **4** was obtained. Some trials with various time and temperature combinations (Table 1) indicated that compound **4** is favored by heating and that the best reagents to obtain OAL (yield 28%) are **2b** and **1b**, which must be stirred for 3 days at room temperature.

The structure of compound **4** was elucidated by ^{1}H NMR and ^{13}C NMR (Table 2). The ^{1}H NMR spectrum shows the presence of a singlet (3 H, 2.3 ppm), and in the ^{13}C NMR spectrum three C=O signals (178.75, 176.80, 169.44) were detected; all of these data and the low value of the carbon chemical shifts suggested the presence of an acetyl group attached to a nitrogen atom and two carboxylic groups.

In a COSY ^{1}H−^{1}H spectrum two distinct sets of sequential relationships were found, one of three protons and the other of seven protons. The former refers

to a CH_2-CH structure, clearly indicated by the large geminal coupling constant (12.0 Hz) between the two well-separated hydrogens of the CH_2 group; the latter refers to a linear four-carbon chain. The chemical shifts of the two terminal CH_2 groups of both chains (4.2 ppm $CH_2\beta$-ala, 3.5 ppm $CH_2\delta$-orn) indicate that they are connected to a nitrogen atom. The position of the acetyl group was established by 2D heteronuclear HMBC and HMQC experiments. In particular, in the HMBC spectrum two connections between the CH_2 groups attached to the nitrogen and the carbonyl of the acetyl group were detected. On the basis of the foregoing, the structure of compound **4** is the N^{δ}-acetyl derivative on the ornithino



N^{δ}-acetylated OAL

residue of OAL. This was confirmed by FAB mass spectrometry, which gave an intense ion at *m/z* 244 corresponding to the loss of H_2O from the quasi-molecular ion M^{+1}.

The removal of the protecting groups had been achieved with 6 N HCl, conditions in which compound **4** is stable. Also, 12 N HCl is practically ineffective, whereas it is possible to obtain OAL from **4** by hydrolysis with 20% NaOH.

As indicated above, compound **4** is formed when the condensation between compounds **1b** and **2a** is conducted at high temperature. In these conditions the competitive acetylation of the very nucleophilic secondary amino group of OAL becomes possible, probably by transacetylation from **1b**, which in the meanwhile is decomposed to pyruvic acid methyl ester. Of course, the detection of compound **4** after hydrolysis is allowed by its unexpected stability toward acids.

With treatment of N^{α}-formyllysine with 2-acetamidoacrylate methyl ester **1b** at high temperature, the formation of a compound analogue to **4** was observed.

NMR Studies. As the structure of the substrates involved in the condensation suggests a scarce kinetic resolution in the addition of lysine and ornithine to DHA, it was expected that the ratio between the isomers could be close to 1, as observed by Liardon et al. (1991) and Friedman et al. (1985), who have studied the diastereoisomeric composition of bound LAL in processed proteins. To establish the diastereoisomeric ratio, it is necessary to use a suitable analytical tool. Cross-linked amino acids are very polar and have a very low absorbance over 210 nm: all of the chromatographic methods require a derivatization at least of the amino groups. In the past, Liardon et al. (1991) and Friedman et al. (1985) have proposed a GC/MS method after derivatization of LAL as *N*-perfluoropropionylisopropyl ester. We decided, instead, to develop a direct NMR method for the determination of the diastereoisomeric ratio without derivatization.

The analysis was started on OAL: with proton resonance it was impossible to observe any peak separation due to the two diastereoisomers even at 600 MHz; on the contrary, with ^{13}C NMR experiments it was possible to observe a 0.01−0.02 ppm separation of most of the peaks also with a 300 MHz instrument (Table 3).

These results prompted us to focus our attention also on LAL (Table 4). It was decided to compare the ^{13}C

Table 3. NMR Data of Ornithinoalanine

position	^1H (ppm)	^{13}C (ppm)
CO ala		176.26
CO orn		174.00
α ala	3.8	56.68
α orn	3.6	52.23
β ala	3.2	50.05/50.00[a]
δ orn	3.0	49.22/49.18[a]
β orn and γ orn	1.5−1.8	29.98 and 24.28/24.22[a]

[a] Values refer to the two diastereoisomers.

