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Reaction of *Nα*-acetyl-L-histidine with diazomethane: A model esterification reaction of carboxylic groups in the presence of imidazole rings

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RESUMEN

Reacción de la *Nα*-acetil-L-histidina con diazometano: Un modelo de la reacción de esterificación de grupos carboxílicos con diazometano en presencia de anillos de imidazol.

La reacción de la *Nα*-acetil-L-histidina con diazometano fue estudiada con objeto de conocer el comportamiento de la reacción de esterificación de un grupo carboxílico en presencia de un anillo de imidazol. La reacción produjo el esperado éster metílico de la *Nα*-acetil-L-histidina **(1)** como producto mayoritario. Sin embargo, también se observó la formación de cantidades importantes de los ésteres metílicos de la [*S*]-*Nα*-acetil-1-metilimidazol-4-alanina **(2)** y la [*S*]-*Nα*-acetil-1-metilimidazol-5-alanina **(3)**. Estos compuestos que pudieron ser detectados por electroforesis capilar y cromatografía en capa fina, fueron separados por cromatografía en columna e identificados por cromatografía de gases acoplada a espectrometría de masas, y por espectroscopía de resonancia magnética nuclear de ^{1}H y ^{13}C. Las estructuras de los compuestos **1-3** fueron confirmadas por electroforesis capilar tras hidrólisis ácida. Los resultados obtenidos indican que el uso de diazometano produce el derivado metílico del anillo heterocíclico en adición al éster metílico. Esta reacción debe ser tenida en cuenta al preparar derivados para análisis cromatográfico.

PALABRAS-CLAVE: Diazometano – Metilación del imidazol – Electroforesis capilar – Productos de reacción entre proteínas y lípidos peroxidados.

SUMMARY

Reaction of *Nα*-acetyl-L-histidine with diazomethane: A model esterification reaction of carboxylic groups in the presence of imidazole rings.

The reaction of *Nα*-acetyl-L-histidine with diazomethane was studied in order to analyze the esterification reaction of a carboxylic group in the presence of an imidazole ring. The reaction produced the expected *Nα*-acetyl-L-histidine methyl ester **(1)** as a major product. However, important amounts of [*S*]-*Nα*-acetyl-1-methylimidazole-4-alanine methyl ester **(2)** and [*S*]-*Nα*-acetyl-1-methylimidazole-5-alanine methyl ester **(3)** were also produced. These compounds, which could be detected by capillary electrophoresis (HPCE) and thin layer chromatography, were fractionated by column chromatography and identified by gas chromatography coupled with mass spectrometry (GC-MS), and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. Structures for compounds **1-3** were confirmed by HPCE after acid hydrolysis. These results indicated that the use of diazomethane produces the methyl derivative of the heterocyclic ring in addition to the methyl ester. This reaction should be considered when preparing derivatives for GC-MS analysis.

KEY-WORDS: Diazomethane - Imidazole methylation - Capillary electrophoresis - Oxidized lipid/amino acid reaction products.

1. INTRODUCTION

Diazomethane is a powerful methylating agent for acidic compounds such as carboxylic acids, phenols, and enols (Pizey, 1974). It has long been used in lipid analysis to quantitatively prepare more volatile, less polar derivatives, previously to be studied by gas chromatography (Hammond, 1993). Usually small-scale procedures are recommended since if sensible precautions are taken, the risks to health are slight, while methyl esters are produced rapidly with virtually no artefact formation and using very mild conditions (Christie, 1993).

The high reactivity of diazomethane, which is one of the reasons for its use as a derivatizing agent, can be at the same time its limitation. Thus, diazomethane has been described to react with carbonyl compounds (Gutsche, 1954; Olías *et al.,* 1989) and olefinic bonds (Huisgen and Eberhard, 1971), among other reactive groups. Therefore, when the derivatization of carboxylic acids is carried out in the presence of other compounds having reactive groups, the formation of unexpected products may be originated. These side-reactions may be important, for example, when studying oxidized lipid/amino acid reaction products (OLAARP).

OLAARP are produced as a consequence of the peroxidation of lipids. Their formation is a recognized pathway of oxidant injury and has been implicated as a deleterious factor in a variety of diseases, including carcinogenesis, mutagenesis, aging, and atherosclerosis (Tappel, 1973; Rice-Evans and Burdon, 1993; Halliwell and Chirico, 1993). The analysis of OLAARP usually implies derivatization of the several reactive groups present in these molecules, which are related to both the oxidized lipids and the amino acids

involved. Among them, the presence of carboxylic (Hidalgo and Zamora, 1995), carbonyl (Kikugawa *et al.,* 1984), amino (Zamora and Hidalgo, 1994), pyrrole (Hidalgo and Zamora, 1993), and imidazole (Uchida and Stadtman, 1992) groups have been described. Therefore, it is important to know the behavior of these groups in the presence of the others in order to use or not a certain derivatizing agent.

