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A convenient synthesis of some cross-linked amino acids and their diastereoisomeric characterization by nuclear magnetic resonance

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Abstract

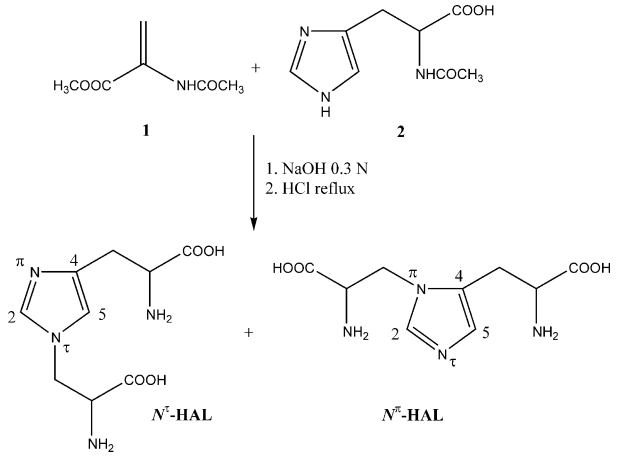
Cross-linked amino acids may be formed in foods submitted to thermal or alkaline conditions during processing. The present paper presents a convenient procedure for the preparation of some known components of this class: histidinoalanine (HAL) and methyl-lysinoalanine (Me-LAL), and of another cross-linked amino acid never prepared before, methyl-ornithinoalanine (Me-OAL). The addition of ornithine and lysine onto the double bond of *N*-acetyldehydroaminobutyric acid methyl ester, the unsaturated intermediate for the synthesis of Me-LAL and Me-OAL, was diastereospecific and gave only the (2*S*,3*R*) or (2*R*,3*S*) alanine derivatives. This procedure is highly reproducible and allows production of pure standards, useful for the quantification of these compounds in foods or for physiological and toxicological studies. In addition, an efficient method (by nuclear magnetic resonance) for the determination of the diastereoisomeric ratio of these cross-linked amino acids is presented. © 2002 Published by Elsevier Science Ltd.

Keywords: Cross-linked amino acids; Histidinoalanine; Methyl-lysinoalanine; Methyl-ornithinoalanine; NMR analysis; Diastereoisomers

1. Introduction

The thermal treatments, necessary for the industrial production and microbiological stabilization of foods, may modify the structure of the side chains of protein-bound amino acids. One of the possibilities is the formation of cross-linked amino acids (Friedman, 1999; Maga, 1984), because a reactive dehydroalanine residue may be formed through elimination of a leaving group from serine, *O*-phosphorylserine, *O*-glycosylserine, or cystine and may undergo Michael addition by the nucleophilic group of another amino acid in a suitable position of the protein chain. For example, the ε-amino group of lysine may react to give a secondary amine, which is normally indicated with the trivial name of lysinoalanine (LAL, N^{ε}-(2-amino-2-carboxyethyl)-lysine).

Analogous reactions may involve ornithine to give ornithinoalanine [OAL, N^{δ}-(2-amino-2-carboxyethyl)-ornithine], cysteine to give lanthionine (LAN), and histidine to produce histidinoalanine (HAL) (Finley & Friedman, 1977). Both nitrogen atoms of histidine may react, giving rise to the regioisomers N^{π}-HAL (α,α'-diamino-1H-imidazole-1,5-dipropanoic acid) and N^{τ}-HAL



Scheme 1. Synthesis of N^{τ}-HAL and N^{π}-HAL.

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(α,α′ - diamino - 1H - imidazole - 1,4 - dipropanoic acid) (Scheme 1; Henle, Walter, & Klostermeyer, 1993).

As well as serine, threonine also (Maga, 1984) may undergo elimination to give a dehydroaminobutyric acid residue (i.e. methyl-dehydroalanine) that reacts with the same amino acids to give methyl-lysinoalanine [Me-LAL, N^{ε}-(2-amino-2-carboxy-1-methylethyl)-lysine], methyl-ornithinoalanine [Me-OAL, N^{δ}-(2-amino-2-carboxy-1-methylethyl)-ornithine], methyl-lanthionine (Me-LAN), and methyl-histidinoalanine (Me-HAL), respectively (Fig. 1).

