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NOTES

SYNTHESIS OF $^{15}N,^{15}N$-IMIDAZOLEACETIC ACID

George D. Prell

Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029

Summary

$^{15}N,^{15}N$-imidazoleacetic acid was synthesized from ^{15}N, ^{15}N-DL-histidine (>99 atom % ^{15}N). The latter, oxidized with sodium hypochlorite, formed $^{15}N,^{15}N$-imidazoleacetonitrile. The free acid was prepared after acid hydrolysis of the nitrile, elution from an anion exchange column with acetic acid, then drying. Formation of by-product(s), produced when other methods were used, and which may contaminate some commercial sources of imidazoleacetic acid, was avoided with this method.

Key words: [$^{15}N,^{15}N$]-histidine, [$^{15}N,^{15}N$]-imidazoleacetonitrile, [$^{15}N,^{15}N$]-imidazoleacetic acid, sodium hypochlorite, imidazoleacetoethylester, mass spectrometry

Introduction

Imidazoleacetic acid (IAA) (Fig.1, III), a metabolite [1] of histidine (Fig.1, I) and histamine, exerts numerous effects in the central nervous system (CNS) [e.g. 2-4]. Although IAA was established to be a potent agonist at γ-aminobutyric acid receptors [e.g. 5-7], it was perceived to have almost no physiological relevance to the CNS since it was thought none existed in brain. We recently demonstrated IAA in brain and cerebrospinal fluid using a gas chromatography-mass spectrometric (GC-MS) method [8,9]. Because IAA is tautomeric, when derivatized for GC-MS analysis, it forms two isomers which are separated on capillary columns [8]. Therefore, the most appropriate internal standard is isotopically labelled IAA. The latter is not available commercially.

Since the first preparation of IAA by oxidation of imidazolelactic acid [10], few schemes for IAA synthesis have been proposed. In the synthesis of histamine, Pyman [11] produced the sodium salt of IAA as a by-product after reduction of imidazoleacetonitrile (ImAcCN) (Fig. 1, II) with metallic sodium, absolute alcohol and concentrated HCl; the sodium salt was then converted to the acid picrate or hydrochloride. Pyman prepared the nitrile from its chloromethyl derivative, the latter synthesized from hydroxymethylimidazole. The most common approach for the preparation of IAA has been to first synthesize ImAcCN [2,12-19]; alkaline hydrolysis of the latter produces the acid. The nitrile has been prepared from histidine after reaction with strong oxidizing agents. Using chloramine-T (sodium N-chlorotoluene-*p*-sulphonamide), Dakin [20] used histidine as an example to show that

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nitriles can be derived by oxidation of their parent amino acids when the oxidizing agent is in two-fold molar excess. Others [15-19] have used commercially available Clorox® (or Javex®) for its active principle, sodium hypochlorite. Once formed, the nitrile was hydrolyzed either in alkaline media, then treated with acid [10,15,16,19], or in acidified aqueous solutions [18]. In both cases, after numerous coevaporations with water, steps presumed to remove all excess HCl, IAA was ultimately extracted into ethanol, with recrystallization yielding IAA-HCl.

Although the exact procedure was not described, ^{15}N-labelled IAA was prepared [19] from ^{15}N-labelled histidine HCl using a published method [15]. Yield from starting material was about 10% and isotopic enrichment, approx. 92%, produced significant amounts of non-isotopic IAA [see 8]. In preparations for the synthesis of highly enriched $^{15}N,^{15}N$-IAA from labelled histidine, it was essential to make changes to existing methods in order to produce in sufficient yield a single intermediate, and ultimately a single product. A method for the synthesis of $^{15}N,^{15}N$-IAA and changes to existing methods of IAA synthesis are reported here.

Experimental

Thin layer chromatograms (40 x 80 mm Polygram Sil G/UV$_{254}$ precoated plates, Macherey-Nagel, Germany) were run throughout the synthesis using the following solvent systems: I: chloroform, methanol, ammonium hydroxide [10,8,1]; II: ethylacetate, methanol, ammonium hydroxide [70,30,7.5]; III; butanol, acetic acid, water [4,1,1]; IV: hexane, ethylacetate, methanol, ammonium hydroxide [16,13,4,1]. To confirm reaction completion and product formation, authentic L-histidine (Degussa), IAA-HCl (Aldrich) and nonlabelled ImAcCN (prepared previously in this laboratory [see below]) were used as reference standards.