As a model compound of some OLAARP produced in reactions involving oxidized lipids and histidine, this study describes the esterification reaction of a carboxylic group using diazomethane in the presence of an imidazole ring, as occurs in the *Nα*-acetyl-L-histidine.

2. EXPERIMENTAL

2.1. Materials

Nα-Acetyl-L-histidine, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (NTSA) and 2-(2-ethoxyethoxy) ethanol were purchased from Aldrich Chemical (Milwaukee, WI). L-Histidine, 1-methyl-L-histidine and 3-methyl-L-histidine were obtained from Signa Chemical Co. (St. Louis, MO). All other chemicals used were analytical grade and were purchased from reliable commercial sources.

2.2. Esterification of *Nα*-acetyl-L-histidine with diazomethane

Nα-Acetyl-L-histidine was suspended in methanol and treated with diazomethane prepared from NTSA according to the procedure of Schlenk and Gellerman (1960). Briefly, gaseous diazomethane was passed through the amino acid solution until a yellow tinge became visible against a white background. The reaction was allowed to continue at room temperature for 5 min, and then the solvent and the excess of diazomethane were removed under vacuum at a temperature below 40°C. The produced compounds were detected by micellar electrokinetic capillary chromatography (MECC) and thin layer chromatography (TLC). The capillary electrophoresis system consisted of a Beckman 2100 P/ACE unit equipped with a UV detector. (The P/ACE unit was linked to a 386/33 computer loaded with System Gold Software for data collection and handling). The capillary consisted of 50 cm in length uncoated silica tubing with 75 μm internal diameter and a detector aperture window of 100 x 200 μm. The sample was introduced into the capillary by pressure injection for 5.0 s. MECC was carried out at 15 kV and 30°C in 100 mM sodium borate buffer, pH 8.4, containing 100 mM SDS. Electrophoretic profiles were monitored by UV absorption at 214 nm. TLC was carried out on silica gel 60 GF_{254} plates (8 x 4 cm), obtained from Macherey Nagel (Düren, Germany), using chloroform-methanol (4:1) as eluent, and iodine vapors to detect the compounds.

Detected compounds were purified by column chromatography on silica gel 60 (Macherey Nagel) using mixtures of chloroform-methanol as eluent. The separation was started with chloroform-methanol (9:1), and the proportion of methanol was increased gradually to facilitate the elution of the most polar products. The final mixture was chloroform-methanol (4:1).

2.3. Characterization of the isolated compounds

Isolated compounds were studied by GC-MS and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. GC-MS analyses were conducted with a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, California) interfaced, *via* an open coupling system, to an AEI-MS/70VG mass spectrometer (VG Analytical, Manchester, UK). A DB-5 fused-silica capillary column (J & W Scientific, Folsom, CA), 30 m x 0.25 mm I.D. was used in all the experiments. The column temperature was programmed from 100 (2 min) to 280°C at 4°C/min. The MS conditions were as follows: ionization by electron impact, 70 eV; accelerating voltage, 4kV; emission current, 100 μA; ion source temperature, 220°C. ^{1}H and ^{13}C NMR at 300 and 75.4 MHz, respectively, were determined in a Bruker AC-300P (Karlsruhe, Germany), with Me_4Si as internal standard. Two-dimensional NMR was used to assign ^{13}C NMR spectra.

Confirmation of the structures was obtained by MECC after acid hydrolysis. Samples were hydrolyzed in 6 N HCl by heating under nitrogen 18 h at 120°C. The resulting solutions were evaporated, and the residues dissolved in 100 mM sodium borate buffer, pH 8.4, containing 100 mM sodium dodecyl sulfate (SDS), and submitted to MECC. MECC was carried out in the same capillary as described above at 15 kV and 30°C in 100 mM sodium borate buffer, pH 8.4, containing 100 mM SDS. Electrophoretic profiles were monitored by UV absorption at 214 nm.