The formation of cross-linked amino acids does not involve the participation of reducing sugars and is particularly extensive when proteins are submitted to aqueous alkali treatments, such as those used in the preparation of soy protein concentrates and in the recovery of proteins from cereal grains, milling by-products, and oilseeds, such as cottonseeds, peanuts, safflower seeds and flaxseeds, and in the separation of sodium caseinate. Other alkali procedures are commonly used for destroying microorganisms, preparing peeled fruits, and inducing fibre-forming properties in textured soybean proteins (Friedman, 1999).

The stability of these compounds in the acidic conditions of protein hydrolysis, makes them useful as molecular markers of the technology applied during food preparation: for example, an analytical method, based on the quantification of LAL, has been proposed for distinguishing natural Mozzarella cheese from imitations (Pellegrino, Resmini, de Noni, & Masotti, 1996).

LAL is certainly the most investigated cross-linked amino acid, but other studies have been focused also on histidinoalanine, detected for the first time in soybean protein isolates treated with alkali (Finley & Friedman, 1977). The HAL contents of heated proteins (bovine serum albumin, bovine tendon collagen, and casein) may be greater than their LAL contents (Fujimoto, 1984), whereas the amounts detected in various milk-protein-containing foods, such as heated skim milk, sterilized milk, and baby formulas, are comparable (Finley & Friedman, 1977; Henle, Schwarzenbolz, & Klostermeyer, 1996; Siegl, Schwarzenbolz, & Henle, 2000). HAL is also present in calcium-binding phosphoproteins derived from extrapallial fluids of certain bivalve molluscs (Marsh & Sass, 1985; Sass & Marsh, 1984). Moreover, it has provoked great interest in medicine, because it has been correlated with the aging of collagen and connective tissues (Fujimoto, Hirama, & Iwashita, 1982) and the formation of brown cataractous lens (Kanayama, Miyanaga, Horiuchi, & Fujimoto, 1987).

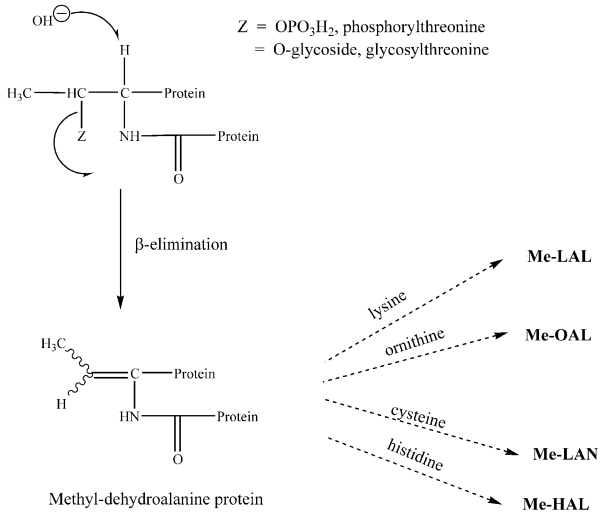


Fig. 1. Formation of Me-LAL, Me-OAL, Me-HAL and Me-LAN.

As for the other cross-linked amino acids, the possible formation of Me-LAL and Me-HAL has been studied in various milk products (Walter, Henle, Haeβner, & Klostermeyer, 1994), whereas Me-OAL has never been cited before in the literature.

Several authors have studied the digestibility of proteins treated with alkali (Savoie, Parent, & Galibois, 1991) and the toxicological and nutritional consequences of LAL formation in foods (Maga, 1984 and literature cited; Friedman, 1999 and literature cited; Jonker, Woutersen, & Feron, 1996): LAL has been shown to provoke lesions in rat kidney cells, causing nephrocytomegaly (Friedman & Pearce, 1989). The fact that most of the investigations were limited to LAL, is certainly related to the absence of commercial standards for the other cross-linked amino acids that has prevented systematic studies of their physiological and toxicological properties.

Although the starting amino acids are enantiomerically pure, in foods these compounds are diastereomeric mixtures because the enantiomerically pure nucleophile may attack the double bond of dehydroalanine on both sides. Thus, for example, LAL is a mixture of (*S*)-lysino-(*S*)-alanine (*S,S*)-LAL and (*S*)-lysino-(*R*)-alanine (*S,R*)-LAL, and OAL is a mixture of (*S*)-ornithino-(*S*)-alanine (*S,S*)-OAL and *S*-ornithino-*R*-alanine (*S,R*)-OAL, being the configuration of lysine and ornithine, respectively, preserved during their formation. This is physiologically relevant because it has been demonstrated that the two diastereoisomers of LAL have different affinities for copper (II) and cobalt (II): in particular, the greater observed nephrotoxicity of (*S,R*)-LAL has been related to its higher Cu(II) affinity (Friedman & Pearce, 1989).