Table 4. NMR Data of Lysinoalanine

position	^1H (ppm)	^{13}C (ppm)
CO ala		175.89
CO lys		173.61
α ala	4.01	56.29
α lys	3.77	52.11
β ala	3.46	50.76
ϵ lys	3.19	49.11/49.09[a]
γ lys	1.93	24.43/24.42[a]
β lys	1.80	32.43
δ lys	1.51	27.98/27.97[a]

[a] Values refer to the two diastereoisomers.

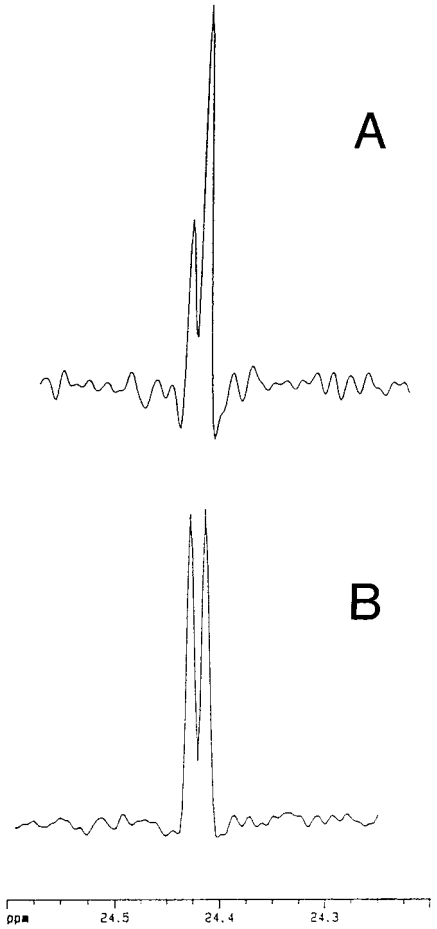


Figure 2. Separation of the signals of C-γ lys of LAL and their relative intensities in (A) a commercial sample and (B) a sample synthesized by us.

NMR spectra of a commercial standard and a sample synthesized by us that had been purified by column chromatography on silica gel and not by crystallization from water. Some signals are clearly resolved in two peaks, for example, that at 24.4 ppm (Figure 2). A very interesting observation is that while in our synthetic sample the peak ratio is ∼1 (Figure 2B), in the com-

mercial standard the ratio is 60:40 (Figure 2A), probably due to a selective crystallization of the less soluble *S*,*R* diastereoisomer (Tas and Kleipool, 1976; Pintauro et al., 1985).

HPLC Analysis. The same samples were submitted to reverse phase HPLC analysis with the method of Pellegrino et al. (1996), which requires derivatization with FMOC-Cl and purification on SPE cartridges. With OAL a broad peak was observed with retention time of 27.3 min, whereas LAL produces two resolved peaks that have a 1:1 ratio in the synthetic sample and a 60:40 ratio in the commercial sample, reproducing exactly the ratios determined by NMR. In the original paper Pellegrino et al. (1996) suggested that the two peaks corresponded to two different FMOC monoderivatives. The coincidence of the ratio determined by NMR without derivatization and the very large excess of FMOC-Cl used [see the discussion of Wood-Rethwill and Warthensen (1980)] suggest an exhaustive acetylation of the three amino groups of LAL and that the two peaks are due to the two diastereoisomers.

CONCLUSIONS

In conclusion, we have presented an optimized procedure for the synthesis of OAL: to our knowledge the yields obtained are the best ever reported. In fact, neither Mirò et al. (1968), who described a synthesis of OAL by condensation between N^{α}-acetyl-L-ornithine and 2-acetamidoacrilate ethyl ester, nor Febrer and Mirò (1969), who condensed 2-(2-phenylacetamido)acrylic acid and N^{α}-carbobenzyloxy-L-ornithine, gave any indication of the yields.

We have also demonstrated that high-field ^{13}C NMR is a good tool for the determination of the diastereoisomeric ratio of these kinds of products: of course, the proposed methodology is applicable to standard samples and not to the analysis of unnatural amino acids in foods.

In the case of foodstuffs, the best analytical method is reverse phase HPLC after FMOC derivatization: this procedure appears to be superior to the dansyl one (Warthesen and Wood-Rethwill, 1984; Moret et al., 1994) because, besides having a very high sensitivity, it permits the determination of the diastereoisomeric ratio of LAL.

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