3. RESULTS AND DISCUSSION

When the esterification reaction of a carboxylic group with diazomethane is carried out in the presence of an imidazole ring, the methyl ester of the acid group is not the only derivative obtained. Thus, when *Nα*-acetyl-L-histidine was treated with diazomethane, three major compounds were detected by MECC and TLC. Fig. 1 shows a typical electropherogram obtained for an esterification mixture of *Nα*-acetyl-L-histidine. Peaks **1, 2** and **3** (which corresponded to compounds **1-3**, respectively), were the major products of reaction, and

were also detected by TLC. Peaks **4** and **5** were minor products, and they could not be characterized.

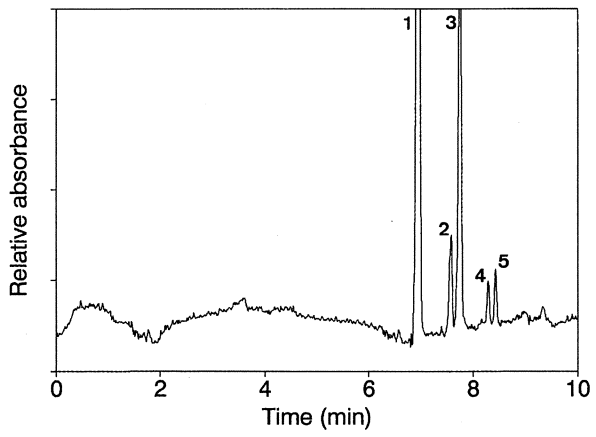

Figure 1
Electropherogram obtained for the esterification mixture of *Nα*-acetyl-L-histidine with diazomethane. Peaks **1-3** were identified as corresponding to *Nα*-acetyl-L-histidine methyl ester **(1)**, [*S*]-*Nα*-acetyl-1-methylimidazole-4-alanine methyl ester **(2)**, and [*S*]-*Nα*-acetyl-1-methylimidazole-5-alanine methyl ester **(3)**. Peaks **4** and **5** are unknown

Major products were isolated by column chromatography, and identified by ^{1}H and ^{13}C NMR and GC-MS as *Nα*-acetyl-L-histidine methyl ester **(1)**, *[S]-Nα*-acetyl-1-methylimidazole-4-alanine methyl ester **(2)**, and [*S*]-*Nα*-acetyl-1-methylimidazole-5-alanine methyl ester **(3)**. Structures for compounds **1-3** are given in Fig. 2.

Compound **1** (71%) was identified as *Nα*-acetyl-L-histidine methyl ester on the basis of its spectral data. R_F 0.46 (chloroform-methanol, 4:1). ^{1}H NMR ($CDCl_3$): δ (ppm) 2.01 *s* (3H, CH_3CO), 3.01 *m* (2H, H-1'), 3.67 *s* (3H, OCH_3), 4.78 *m* (1H, H-2'), 6.81*s* (1H, H-5), 7.55 *s* (1H, H-2), 7.45*d* (1H, $J_{NH,2'}$=7.5 Hz, NH), and 8.9 *br* (1H, NH). ^{1}H NMR (CD_3OD): δ (ppm) 1.94 *s* (3H, CH_3CO), 2.97 *dd* (1H, $J_{1'a,2'}$=8.4 Hz, $J_{1'a,1'b}$=14.9 Hz, H-1'a), 3.09 *ddd* (1H, $J_{1'b,5}$=0.7 Hz, $J_{1'b,2'}$=5.7 Hz, $J_{1'b,1'a}$=14.9 Hz, H-1'b), 3.69 *s* (3H, OCH_3), 4.66 *dd* (1H, $J_{1'b,2'}$=5.7 Hz, $J_{1'a,2'}$=8.4 Hz, H-2'), 6.86 *d, br* (1H, $J_{2,5}$=0.9 Hz, H-5), and 7.59 *d* (1H, $J_{2,5}$=0,9 Hz, H-2). ^{13}C NMR ($CDCl_3$): δ(ppm) 22.82 *q* (CH_3CO), 28.90 *t* (C-1'), 52.12 *d* (C-2'), 52.47 *q* (OCH_3), 115.68 *d* (C-5), 133.92 and 135.09 (C-2 and C-4), 170.45 *s* (ester), and 171.87 *s* (amide). ^{13}C NMR (CD_3OD): δ (ppm) 22.28 *q* (CH_3CO), 30.11 *t* (C-1'), 52.72 and 54.13 (C-2' and OCH_3), 117.99 *d* (C-5), 134.52 and 136.35 (C-2 and C-4), 173.19 and 173.46 (ester and amide). MS *m/z* (relative intensity, ion structure): 212 (5, M^+ + 1), 211 (13, M^+), 196 (2, $M^+ - CH_3$), 179 (5, $M^+ - CH_3OH$) 168 (9, $M^+ - CH_3CO$), 152 (82, $M^+ - CH_3OCO$ or $M^+ - CH_3CONH_2$), 121 (23, 152 – CH_3O), 120 (22, 152 – CH_3OH), 110 (58, 152 – CH_2CO), 82 (100, 4-methylimidazole), and 81 (94, 4-methylimidazole – 1).