Recently, we have reported an improved synthesis of LAL and OAL and an analytical method for the determination of their diastereoisomeric ratio by ^{13}C-NMR (Boschin, Scaglioni, & Arnoldi, 1999), which is particularly reliable because it does not require any derivatisation. In this paper, both procedures were extended to HAL, Me-LAL, and Me-OAL, with the aim of producing standard samples with known diastereoisomeric

ratio to be used for the quantification of these compounds in foods.

2. Materials and methods

2.1. Materials

N^{α}-acetyl-S-lysine, N^{α}-acetyl-S-ornithine and N^{α}-acetyl-S-threonine methyl ester were purchased from Bachem, N^{α}-acetyl-S-histidine monohydrate from Aldrich, 2-acetamidoacrylate methyl ester **1** from Lancaster, *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride from Fluka. *N*-acetyldehydroaminobutyric acid methyl ester **3** was prepared as described by Henle et al. (1993).

2.2. Nuclear magnetic resonance (NMR)

^{1}H-NMR and ^{13}C-NMR spectra were recorded in D_2O, using tetramethylsilane as internal standard on a Bruker AMX-300 (300 MHz). Chemical shifts were expressed in parts per million (δ).

2.3. Mass spectrometry

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

2.4. Preparation of the two isomers of histidinoalanine, N^{τ}- and N^{π}-(2-amino-2-carboxy-ethyl)-S-histidine

NaOH (0.6 N, 1.6 ml) was added to a 0.6 N solution of N^{α}-acetyl-S-histidine monohydrate (200 mg, 0.93 mmol) in distilled water (1.6 ml). After the addition of 2-acetamidoacrylate methyl ester 1 (198 mg, 1.38 mmol) at 0 °C, the solution was stirred at room temperature for 10 days. The solvent was removed under vacuum, and the residue was hydrolysed with 6 N HCl (5 ml) at reflux for 6 h. The crude residue was purified by flash chromatography on silica gel with $MeOH:CH_2Cl_2:H_2O:NH_3 = 5:4.5:1:0.5$ as eluent. The pure isomers were recovered as oils, which were solidified by addition and evaporation of several portions of ethanol, giving white solids. N^{τ}-HAL was obtained in 66% yield and N^{π}-HAL in 28% yield. N^{τ}-HAL: m.p. = 208–215 °C (dec.); MS-FAB (thioglycerol) *m*/*z* (%): 243 (48, M + 1). N^{π}-HAL: m.p. = 206–210 °C; MS-FAB (thioglycerol) *m*/*z* (%): 243 (100, M + 1).

2.5. Preparation of Me-LAL, N^{ε}-(2-amino-2-carboxy-1-methyl-ethyl)-S-lysine

A 0.6 N solution of N^{α}-acetyl-S-lysine (74 mg, 0.39 mmol) in distilled water (650 μl) was prepared, and NaOH was added (0.6 N, 650 μl). After the addition of compound **3** (80 mg, 0.51 mmol) at 0 °C, the solution was stirred at room temperature for 10 days. The solvent was removed under vacuum, and the residue was hydrolysed with 6 N HCl (5 ml) at reflux for 6 h. The crude product was purified by flash chromatography on silica gel with $MeOH:CHCl_3:H_2O:NH_3 = 22:20:5:5$ as eluent. The pure product was recovered as an oil that was treated with several portions of ethanol, to give a white solid. Me-LAL (mp 196 °C) was obtained with 33% yield, whereas other column fractions contained the N^{ε} acetyl derivative *N*-Ac-Me-LAL (yield = 15%).