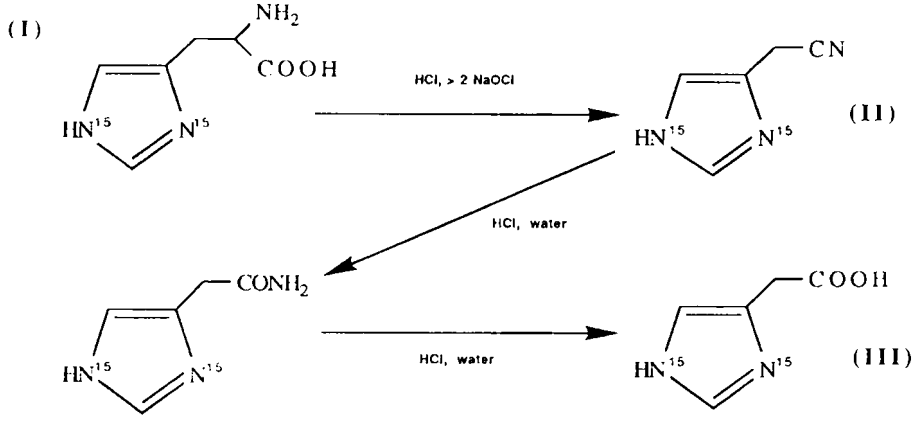

Figure 1. Preparation of $^{15}N,^{15}N$-IAA. The isomers shown each bear a proton on the tele-nitrogen.

The reaction scheme is depicted in Fig. 1. A vessel containing 1.11 mmol (175.1 mg) DL-histidine-1,3-$^{15}N_2$ (>99.2 atom % ^{15}N) (MSD Isotopes) dissolved in 5 ml of 0.3 N HCl, and stirred constantly, was cooled in an ice bath. When at 4°C, 4.42 ml of NaOCl (5.7%; Aldrich Chemicals) solution (3.38 mmol) was added dropwise such that the temperature did not exceed 10°C.

Within 10 min, the solution became intensely yellow, then orange, before it turned dark brown with evolution of CO_2 and formation of a bright orange precipitate. After 2 h, aliquots were subjected to thin layer chromatography (TLC) and visualized with sublimated iodide which indicated formation of ImAcCN [R_f: 0.69(I), 0.60(II), 0.46(III), 0.30(IV)], the most abundant product, and another *less polar, transient* product [R_f: 0.71(I), 0.65(II), 0.36(IV)]. No unreacted histidine was detected. After 12 h, only ImAcCN was observed. Two hours later, pH of the reaction mixture was raised from 4.5 to 8.5 with addition of 75 mg anhydrous sodium carbonate. The basified mixture was dried at room temperature in a rotary film evaporator (Büchi, Switzerland) brought to 200 mtorr then heated at reflux for 60 min with 10 ml of freshly distilled ethylacetate. The mixture was cooled, filtered by gravity through Whatman 40 paper, then dried under reduced pressure. These steps were repeated three times and the extracts combined; further extracts of the original residual material were devoid of ImAcCN when applied to TLC. Because this investigator's previous work showed presence of contaminants in pooled, dried extracts (one highly soluble in methanol, another rich in sodium), the pooled extracts were refluxed for 30 min in 10 ml ethylacetate, filtered, then dried. Material scraped from the vessel weighed > 75 mg, suggesting a maximum theoretical yield of 63%. Thermal Spray-Mass Spectrometry indicated a strong molecular ion at 109, i.e. the mass of ImAcCN + 2 mass units of label.

To remove traces of ethylacetate, the extract was dissolved in 25 ml distilled water then dried under reduced pressure; the residue was heated for 30 min at 100°C, redissolved in 25 ml water and evaporated to dryness. The latter steps were repeated twice before addition of 4 ml of 6 N HCl. The solution was heated at reflux, its color changed from dark red to bright orange. After 5 h, 100 µl aliquot was removed; TLC analysis showed IAA [R_f: 0.24(I), 0.07(II), 0.36(III), 0(IV)] to be the sole product. A portion analyzed [8,9] by GC-MS confirmed the formation of $^{15}N,^{15}N$-IAA. The remaining solution was hydrolyzed an additional hour, dried at 200 mtorr, then coevaporated three times, each with 20 ml water, before addition of 5 ml of 0.5 N NaOH. The alkaline solution (pH> 12) was applied to an ion exchange column (AG 1-X4, 100-200 mesh, converted to the acetate form; 7 mm i.d. x 140 mm). The eluate returned to neutrality after washing with 25 ml water; $^{15}N,^{15}N$-IAA was eluted with 35 ml 0.1 N acetic acid. The eluate was evaporated (60°C, 200 mtorr); the residue, after further drying (2h, 110°C), yielded $^{15}N,^{15}N$-IAA as nearly pure free acid. Additional product was eluted with 1 N acetic acid. Since the dried residue was a dull white that differed from the light flakes of purified, non-labelled IAA synthesized and recrystallized in pilot studies, the dried material was basified and re-chromatographed on another AG column. Elution with 0.1 N acetic acid and drying (as described above) yielded pure product, confirmed by TLC (Table 1), Thermal Spray-MS (data not shown), and GC-MS analysis [8] of the *n*-butyl-(*N*-ethoxycarbonyl) derivative of $^{15}N,^{15}N$-IAA.

