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Note

Chromatographic determination of 4-nitro-L-histidine

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The synthetic amino acid 4-nitro-L-histidine was first prepared by Tautz *et al.*[1] by direct nitration of histidine. It has been recently used in our laboratory in the solid-phase synthesis of two modified hypothalamic peptide hormones, namely [4-nitro-L-histidine]^2-thyrotropin releasing factor (nitro-TRF) and [4-nitro-L-histidine]^2-luteinizing hormone releasing factor[2,3]; substitution of 4-nitro-L-histidine for L-histidine enhances the acidity of the imidazole side chain, thus providing a tool for studying the role played by histidine in the biological activity of natural peptides.

Acid hydrolysis followed by automatic ion-exchange chromatographic analysis is the usual way to control the synthesis and check the purity of the synthetic peptides[4]. Fig. 1 shows the chromatogram corresponding to a nitro-TRF hydrolysate obtained from a Beckman 120-C autoanalyzer equipped with a M 82 resin and working at standard conditions. Peaks corresponding to 4-nitro-L-histidine and proline are eluted simultaneously and therefore not resolved. Similar results were obtained when using a Beckman-Unichrom autoanalyzer equipped with a M 72 resin. In view of these difficulties, we have studied in detail the problem of chromatographic determination of 4-nitro-L-histidine.

EXPERIMENTAL

4-Nitro-L-histidine was synthesized by treating L-histidine with a sulphuric acid–fuming nitric acid mixture as described by Tautz *et al.*[1]. The product was precipitated at its isoelectric point and recrystallized twice from water: m.p. 197–198°; $[\alpha]_{25}^{D} = -23.5°$ (*c* 1, 5 *N* HCl). NMR spectrum (^2H_2O, NaO^2H): 8.56 ppm (s, 1 H, imidazole proton); $\delta_X = 3.72$ ppm, $\delta_A = 3.15$ ppm, $\delta_B = 3.34$ ppm, $J_{AX} = 11$ Hz, $J_{BX} = 3.5$ Hz, $J_{AB} = 13.8$ Hz) (3H, ABX system of protons from C_α and C_β).

Gas chromatography

A Perkin-Elmer F-11 apparatus equipped with a flame ionization detector was used. The carrier gas (nitrogen) flow-rate was 34 ml/min. Chromatograms were recorded between 60 and 210° employing a temperature gradient of 5°/min. The col-

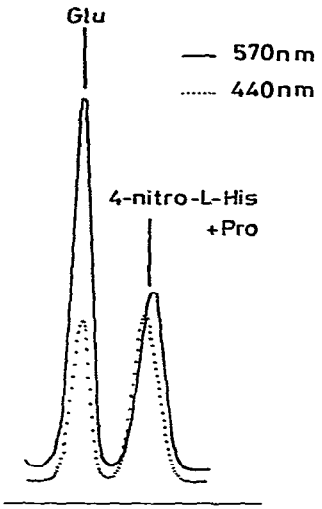

Fig. 1. Ion-exchange chromatogram corresponding to an hydrolysate of nitro-TRF. The analysis was performed on a Beckman 120-C autoanalyzer equipped with a M 82 resin. Temperature, 55°; sodium citrate buffers, pH 3.25 (0.2 *N* Na^+, 0.2 *N* citrate), pH 4.12 (0.4 *N* Na^+, 0.2 *N* citrate), pH 6.40 (1.0 *N* Na^+, 0.2 *N* citrate).

umns contained either Chromosorb–4% poiy(phenyl ether) or Chromosorb–0.65% ethyleneglycol adipate. N-Trifluoroacetyl-*n*-butyl derivatives of amino acids were prepared by treatment of the amino acid with 3 *N* hydrogen chloride–butanol and 25% trifluoroacetic anhydride–methylene chloride following standard procedures[5].

Spectrophotometric determination

Spectra were recorded on a Perkin-Elmer 124 spectrophotometer. The molar extinction coefficient of 4-nitro-L-histidine was determined from the absorptions at 295 nm of 14 solutions havnig concentrations ranging from $8.35 \cdot 10^{-6}$ to $1.67 \cdot 10^{-4}$ *M* (linear regression coefficient, 0.9982).

Ion-exchange chromatography

Acid hydrolyses of peptides were performed with 6 *N* hydrochloric acid in vacuum-degassed sealed tubes at 110° for 24 h, norleucine being added as the internal standard. The resolution of proline and 4-nitro-L-histidine was achieved by using a Beckman 119-C autoanalyzer equipped with an AA 20 resin and operating under the following conditions: column, 46 × 0.6 cm; flow-rates, 35 ml/h (buffer), 17.5 ml/h (ninhydrin); sodium citrate buffers, pH 3.25 (0.2 *N* Na^+, 0.2 *N* citrate), pH 4.12 (0.4 *N* Na^+, 0.2 *N* citrate), pH 6.40 (1.0 *N* Na^+, 0.2 *N* citrate); temperature, 50°. 4-Nitro-L-histidine was quantitated at 570 nm, using a colorimetric constant of 0.530 relative to leucine[6].