2.6. N-Ac-Me-LAL

mp 200 °C; ^{1}H-NMR (300 MHz, D_2O): 1.2 (3H, d, CH_3), 1.3 (2H, m, CH_2 γ-lys), 1.55 (2H, m, CH_2 δ-lys), 1.75 (2H, m, CH_2 β-lys), 2.15 (3H, s, CH_3CO), 3.3–3.5 (2H, m, CH_2 ε-lys), 3.6 (1H, m, CH β-ala), 4.0 (1H, d, J = 9 Hz, CH α-ala), 4.1 (1H, m, CH α-lys). ^{13}C-NMR (300 MHz, D_2O): 14.37, 20.93, 23.96/24.27[a], 28.97/29.05[a], 32.51/32.63[a], 45.41, 56.92, 63.81/63.91[a], 67.24, 168.34, 176.91, 178.34 ([a] values refer to two diastereoisomers).

2.7. Preparation of Me-OAL, N^{δ}-(2-amino-2-carboxy-1-methyl-ethyl)-S-ornithine

A 0.6 N solution of N^{α}-acetyl-S-ornithine (77 mg, 0.44 mmol) in distilled water (750 μl) was prepared, and 0.6 N NaOH was added (750 μl). After the addition at 0 °C of compound **3** (90 mg, 0.57 mmol), the solution was stirred at room temperature for 10 days. The solvent was removed under vacuum, and the residue was hydrolysed with 6 N HCl (5 ml) at reflux for 6 h. The crude product was purified by flash chromatography on silica gel with $MeOH:CHCl_3:H_2O:NH_3 = 22:20:5:5$ as eluent. Unfortunately, Me-OAL was obtained in mixture with the N^{δ}-acetylated product (*N*-Ac-Me-OAL). Without separation, the mixture was dissolved in 5 N NaOH and heated under reflux for 6 h. The solvent was removed in a vacuum and a yellowish solid was obtained. Purification was performed on the cation-exchange resin Dowex 50 × 8 (H^+ form). Me-OAL (mp 193 °C) was obtained in 40% yield and the N^{δ}-acetyl derivative *N*-Ac-Me-OAL in 30% yield.

2.8. N-Ac-Me-OAL

mp 195 °C; ^{1}H-NMR (300 MHz, D_2O): 1.4 (3H, d, CH_3), 1.6–1.8 (4 H, m, CH_2 β-orn + CH_2 γ-orn), 2.2 (3H, s, CH_3CO), 3.4 (2H, m, CH_2 δ-orn), 3.7 (1H, m, CH β-ala), 4.05 (1H, d, J = 9 Hz, CH α-ala), 4.15 (1H, t, CH α-orn). ^{13}C-NMR (300 MHz, D_2O): 14.38, 21.07, 25.51, 30.04, 45.25, 60.10, 64.09, 67.45, 168.54, 176.51, 178.49.

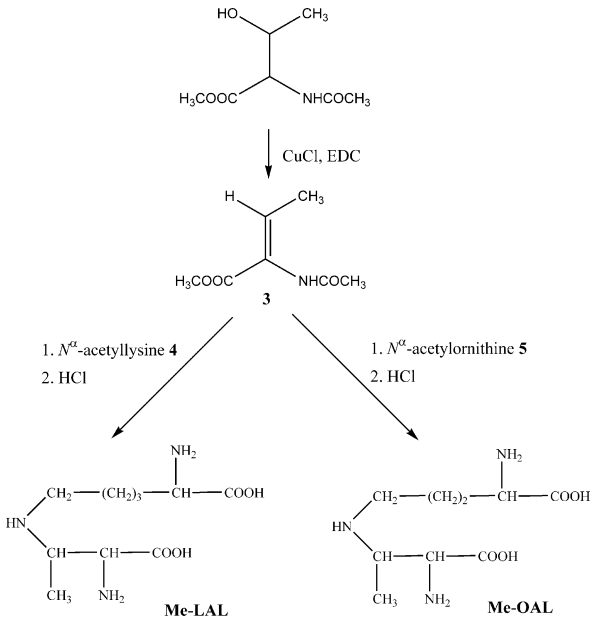
3. Results and discussion

3.1. Synthesis

The strategy generally applied for 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). Following this strategy, we have recently reported an improved method for the preparation of LAL and OAL (Boschin et al., 1999). The preceding methodology (Pintauro, Philipossian, Finot, & Lee, 1985) was based on the reaction of N^{α}-formyl amino acids with 2-acetamidoacrylate ethyl ester, a suitable *N*-protected form of dehydroalanine. Our modification consisted in the use of the commercial derivatives N^{α}-acetyl-S-lysine, N^{α}-acetyl-S-ornithine and 2-acetamidoacrylate methyl ester **1**. However, these reagents react very slowly and, when heating at reflux was used in order to increase the reaction rate, acetylation of the N^{ε} of lysine (or N^{δ} of ornithine) took place and the yields strongly impaired. Only stirring the reagents at room temperature for very long times (some days) allowed satisfactory yields and purity.