Comments

The small amounts of $^{15}N,^{15}N$-DL-histidine available encouraged scale-down of the reaction steps using non-labelled starting material. Following methods from the literature resulted in poor yields and significant side reactions that required further modifications in order to overcome these drawbacks. Oxidation of histidine *free base* consistently formed a stable by-product(s) unless (a) hydrochloric acid was in molar excess of histidine, and (b) the oxidizing agent was present in greater than 2-fold molar excess. Reaction of free base with excess hypochlorite yielded a product *more polar*

[R_f: 0.64(I), 0.54(II)] than ImAcCN. Histidine HCl, after reacting with less than 2 molar proportions of hypochlorite, yielded a less polar but *stable* substance, possibly imidazole-acetaldehyde [21,22]. This by-product, formed when insufficient oxidant was used, was separated from ImAcCN by flash chromatography [23] and eluted before ImAcCN on silica gel (30-60 mesh, MC&B Chemists) using ethylacetate, methanol, *n*-hexane (3,1,1) saturated with NH_4OH (28%). It was not identified. That stable by-product is distinct from the *transient,* less polar *intermediate* (probably the dichloro-amino derivative [20]) that was consistently observed on TLC during the first few hours of histidine's oxidation when oxidant was in greater than 2-fold excess (see experimental). Oxidation reactions using either isomer of histidine each formed ImAcCN. But since oxidation of D-histidine (Sigma) appeared to be slower than for the L-isomer, reaction time was extended for the racemate.

Clorox® (5.25% NaOCl) was abandoned as a source of sodium hypochlorite since this preparation, unlike reagent grade material, contains additional salts [24], some of which were extracted into ethylacetate. Since few investigators have purified ImAcCN, such contamination may have escaped attention. Consequently, yields of ImAcCN may have been overestimated. For example, this investigator's replication of a method [18], in which extractions were made with Soxhlet apparatus, and which claimed yields >98%, yielded salts rich in sodium. Admixture of that product with purified ImAcCN showed melting point depression. In the present study, almost all contaminants were purged by additional extraction of pooled, dried extracts. Dried material had a sharp melting point (135-136°C, uncorr.) (cf. 138°C [15, 20]), unchanged when mixed with recrystallized ImAcCN. Thermal spray-MS scans ranging between 50-250 amu's showed ImAcCN abundance > 99% (not shown). Note that new or freshly distilled ethylacetate was required. Refluxing with older solvent yielded oils that were resistant to drying (even at 70°, <200 mtorr). The latter likely consisted of reaction products derived from acetic acid or ethanol, contaminants that form when ethylacetate is exposed to air.

After synthesis of the acid hydrochloride, excess acid must be removed to prevent esterification during ethanol extraction. In previous methods, this was claimed to have occurred when water was added and coevaporated successively under reduced pressure. However, numerous attempts, including 12 successive coevaporations, failed to abolish quantitatively formation of mixtures of IAA and IAA-ethylester [R_f: 0.80(I), 0.62(II), 0.48(III), 0.38(IV)] (confirmed by NMR and GC-MS). Others have noted similar difficulties [2,11,18]. In fact, IAA-HCl available commercially from many suppliers is contaminated (verified by GC-MS) with imidazoleacetoethylester (unpublished).

Isolation of IAA by ion exchange vitiated ester formation. The high ionic strength of the hydrolysis mixture, which would likely confound ion exchange separation, was reduced by successive coevaporations with water. In parallel experiments using identical amounts of non-labelled material, IAA was eluted with 0.1 N acetic acid. This free IAA discolored sharply at 214°C and melted with decomposition at 216-219°C (uncorr) (cf. corr 217-219 [13], 222°C [11]), values that were unchanged after recrystallization (acetone and water [13]). Recovery of $^{15}N,^{15}N$-IAA from the column was quantitative when eluted with 1N acetic acid; but this higher acetate concentration eluted another substance(s). Surprisingly, IAA could be selectively eluted when re-chromatographed using 0.1 N acetic acid. Total yield from labelled histidine was about 33%.

As an internal standard for GC-MS analysis, previously prepared $^{15}N,^{15}N$-IAA caused significant carryover (7-10%) of non-isotopic IAA which contaminated the standard [8] and thereby

reduced sensitivity of the assay for IAA. Preparation of highly enriched $^{15}N,^{15}N$-IAA attenuated this carryover such that 10-30 pg of authentic IAA can now be measured routinely (unpublished). With discovery of an acid-hydrolyzable conjugate(s) of IAA in brain [9,25] and cerebrospinal fluid [9,25,26], it is imperative that $^{15}N,^{15}N$-IAA of high purity, added to biological samples before hydrolysis, be available for GC-MS analysis of the hydrolysates.

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