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Note

Specific method for the determination of 1,4-thiomorpholine-3,5-dicarboxylic acid and its unsaturated analogue lanthionine ketimine

L. PECCI and M. COSTA

Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza", 00185 Rome (Italy)

F. PINNEN

Istituto di Chimica Farmaceutica, Università di Roma "La Sapienza", 00185 Rome (Italy)

A. ANTONUCCI

Centro di Biologia Molecolare del CNR, 00185 Rome (Italy)

and

D. CAVALLINI*

Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza", 00185 Rome (Italy)

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1,4-Thiomorpholine-3,5-dicarboxylic acid (TMDA) is the reduction product of lanthionine ketimine (LK) that has been recently identified, together with its seven-membered homologue cyclothionine (1,4-hexahydrothiazepine-3,5-dicarboxylic acid), in bovine brain [1,2] and in normal human urine [3]. The detection of these compounds in biological samples raised the problem of their quantitation by a specific and fast method. We have found that TMDA and LK react with the Edman reagent phenyl isothiocyanate (PITC) to produce derivatives suitable for their determination by high-performance liquid chromatography (HPLC). Moreover, the phenylthiohydantoin (PTH) derivatives of TMDA and LK exhibit specific properties that are useful for their discrimination from the PTH derivatives of other amino acids.

EXPERIMENTAL

Reagents

TMDA and LK were prepared according to refs. 4 and 5, respectively. PITC was purchased from Fluka (Buchs, Switzerland). Other compounds were of the best available commercial quality.

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Spectral analyses

UV spectra were recorded with a Varian DMS 90 spectrophotometer (Mulgrave, Australia). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer operating at 300 MHz and 75.43 MHz, respectively. Mass spectra were determined with a Hewlett-Packard 5980 A (Palo Alto, CA, U.S.A.) operating at 70 eV.

Chromatography

Analyses were performed with a Violet (Rome, Italy) gradient liquid chromatograph consisting of a Model Clar 002 solvent pump, a Model Clar 001 controller programmer and a Model Clar 004 variable-wavelength detector. The analytical column was a stainless-steel tube (250×4 mm I.D.) packed with 5-μm particles of Spherisorb ODS2 (Phase Separations, Queensferry, U.K.). The mobile phases were: A, 0.05 *M* amonium acetate; B, a mixture of 0.05 *M* ammonium acetate and acetonitrile (65:35, v/v); C, acetonitrile–water (70:30, v/v). After washing with solvent C for 10 min, the column was preconditioned with solvent A for 15 min before sample loading. Then a linear gradient from A to 100% B for 30 min was produced; flow-rate: 1.0 ml/min at room temperature.

Preparation of derivatives

The derivatives of TMDA and LK with PITC were prepared according to the Edman procedure [6]: 2 mmol of the compound were dissolved in 10 ml of 50% aqueous pyridine, adjusted to pH 8.6 with 2 *M* sodium hydroxide and stirred for 30 min at 40°C with 0.5 ml of PITC. The reaction mixture was extracted three times with benzene and the aqueous solution was cooled at 0°C and adjusted to pH 1.5 with 2 *M* hydrochloric acid. After 2 h the sample was centrifuged and the precipitate dried under vacuum. The residue was purified by crystallization or preparative thin-layer chromatography (TLC) on silica gel with benzene–ethyl acetate (95:5, v/v). Treatment of TMDA and LK derivatives with diazomethane in methanol–ethyl acetate gave the corresponding methyl esters.

RESULTS AND DISCUSSION

Reaction with phenyl isothiocyanate

Phenylthiocarbamyl (PTC) and PTH derivatives of most of the amino acids are distinguishable on the basis of their UV spectra: the absorption maximum at 240 nm is indicative of PTC amino acids and that at 265–270 nm of the PTH amino acids [7].

When TMDA reacts with PITC in weakly alkaline media, the maximum absorbance is observed at 270 nm instead of 240 nm, suggesting the formation of the PTH derivative without an acidification and heating step. Acidification and heating do not modify or increase the absorbance at 270 nm.