It was decided to apply the same procedure to the synthesis of HAL, the starting reagents being N^{α}-acetyl-S-histidine and acetamidoacrylate methyl ester **1** (Scheme 1). The reaction of these two compounds at room temperature for 10 days in the presence of 0.3 N NaOH increased the yields up to 94% while a preceding literature procedure (Henle et al., 1993), which included treatment for 24 h at 90 °C at pH 9, gave only 50% yields. Both nitrogen atoms of the imidazole of histidine can react, giving rise to two regioisomers, N^{τ}-HAL and N^{π}-HAL: at room temperature the N^{τ}-HAL/N^{π}-HAL ratio is 5/2, whereas it becomes 7/1 at 90 °C (Henle et al., 1993). Other differences in the two procedures are related to the separation and purification of the two regioisomers: Henle et al. (1993) used ion-exchange chromatography, while we preferred a flash silica gel column, using $MeOH:CH_2Cl_2:H_2O:NH_3 = 5:4.5:1:0.5$ as eluent. This procedure was faster and gave products with a purity higher than 97%, as shown by spectroscopic and chromatographic analysis (NMR, TLC). The structures of the two regioisomers were assigned with ROESY experiments, similar to those reported by Henle et al. (1993).

The second part of the research was devoted to Me-LAL and Me-OAL. In the case of these methyl derivatives, the unsaturated acceptor was *N*-acetyldehydroaminobutyric acid methyl ester **3** that was reacted with N^{α}-acetyl-S-lysine **4** and N^{α}-acetyl-S-ornithine **5**, respectively (Scheme 2). The unsaturated ester **3** was prepared by treating N^{α}-acetyl-S-threonine methyl ester with copper(I) chloride and *N*-ethyl-*N*′-(3-dimethylamino-propyl)carbodiimmide hydrochloride (Henle et al., 1993). The very simple NMR spectrum suggested that only one diastereoisomer had been produced, whose correct (*E*) structure was assigned by comparison of our data with those of Miyata, Shinada, Ninomiya, and Naito (1990) who converted (*Z*)-2-alkenoic esters in to (*E*)-isomers. With the sequence of reaction already applied to HAL, the addition of compounds **4** and **5** onto **3**, followed by deprotection with HCl or NaOH, gave Me-LAL and Me-OAL in 43 and 44% yields, respectively (purity higher than 97%). As already observed in the case of LAL and OAL (Boschin et al., 1999), in both condensations, minor amounts of N^{ε}-acetylated Me-LAL and N^{δ}-acetylated Me-OAL were formed: they were stable towards HCl hydrolysis and could be hydrolysed only with bases. These products were completely characterized by NMR spectroscopy (see Section 2). Their yields are in direct correlation with the reaction temperature and they are formed by competitive acetylation of the very nucleophilic secondary amino group, probably by trans-acetylation from compound **3**, which, in the meanwhile, is decomposed to 3-ketobutanoic acid methyl ester. The same phenomenon was also observed in the synthesis of LAL and OAL (Boschin et al., 1999).



Scheme 2. Synthesis of methyl-LAL and methyl-OAL.

3.2. Determination of the diastereomeric ratio with NMR experiments

The structure of the substrates involved in the condensation suggests a scarce kinetic resolution in the addition of these nucleophiles onto the unsaturated derivatives **1** and **3**, as has been already observed either by the addition of lysine and ornithine to compound **1** (Boschin et al., 1999), for the synthesis of LAL and

OAL, or by other authors, who have studied the diastereoisomeric composition of bound LAL in processed foods (Friedman & Liardon, 1985; Liardon, Friedman, & Philippossian, 1991). To establish the diastereoisomeric ratio, we have used a ^{13}C-NMR methodology (Boschin et al., 1999), which has the advantage of not requiring any derivatisation of LAL and OAL, a fact particularly useful while determining the diastereoisomeric ratio of analytical standards. The application to LAL verified that a commercial sample had a 60:40 composition, whereas another, synthesized by us without crystallization from water, had a 50:50 ratio and indicated that our sample was a better standard, because it clearly matched the LAL composition in processed foods determined by HPLC after derivatisation (Friedman & Liardon, 1985; Liardon et al., 1991; Pellegrino et al., 1996; Arnoldi, Rinaldi, Boschin, & D'Agostina, 2000).