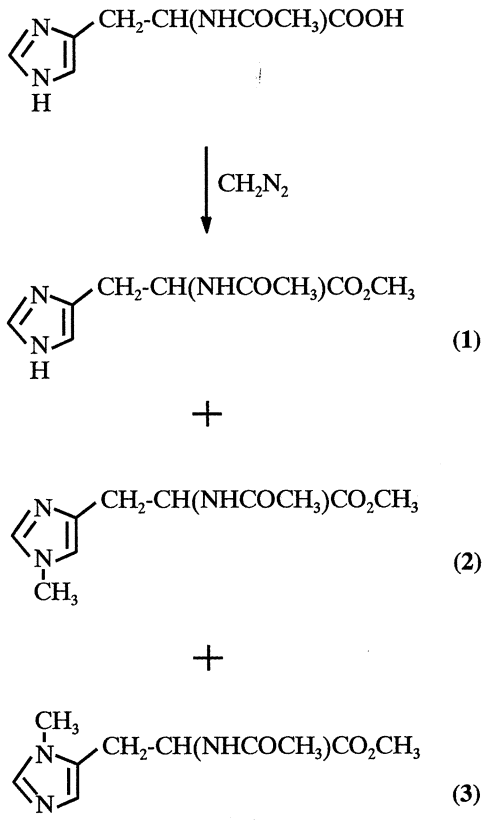

Figure 2
Scheme for the derivatization of *Nα*-acetyl-L-histidine with diazomethane. Compounds were isolated by column chromatography and identified by gas chromatography coupled with mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy

Compound **2** (11%) was identified as [*S*]-*Nα*-acetyl-1-methylimidazole-4-alanine methyl ester on the basis of its spectral data. R_F 0.68 (chloroform-methanol, 4:1). GC, R_t 27.1 min. ^{1}H NMR ($CDCl_3$): δ (ppm) 2.03 *s* (3H, CH_3CO), 2.98 *ddd* (1H, $J_{1'a,5}$= 0.6 Hz, $J_{1'a,2'}$-5.2 Hz, $J_{1'a,1'b}$=14.9 Hz, H-1'a), 3.08 *dd* (1H, $J_{1'b,2'}$= 5.2 Hz, $J_{1'b,1'a}$=14.9 Hz, H-1'b), 3.64 *s* (3H, NCH_3), 3.69 *s* (3H, OCH_3), 4.78 *m* (1H, H-2'), 6.67 *s, br* (1H, H-5), 7.34 *s* (1H, H-2), and 7.38 *d* (1H, $J_{NH,2'}$=7.7 Hz, NH). ^{13}C NMR ($CDCl_3$): δ (ppm) 23.43 *q* (CH_3CO), 29.46 *t* (C-1'), 33.38 *q* (NCH_3), 52.21 *q* (OCH_3), 52.45 *d* (C-2'), 117.81 *d* (C-5), 137.59 and 137.63 (C-2 and C-4), 170.17 *s* (ester), and 172.09 *s* (amide). GC-MS *m/z* (relative intensity, ion structure): 225 (12, M^+), 182 (8, $M^+ - CH_3CO$), 166 (38, $M^+ - CH_3CONH_2$), 124 (33, 166 – CH_2CO), 96 (33, 1,4-dimethylimidazole), 95 (76, 1,4-dimethylimidazole – 1), and 44 (100).

Compound **3** (10%) was identified as [*S*]-Nα-acetyl-1-methylimidazole-5-alanine methyl ester on the basis of its spectral data. R_F 0.59 (chloroform-methanol, 4:1). GC, R_t 26.2 mim. ^{1}H NMR ($CDCl_3$): δ (ppm) 2.00 *s* (3H, CH_3CO), 3.10 *m* (2H, H-1'), 3.58 *s* (3H, NCH_3), 3.74 *s* (3H, OCH_3), 4.83 *m* (1H, H-2'), 6.72 *s* (1H, H-4), 7.34

s (1H, H-2), and 7.75 *d* (1H, $J_{NH,2'}$=7.7 Hz, NH). ^{13}C NMR ($CDCl_3$): δ (ppm) 22.76 *q* (CH_3CO), 26.27*t* (C-1'), 31.36 *q* (NCH_3), 51.69 *d* (C-2'), 52.51 *q* (OCH_3), 126.87 *s* (C-5), 127.79 *d* (C-4), 138.12 *d* (C-2), 170.36 *s* (ester), and 171.77 *s* (amide). GC-MS *m/z* (relative intensity, ion structure): 225 (4, M^+), 166 (20, $M^+ - CH_3CONH_2$), 124 (7, 166 – CH_2CO), 96 (18, 1,5-dimethylimidazole), 95 (40, 1,5-dimethylimidazole – 1), and 44 (100).

