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Identification of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptopyruvic acid with a metabolic intermediate between *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine and *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptolactic acid

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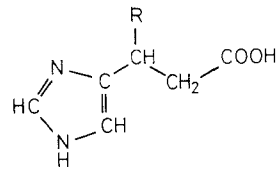
Summary. *S*-[2-Carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptopyruvic acid (**I**) was chemically synthesized in 15% yield by incubating a reaction mixture of *trans*-urocanic acid and 3-fold excess of 3-mercaptopyruvic acid at 45°C for 6 days. The synthesized compound was characterized by fast-atom-bombardment mass spectrometry and high-voltage paper electrophoresis. Compound **I** was identified with a product of an enzymatic reaction of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine (**II**) with rat liver homogenate in a phosphate buffer, pH 7.4. Compound **I** was degraded to *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptolactic acid (**III**), a compound previously found in human urine [Kinuta et al. (1994) Biochem J 297: 475–478], by incubation with rat liver homogenate. From these results, we suggest that compound **I** is a metabolic intermediate for the formation of compound **III** from compound **II**. The present pathway follows a formation of compound **II** from *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]gluthathione [Kinuta et al. (1993) Biochim Biophys Acta 1157: 192–198], a proposed metabolite of L-histidine.

Keywords: Amino acids – Imidazole compound – Mercaptopyruvic acid – Urocanic acid – Histidine – Mass spectrometry – Paper electrophoresis

Introduction

In the first step of L-histidine catabolism, urocanic acid is formed by the action of histidine ammonia-lyase (EC 4.3.1.3). In liver, urocanic acid is metabolized by urocanate hydratase (EC 4.2.1.49) to 4,5-dihydro-4-oxo-5-imidazolepropanoic acid, and this compound is further catabolized ultimately to CO_2 and water. In epidermis, however, L-histidine metabolism terminates with the formation of urocanic acid, since urocanate hydratase is absent from the skin (Barden and Pathak, 1967; Scott, 1981). On the other hand, it has



Compound I : R = -S-CH_2-CO-COOH
Compound II : R = -S-CH_2-CH(NH_2)-COOH
Compound III : R = -S-CH_2-CH(OH)-COOH

Fig. 1. Structural formulae of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptopyruvic acid (**I**), *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine (**II**) and *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptolactic acid (**III**)

been suggested that histidine is metabolized in part *via* an alternative pathway which is initiated by the adduction of natural thiol compounds such as L-cysteine and gluthathione to urocanic acid (Kinuta et al., 1991a; 1991b; 1992; 1993; 1994; 1996). In these studies, *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptopyruvic acid (**I**) {3-[(2-oxo-2-carboxyethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid} is proposed to be an intermediate of the metabolism from *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine (**II**) to *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptolactic acid (**III**) {3-[(2-hydroxy-2-carboxyethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid} (Kinuta et al., 1994). However, we had no direct evidence showing the identification of the intermediate with compound **I** because of an unprepared compound **I**. The intermediate has now been purified, and determined to be compound **I**. The present paper describes the chemical synthesis and characterization of compound **I**, and the demonstration of this compound to be a metabolic intermediate between compound **II** and **III**, compounds previously found in normal human urine (Kinuta et al., 1991b; 1992; 1994). Figure 1 shows the structural formula of compounds **I**, **II** and **III**.

Materials and methods

Chemicals

Ion exchangers and chemicals used in the present work were as described previously (Kinuta et al., 1991a). Sodium 3-mercaptopyruvate was purchased from Aldrich Chemical Co. (Milwaukee. WI., U.S.A.); *trans*-urocanic acid [*trans*-3-(1*H*-imidazol-4-yl)acrylic acid] were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Compound **II** was synthesized by the addition of L-cysteine to urocanic acid (Kinuta et al., 1992). Compound **III** was made by the reaction of compound **II** with $NaNO_2$ in HCl (Kinuta et al., 1994). Pauly's reagent (Pauly, 1904; Macpherson, 1946) which is a diazotized sulphanilic acid reagent for the detection of imidazole compounds, chloroplatinate reagent (Toennis and Kolb, 1951) for the detection of sulphur-containing compounds and ninhydrin (triketohydrindene hydrate) reagent (Kinuta et al., 1993) were used as described previously (Kinuta et al., 1991a).

Spectroscopy

Fast-atom-bombardment mass spectrometry (FAB-MS) and chemical-ionization mass spectrometry (CI-MS) with a direct-inlet system were carried out on a Shimadzu 9020-DF gas chromatography-mass spectrometer equipped with a Shimadzu SCAP 1123 data system and a Hewlett-Pachard 7240A plotter printer according to a previous method (Kinuta et al., 1992).