This method was extended to the determination of the diastereomeric composition of HAL, Me-LAL and Me-OAL. With ^{1}H-NMR, even at 600 MHz, it was not possible to observe any significant chemical shift difference and only ^{13}C-spectra showed a 0.01–0.02 ppm separation of some peaks. In the case of N^{τ}-HAL (Fig. 2) the split carbons were C-2 (Fig. 3) and C-4 of the imidazole ring and CH_2-β of histidine (Table 1) whereas in the case of N^{π}-HAL, the split signals were those of C-2 and C-5 of the imidazole ring (Table 2). These samples had a diastereoisomeric ratio equal to 1.

Me-LAL and Me-OAL have three stereo centres; therefore four diastereoisomers are expected when (*S*)-ornithine and (*S*)-lysine are reacted with the unsaturated substrate **3**. Nevertheless, the ^{1}H-NMR showed only one signal, which could be attributed to the H-2 of alanine, indicating that only one couple of diastereoisomers on the alanine residue had been formed. This hydrogen had a coupling constant of 9 Hz with H-3, indicating that the two H atoms have an anti arrangement.

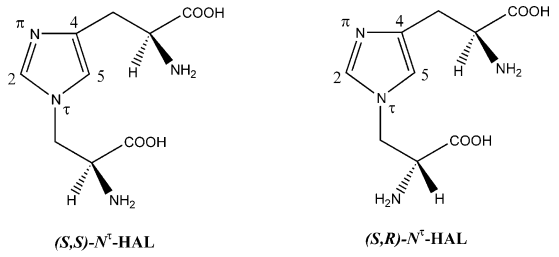


Fig. 2. Structure of N^{τ}-HAL diastereoisomers.

Table 1
NMR data of N^{τ}-HAL

Position	^{1}H (ppm)	^{13}C (ppm)
CO his		176.43
CO ala		174.09
C-4		141.24/141.21[a]
CH-2	7.55	138.29/138.25[a]
CH-5	6.8	121.10
α his	3.85	57.31
β his	2.9–3.1	31.27/31.21[a]
α ala	4.0	57.56
β ala	4.4	49.59

[a] Values refer to the two diastereoisomers.

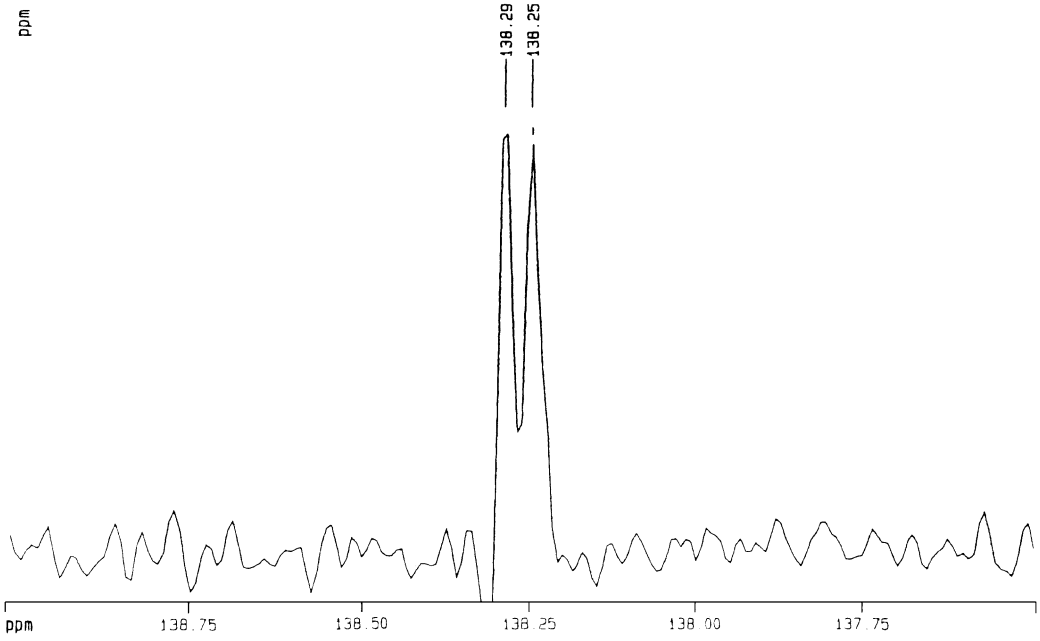


Fig. 3. ^{13}C-NMR of C-2 of N^{τ}-HAL.