The above results show that the esterification reaction of a carboxylic group with diazomethane in the presence of an imidazolic ring may be accompanied by the methylation of the imidazolic nitrogen atoms. In addition, and because of the tautomerism existing in the imidazole ring, both nitrogen atoms are susceptible to react with diazomethane producing the corresponding isomers. These compounds could be easily fractionated by GC or TLC under standard conditions and columns, but their identification was difficult because they had very similar mass spectra and ^1H NMR spectra. The best procedure to distinguish between them was the use of ^{13}C NMR spectroscopy. This technique produced two different patterns of signals for the two isomers, which could be interpreted in accordance with the spectra of model compounds collected in the literature. Thus, the ^{13}C NMR spectra of 1,4-dimethylimidazole showed signals at δ 116.2, 136.5 and 138.0 ppm, for carbons 5, 2 and 4, respectively (Aoyagi *et al.,* 1992). On the contrary, the ^{13}C NMR spectra of 1,5-dimethylimidazole showed signals at δ 125.6, 126.7 and 136.4 ppm, for carbons 4, 5 and 2, respectively (Aoyagi *et al.,* 1992). The same pattern was observed for the obtained compounds **2** and **3.** Compound **2** (1,4-isomer) showed the imidazolic carbon signals at δ 117.81, 137.59 and 137.63 ppm, and compound **3** (1,5-isomer) showed the signals at δ 126.87, 127.79 and 138.12 ppm.

An additional confirmation of the proposed structures was obtained by MECC after acid hydrolysis of the esterification mixture. Acid hydrolysis of compounds **1-3** produced L-histidine **(a)**, 3-methyl-L-histidine **(b)** and 1-methyl-L-histidine **(c)**, respectively, which could be compared with commercial standards. Fig. 3 shows a typical electropherogram obtained with a hydrolysate (Fig. 3A), and the electropherogram obtained using commercial L-histidine, 1-methyl-L-histidine and 3-methyl-L-histidine (Fig. 3B).

Although it is not very common to esterify carboxylic acids in the presence of imidazole rings, as occurs when studying OLAARP, the results obtained in this study show that the use of diazomethane may produce the methyl derivative of the heterocyclic ring in addition to the methyl ester. This side-reaction should be considered when preparing derivatives for GC-MS analysis.

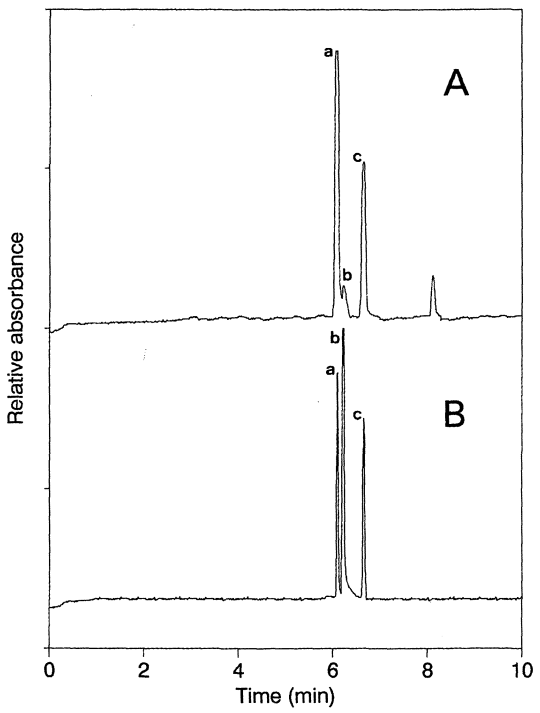

Figure 3
Electropherograms obtained for A: the acid hydrolysate of an esterification mixture of Nα-acetyl-L-histidine with diazomethane; and B, a standard mixture of L-histidine **(a)**, 3-methyl-L-histidine **(b)**, and 1-methyl-L-histidine **(c).**

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