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UNUSUAL CONJUGATES IN BIOLOGICAL PROFILES ORIGINATING FROM CONSUMPTION OF ONIONS AND GARLIC

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SUMMARY

After consumption of onions or garlic, biological profiles of human urine samples show, in the methylated conjugate fraction, peaks corresponding to the methylates of N-acetyl-S-(2-carboxypropyl)cysteine (**1**), N-acetyl-S-allylcysteine (**2**) and hexahydrohippuric acid (**3**). The compounds **1** and **2** are metabolites of peptides introduced with onions or garlic into the body.

INTRODUCTION

Biological profiles [1,2] are widely used for the identification of inherited errors of metabolism [3–5]. However, "unusual" peaks due to nutritional status are sometimes detected in such samples. Since the "food" originates from various sources it is often rather time-consuming to identify the precursors of such "misleading" peaks. We report here on the identification of some conjugates originating from the consumption of garlic and onions.

EXPERIMENTAL

Synthesis of reference compounds (Fig. 1)

N-Acetyl-S-(2-carboxypropyl)cysteine. A solution of 163 mg (1 mmol) of N-acetylcysteine (Sigma, Deisenhofen, F.R.G.) and 86 mg (1 mmol) of methacrylic acid (Merck, Darmstadt, F.R.G.) dissolved in 10 ml of water was brought to pH 10 with 2 *M* sodium hydroxide. After stirring for 10 h at room temperature, the mixture was acidified with 2 *M* hydrochloric acid to pH 2 and extracted with two 20-ml portions of ethyl acetate. The organic layer was evaporated, and 1 mg of the residue was treated with 2 ml of an ethereal solution of diazomethane. After

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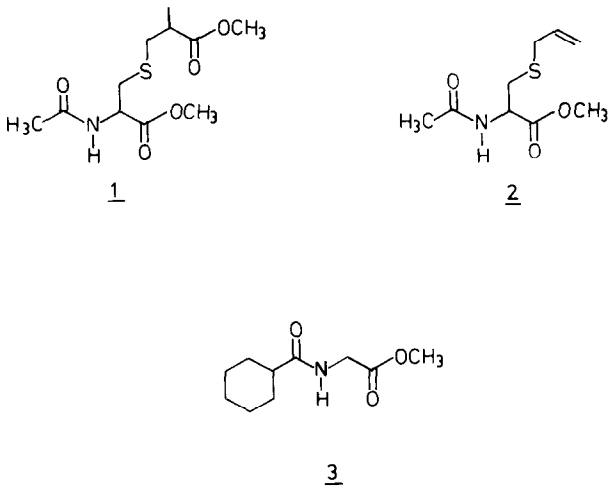


Fig. 1. Structures of N-acetyl-S-(2-carboxypropyl)cysteine (**1**), N-acetyl-S-allylcysteine (**2**) and hexahydrohippuric acid (**3**) as methyl esters.

removal of the solvent, the compound was ready for analysis by gas chromatography–mass spectrometry (GC–MS).

N-Acetyl-S-allyl-cysteine. A solution of 81.5 mg (0.5 mmol) of N-acetylcysteine (Sigma) and 60.5 mg (0.5 mmol) of allyl bromide (Merck) in 5 ml of water was brought to pH 10 with 2 *M* sodium hydroxide. Ethanol was added to this mixture until the solution became clear. After stirring at room temperature for 4 h, the solvent was evaporated. The residue was dissolved in 10 ml of water, brought to pH 2 with 2 *M* hydrochloric acid, and extracted with two 20-ml portions of ethyl acetate. The organic layer was evaporated and 1 mg of the residue was prepared for GC–MS analysis.

Hexahydrohippuric acid. A solution of 75 mg (1 mmol) of glycine (Sigma) dissolved in 1 ml of 1 *M* sodium hydroxide, and 146 mg (1 mmol) of cyclohexanecarboxylyl chloride (Fluka, Neu-Ulm, F.R.G.) in 10 ml of dioxan, was stirred vigorously for 30 min at room temperature. The solvent was evaporated, and the residue dissolved in 10 ml of water. This solution was acidified to pH 2 with 2 *M* hydrochloric acid and extracted with two 20-ml portions of ethyl acetate. After evaporation of the organic layer, a 1-mg sample of the residue was used, after treatment with diazomethane, for GC–MS analysis.

Isolation of the conjugate fractions from human urine

A 100-ml volume of 24-h urine was acidified to pH 2.0 with 1 *M* hydrochloric acid and extracted with three 150-ml portions of ethyl acetate. The combined organic layers were evaporated to dryness and treated with an ethereal diazomethane solution. The solvent and the excess of diazomethane were evaporated by a stream of nitrogen. The dry residue (150 mg) and 300 mg of silica gel were suspended in 5 ml of methanol and, after 10 min, the solvent was removed again. The residue was placed on the top of a column filled with 5 g of silica gel suspended in cyclohexane.