Table 2
NMR data of N^{π}-HAL

Position	^{1}H (ppm)	^{13}C (ppm)
CO his		175.25
CO ala		173.22
C-4		131.02
CH-2	7.65	140.25/139.93[a]
CH-5	6.9	124.78/124.47[a]
α his and α ala	3.9	55.77 and 56.66
β his	3.0–3.2	27.20
β ala	4.35	48.24

[a] Values refer to the two diastereoisomers.

Table 3
NMR data of Me-LAL

Position	^{1}H (ppm)	^{13}C (ppm)
CO ala		177.19
CO lys		173.03
α ala	4.0	57.05
β ala and α lys	3.5–3.7	56.68/56.38[a] and 54.89
CH_3 ala	1.2	14.05
β lys	1.8	32.43
γ lys	1.3	24.14/24.10[a]
δ lys	1.65	28.00/27.91[a]
ε lys	3.1	48.11

[a] Values refer to the two diastereoisomers.

Table 4
NMR data of Me-OAL

Position	^{1}H (ppm)	^{13}C (ppm)
CO ala		176.06
CO orn		174.69
α ala	3.7	60.02
β ala	3.4	56.70/56.20[a]
CH_3	1.1	14.42/14.24[a]
α orn	3.55	57.39
β orn and γ orn	1.6–1.9	25.53 and 24.85/24.55[a]
δ orn	2.9	47.70

[a] Values refer to the two diastereoisomers.

MM2 minimization and molecular dynamics, performed with Chem3D® Pro by CambridgeSoft, indicated that H-2 and H-3 can assume an arrangement consistent with the experimental coupling constant only when the alanine residue has (2*R*,3*S*) or (2*S*,3*R*) configurations. The diastereospecificity of the Michael addition of ornithine or lysine on the (*E*) unsaturated substrate **3** is probably governed by thermodynamic factors.

Having established the relative stereochemistry of C-2 and C-3 of the alanine residue, ^{13}C-NMR experiments distinguished out some signals useful for the determination of the ratio between the (2′*S*,2*R*,3*S*) and (2′*S*,2*S*,3*R*) diastereoisomers. In Me-LAL, the carbons whose chemical shifts have a separation of 0.01–0.02 ppm are C-δ and C-γ of lysine (Table 3) whereas, in Me-OAL, they are C-β of alanine and C-γ of ornithine. In the latter, a good separation is also shown by the signals of the CH_3 group (Table 4). Either the diastereoisomeric ratios of Me-OAL or Me-LAL were 1.

4. Conclusions

In conclusion, this paper presents a convenient procedure for the preparation of N^{τ}-HAL, N^{π}-HAL, Me-LAL, and Me-OAL. This procedure is highly reproducible and yields highly pure standards, useful for the quantification of these compounds in foods or for physiological and toxicological studies. In the synthesis of Me-LAL and Me-OAL, the addition of ornithine and lysine on to *N*-acetyldehydroaminobutyric acid methyl ester is stereospecific for the formation of (2*S*,3*R*) or (2*R*,3*S*) alanine residues.

It is noteworthy that our low temperature procedure is particularly useful for the preparation of N^{π}-HAL and we have demonstrated that, in the addition of histidine onto the double bond of dehydroalanine, the relative amount of the two possible regioisomers depends on the reaction conditions. N^{π}-HAL has been cited in literature only five times (Chin, 1984; Henle et al., 1996; Marsh, 1986; Marsh & Sass, 1985; Sass & Marsh, 1984) and, generally, it is not quantified in foods, because, at least in dairy products, it is superimposed with other peaks (Henle et al., 1996). Therefore the reported quantifications in foods are probably unpredictably low, considering that the relative amounts of the two regioisomers seem to depend on the conditions.

In addition, it is confirmed that ^{13}C-NMR is an efficient method for the determination of the diastereoisomeric ratio of these cross-linked amino acids in standard samples, useful for their quantification in foods.

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