Paper electrophoresis

High-voltage paper electrophoresis was performed on Whatman 1Chr paper (Whatman, Maidstone, Kent, U.K.) in a buffer, pH 3.1, consisting of pyridine/acetic acid/water) 1:20:179, by vol.) (Ubuka, 1962) at a potential gradient of 100 V/cm for 45 min (Kinuta et al., 1994). Relative mobilities of compounds on the paper electrophoretogram, termed the mR_{ILA} value, were determined by comparison of the mobility for imidazol-4-yl-lactic acid as 1.00 (Kinuta et al., 1991a).

Enzymatic reactions

Male Wistar rats (Clea Japan, Tokyo) weighing 280–350 g were maintained on a laboratory diet, MF (Oriental Yeast Co, Tokyo, Japan). Rat liver homogenate in 0.1 M potassium phosphate buffer, pH 7.4, containing 5 mM EDTA was prepared in accordance with a previous method (Kinuta et al., 1991a). The homogenate was used as the enzyme source without further purification. Enzymatic degradation of compound **II** (1 mmol) was carried out by a previous method (Kinuta et al., 1994), and the products were desalted on a Dowex 50 column (H^+ form, 2 cm × 20 cm) (Kinuta et al., 1993; 1994). Enzymatic degradation of compound **I** (1 mmol) with rat liver homogenate in the phosphate buffer, pH 7.4, was performed by using this compound instead of compound **II** as the enzyme substrate in the same manner as that described above. The products were desalted and further chromatographed on a Dowex 1 column (acetate form, 1.5 cm × 15 cm) by a previous method (Kinuta et al., 1994) employed for the purification of compound **III**. Reaction mixtures without the substrate or with homogenate preheated at 100°C for 3 min instead of the above homogenate were used as contrals.

Results

Chemical synthesis and characterization of compound I

Compound **I** was synthesized by the addition of 3-mercaptopyruvic acid to *trans*-urocanic acid in a similar manner to that employed for the synthesis of compound **II** (Kinuta et al., 1992); a reaction mixture (pH 8.3) of *trans*-urocanic acid (2 mmol) and sodium 3-mercaptopyruvate (6 mmol) in 4 ml of 2.25 M Na_2CO_3 was incubated anaerobically at 45°C for 6 days, using a sealed glass tube. Reaction products were desalted on a Dowex 50 column (H^+ form, 1.5 cm × 10 cm). The desalted products were further chromatographed on a Dowex 1 column (acetate form, 1 cm × 25 cm) with the successive eluents (100 ml of each) water, 0.02 M, 0.05 M, 0.1 M, 0.5 M, 1 M and 2 M acetic acid, with 5 ml portions being collected. All the isolation procedures were performed in the cold room at 4°C to avoid degradation of the products. As checked by paper electrophoresis, compound **I** was eluted between 35 and 75 ml of the 1 M acetic acid. Crystals of compound **I** (yield 15.2%) were obtained from the eluates. Analytical data culculated for $C_9H_{10}N_2O_5S$: C, 41.86; H, 3.90; N, 10.85%. Found: C, 42.08; H, 4.02; N, 10.63%.

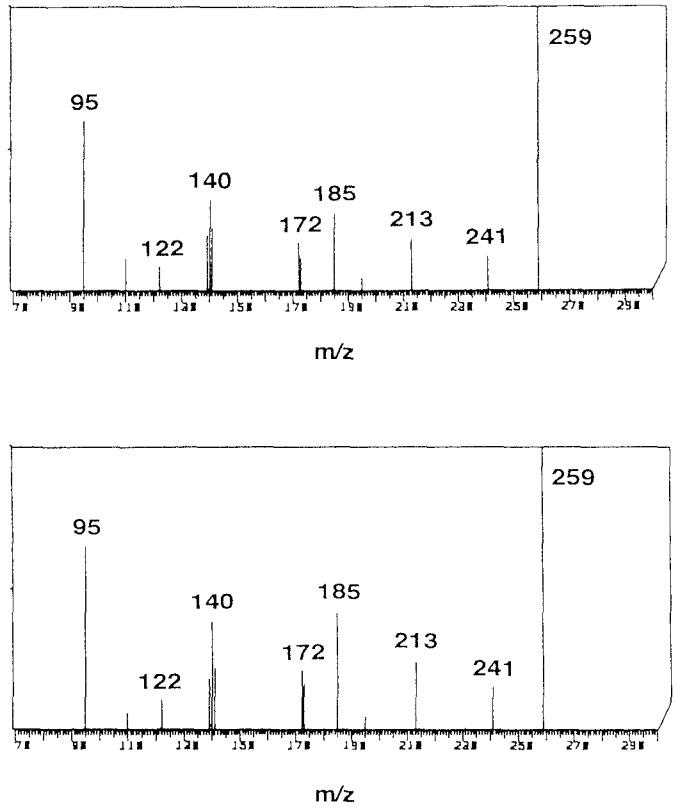


Fig. 2. Fast-atom-bombardment mass spectra of the chemically synthesized compound **I** (the upper) and the isolated product (the lower) of enzymatic reaction of compound **II** with rat liver homogenate