The spectral data of PTH-TMDA are in agreement with the assigned structure (Fig. 1). This compound was characterized as a methyl ester (m.p. 160–165°C from ethyl acetate–*n*-hexane). Spectral data: mass spectrometry, *m/z* 322 (M^+, 100%), 263 (76), 100 (29); ^{1}H NMR (C^2HCl_3) δ = 2.5–3.4 (m, 4 H), 3.85 (s, 3 H), 4.70 (dd, 1 H), 6.10 (dd, 1 H), 7.2–7.7 ppm (m, 5 H); ^{13}C NMR (C^2HCl_3)

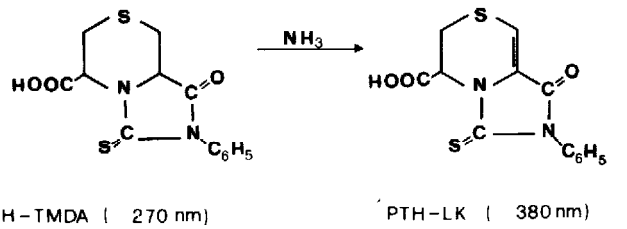

Fig. 1. Structures of phenylthiohydantoin derivatives of 1,4-thiomorpholine-3,5-dicarboxylic acid (TMDA) and of lanthionine ketimine (LK).

$\delta = 29.64, 29.69, 53.17, 55.07, 58.36, 128.18, 129.00, 129.36, 132.71, 168.39, 170.75, 181.74$ ppm.

If LK, the unsaturated form of TMDA, is treated with PITC at pH 8.6 a compound with a totally different UV spectrum is produced. The spectrum exhibits a maximum at 380 nm that is not observed with other amino acids. Treatment of this ketimine derivative with 1 *M* hydrochloric acid at 80 °C for 10 min does not lead to any change of the absorption spectrum.

The spectral data for the LK derivative are in agreement with a PTH structure (Fig. 1). This compound was characterized as a methyl ester (m.p. 154–155 °C from ethyl acetate–*n*-hexane). Spectral data: mass spectrometry, *m/z* 320 (M^+, 60%), 261 (11), 126 (100); ^{1}H NMR (C^2HCl_3) $\delta = 3.3$–3.7 (m, 2 H), 3.85 (s, 3 H), 5.95 (dd, 1 H), 6.90 (dd, 1 H), 7.35–7.60 ppm; ^{13}C NMR (C^2HCl_3) $\delta = 28.09$, 52.89, 53.51, 112.49, 124.68, 128.23, 129.14, 129.25, 132.57, 159.11, 166.81, 176.07 ppm.

Ammonia treatment

Solutions of PTH-TMDA in 1 *M* ammonia show the appearance of a new absorption maximum at 380 nm, which increases with time (Fig. 2). The reaction rate is temperature-dependent and the maximum value could be reached within 1 min by heating at 100 °C. Increase of the ammonia concentration to 5 *M* did not change significantly the absorbance measured in 1 *M* ammonia. When PTH-TMDA was treated with 1 *M* ammonia in the absence of oxygen (in Tunberg cells under vacuum), no increase of the absorbance at 380 nm was observed after 1 h at 30 °C. The addition of 1 m*M* EDTA decreased the reaction rate at 30 °C in air: after 1 h it was 10% of that observed in the absence of EDTA.

As reported in the previous section, LK reacts with PITC to produce the same absorbance at 380 nm that is produced by treating PTH-TMDA in ammonia at 100 °C. This fact, together with the need for oxygen and traces of a metal, suggests that the ammonia treatment converts the PTH-TMDA into PTH-LK by an oxidative step.

The identity of PTH-LK and the product obtained after treatment of PTH-TMDA with ammonia is supported further by HPLC: the two compounds coelute with a retention time that is close to that of PTH-TMDA (Fig. 3).