The spectra shown in Fig. 3 were recorded after heating at 100° for 20 min a mixture of 1 ml of the ninhydrin solution used by the analyzer and 1 ml of a solution of each amino acid in the initial citrate buffer.

RESULTS AND DISCUSSION

Gas chromatographic analysis

Gas chromatography of N-trifluoroacetyl-4-nitro-L-histidine *n*-butyl ester using columns of either Chromosorb–ethyleneglycol adipate or Chromosorb–poly-(phenyl ether) under the conditions described by Kaiser *et al.*[5] led to no definite peaks. This behaviour is probably due to decomposition of the derivatized 4-nitro-L-histidine

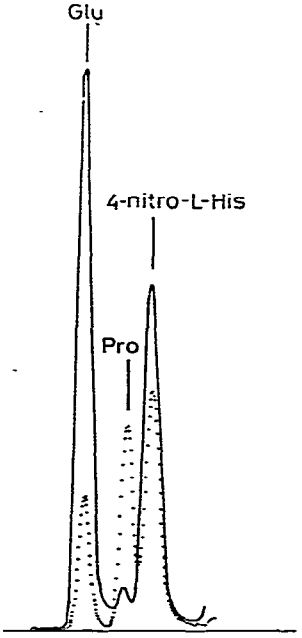

Fig. 2. Ion-exchange chromatogram of a hydrolysate of nitro-TRF obtained using a Beckman 119-C autoanalyzer equipped with a AA 20 resin. For conditions see Experimental section.

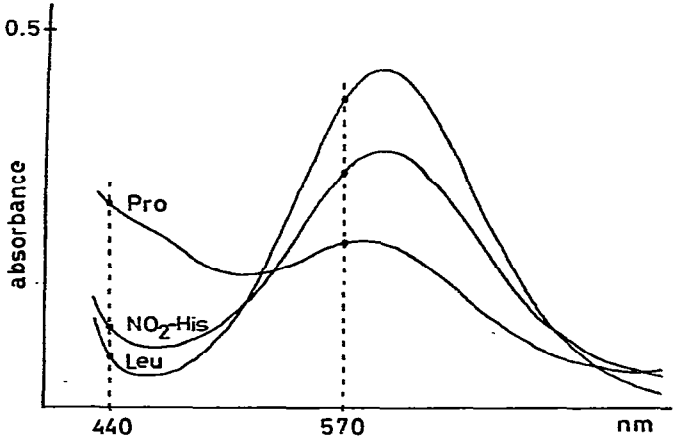

Fig. 3. Visible spectra of the products of reaction of ninhydrin with proline, leucine and 4-nitro-L-histidine.

on the column. An analogous decomposition of derivatized L-histidine has been previously described[7]. However, the application of this method to the analysis of hydrolysates of nitrated analogues of hypothalamic hormones allows the determination of amino acids other than 4-nitro-L-histidine. Proline can also be determined by gas chromatography, thus avoiding the difficulties encountered in its ion-exchange chromatographic separation from 4-nitro-L-histidine (Fig. 1).

Spectrophotometric determination

The combination of gas chromatography and UV spectroscopy allows analysis of most nitrated hypothalamic hormone hydrolysates. The UV spectrum of 4-nitro-L-histidine in acid solution shows an absorption maximum at 295 nm ($\varepsilon = 5661$, 1 *N* HCl) corresponding to the nitroimidazole side chain. Beer's law is obeyed over the whole range of concentrations examined (up to $1.67 \cdot 10^{-4}$ *M*). Thus, 4-nitro-L-histidine can be determined spectrophotometrically from peptide hydrolysates using 1 *N* hydrochloric acid as solvent.

Ion-exchange chromatography

As mentioned in the introduction, proline and 4-nitro-L-histidine are eluted simultaneously when M 82 and M 72 resins are used in Beckman analyzers under standard conditions. We have thoroughly varied the experimental parameters (temperature, buffer pH, buffer ionic strength) but could not resolve the two amino acids. However, the use of a Beckman AA 20 resin in a Beckman 119-C autoanalyzer under standard conditions (see Experimental section) provides a fairly good separation (Fig. 2).

The 570 nm:440 nm absorption ratio at the peak corresponding to 4-nitro-L-histidine in Fig. 2 is lower than the values usually found for other amino acids containing primary amine functions. The visible spectrum of a previously heated mixture of ninhydrin and 4-nitro-L-histidine is shown in Fig. 3 and compared with those of leucine and proline. Although the spectrum is very similar to that of leucine, the 570 nm:440 nm absorption ratio is lower for 4-nitro-L-histidine than for leucine. This suggests that the anomalous shape of the 4-nitro-L-histidine peak in Fig. 2 is not due to an inefficient separation from proline but rather to the chromophoric properties of the reaction product of ninhydrin and 4-nitro-L-histidine.

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