In the FAB-mass spectrum (the upper in Fig. 2), signals were assigned as follows: *m/z* 259 [MH^+ (the molecular ion plus proton)], 241 ($MH^+ - H_2O$), 213 [$MH^+ - (COOH + H)$], 185 [$MH^+ - (CO\text{-}COOH + H)$], 172 [$MH^+ - (CH_2\text{-}CO\text{-}COOH)$], 140 [$MH^+ - (S\text{-}CH_2\text{-}CO\text{-}COOH)$], 122 ($140 - H_2O$) and 95 ($140 - COOH$). In the CI-mass spectrum, a signal at *m/z* 259 was small (2.8%); instead, a signal of $MH^+ - H_2O$, $MH^+ - CO_2$, $MH^+ - (HS\text{-}CH_2\text{-}CO\text{-}COOH)$ and $[C_3H_3N_2\text{-}CH = CH_2]H^+$ were given at *m/z* 241 (9.2%), 215 (18.4%), 139 (base) and 95 (86.3%), respectively. The synthesized compound gave a reddish band at mR_{ILA} 0.30 on the paper electrophoretogram sprayed with Pauly's reagent. This compound gave a positive reaction with the chloroplatinate reagent, but the ninhydrin test was negative.

*Identification of the metabolite of compound **II** with compound **I***

Enzymatic degradation of compound **II** (1 mmol) was performed by incubation with rat liver homogenate in the phosphate buffer, pH 7.4, at 37°C. The desalted products were chromatographed on a Dowex 1 column (acetate form, 1 cm × 25 cm) in the cold room with the successive eluents of acetic acid

at concentrations described above. The desired compound having mR_{ILA} 0.3 was found in eluates of the 1M acetic acid. Futher purification of the compound was carried out by high-voltage paper electrophoresis. After electrophoresis, a part of the paper containing the desired compound was cut out with scissors and then cut into small pieces. After repitition of the paper electrophoresis about 20 times, the compound was extracted from the pieces with water. The extracts were combined, concentrated to an appropriate volume and applied to a Dowex 50 column (H^+ form, 0.7cm × 8cm). The desired compound was obtained with little contamination from eluates of 2M ammonia after the eluates had been evaporated to dryness under the reduced pressure at 35°C. A purity of the isolated compound was determined by high-voltage paper electrophoresis. The compound gave positive reactions with Pauly's reagent and the chloroplatinate reagent, whereas no reaction was observed with the ninhydrin reagent. The mR_{ILA} value coincided with that of compound **I**. The FAB-mass spectrum of the isolated compound (the lower in Fig. 2) was also identical with that of compound **I**. From these results, the product of enzymatic degradation of compound **II** was determined to be compound **I**.

*Enzymatic formation of compound **III** from compound **I***

Enzymatic degradation of compound **I** (1mmol) was carried out in the same manner as that described above except for using compound **I** instead of compound **II** as the enzyme substrate. After reaction, a product having mR_{ILA} 0.32 (Kinuta et al., 1994) was isolated from the reaction mixture essentially by a previous method (Kinuta et al., 1994). The isolated product was found to be compound **III** by FAB- and CI-MS and high-voltage paper electrophoresis, confirning their data to those of compound **III** (Kinuta et al., 1994).

Discussion

From these results, compound **I** was found to be a metabolic intermediate for the formation of compound **III** from compound **II**. In the previous study on the enzymatic formation of compound **II** (Kinuta et al., 1994), a compound having a signal at *m/z* 259 on the FAB-mass spectrum was detected. This compound has now been identified with compound **I**. The other compound having the signal at *m/z* 215 (Kinuta et al., 1994) has not been specified, although this compound is postulated to be a precursor of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-2-mercaptoacetic acid {3-[(carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid}, a compound previously isolated from human urine (Kinuta et al., 1991a). Results here approve of a suggestion (Kinuta et al., 1994) that the cysteine moiety of compound **II** generates the mercaptolactate part of compound **III**, *via* compound **I**, possibly like a metabolism of L-cysteine *via* 3-mercaptopyruvate pathway (transamination pathway) (Ubuka et al., 1992). On the other hand, *N*-acetyl-*S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine also was isolated from normal human

urine (Kinuta et al., 1996) and this suggests that compound **II** is introduced in part into the *N*-acetylating pathway *i.e.* mercapturate formation (Kinuta et al., 1996).

These findings support the alternative pathway of L-histidine metabolism. In the metabolic pathway, *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]gluthathione, an analogue of urocanic acid adducted with glutathione, is formed possibly (Kinuta et al., 1991a; 1993). Compound **II** was previously demonstrated (Kinuta et al., 1993) to be a product of enzymatic degradation of the glutathione adduct possibly *via* a metabolic intermediate, *N*-{*S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteinyl}glycine. Thus, the present pathway from compound **II** to **III** succeeds to the metabolism of the gluthathione adduct. A physiological role of the pathway has not been established, although it has been discussed (Kinuta et al., 1991a; 1992) that the formation of compound **II** may participate in part in the accumulation and the elimination of the epidermal urocanic acid under conditions of sunlight irradiation.

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