The absorbance shift at 380 nm after ammonia treatment is characteristic of the PTH derivative of TMDA. Mixtures of common amino acids that were de-

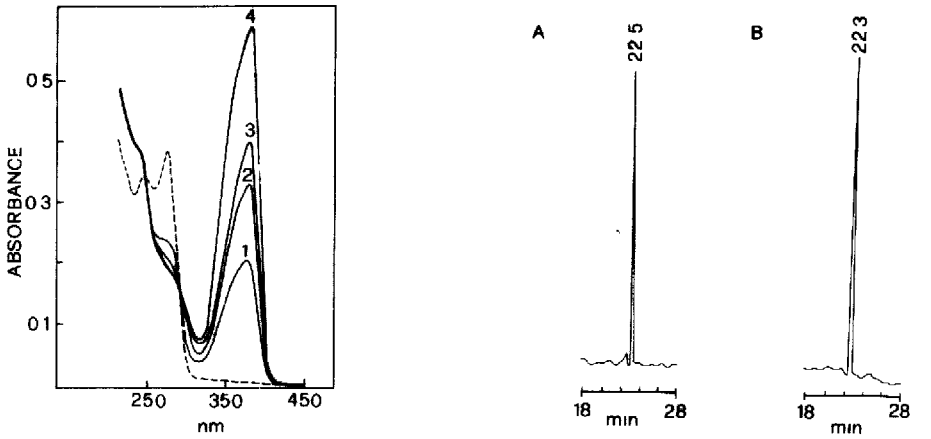

Fig. 2. Changes of absorption characteristics of PTH-TMDA on treatment with 1 *M* ammonia. Absorption spectrum of PTH-TMDA in 0.01 *M* phosphate buffer pH 8 (broken curve). Curves 1, 2 and 3 were registered after 5, 20 and 30 min of incubation at 30 °C in 1 *M* ammonia, respectively. Curve 4 was obtained after 1 min at 100 °C in 1 *M* ammonia.

Fig. 3. HPLC profile of PTH-TMDA (0.5 μg) detected at 254 nm (A) and of PTH-TMDA (0.2 μg) treated in ammonia plus PTH-LK (0.2 μg) detected at 380 nm (B).

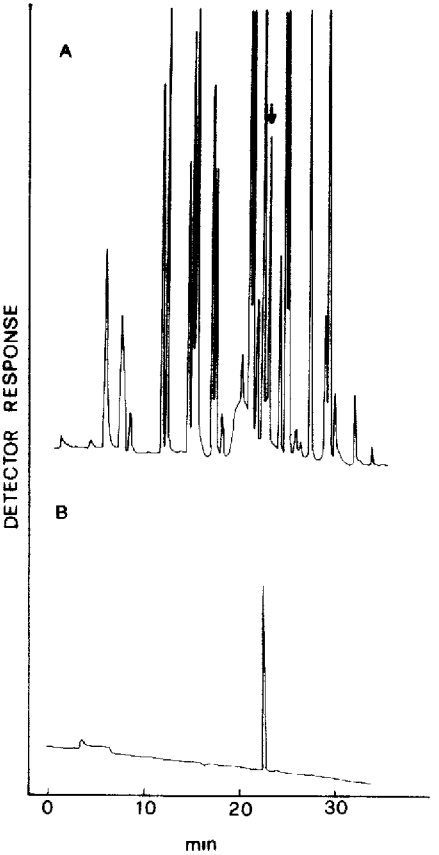

Fig. 4. HPLC separation of a mixture of standard amino acids (Pierce kit) plus 0.5 μg of TMDA treated with PITC. (A) Detection at 254 nm (0.08 a.u.f.s.); the arrow indicates the position of the TMDA derivative. (B) Detection at 380 nm (0.16 a.u.f.s.) after treatment with 1 *M* ammonia for 1 min at 100 °C.

rivatized to form either PTC or PTH did not exhibit any absorbance at 380 nm after ammonia treatment. Fig. 4 illustrates the HPLC separation monitored at 254 and 380 nm (before and after treatment with ammonia solution, respectively) of the standard amino acid mixture with added TMDA derivatized with PITC according to ref. 8. The main feature of the chromatogram obtained at 380 nm is the appearance of a single peak corresponding to the derivative of TMDA. This property is of value for the distinction of TMDA from other amino acids.

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