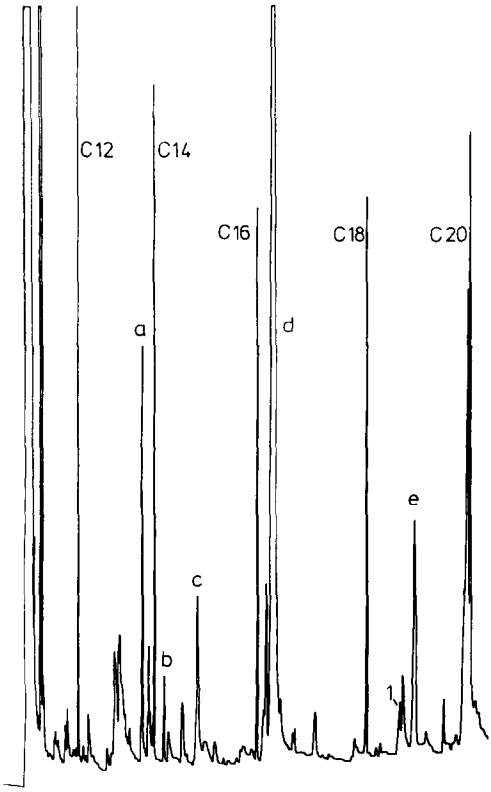


Fig. 2. Glass capillary gas chromatogram of urinary organic acids, separated as their methyl esters. Peaks: a = *p*-methoxyphenylacetic acid; b = citric acid; c = unknown; d = hippuric acid; e = tetramethyluric acid; 1 = N-acetyl-S-(2-carboxypropyl)cysteine.

Chromatography was carried out with the following solvents: fraction 1: 25 ml of cyclohexane–ethyl acetate (95:5, v/v); fraction 2: 25 ml of cyclohexane–ethyl acetate (80:20, v/v); fraction 3: 25 ml of cyclohexane–ethyl acetate (70:30, v/v); fraction 4: 25 ml of cyclohexane–ethyl acetate (50:50, v/v); fraction 5: 25 ml of ethyl acetate. The conjugates were found in fraction 5.

Gas chromatography–mass spectrometry

Gas chromatograms were obtained with a Packard 438S gas chromatograph, equipped with a flame-ionization detector and a WCOT glass capillary column (30 m × 0.3 mm I.D.) coated with OV-101. The gas chromatograph was connected to a Shimadzu C-R3A integrator. The carrier gas was hydrogen at 0.5 kg/cm^2. The injector temperature was 270°C, the detector temperature 280°C and the column programme 100°C for 3 min followed by 100–280°C at 3°C/min. Mass spectra were obtained with a Varian-MAT 312 mass spectrometer equipped with a MAT SS 200 data system (PDP 11/34 computer). The ionization energy was 70 eV. The mass spectrometer was connected to a Varian 3700 gas chromatograph equipped with the glass capillary column mentioned above.

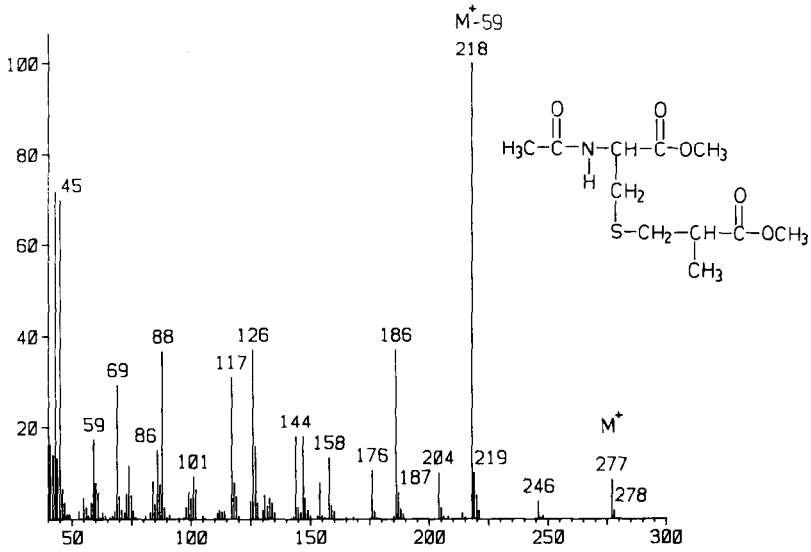
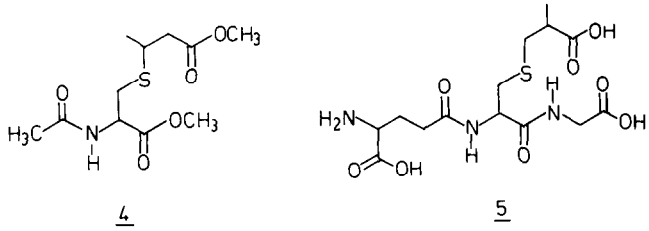


Fig. 3. Mass spectrum of peak 1 in Fig. 2.



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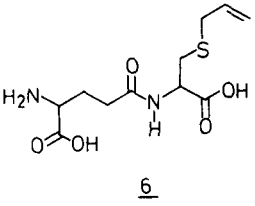

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Fig. 4. Structures of N-acetyl-S-(1-methyl-2-carboxyethyl)cysteine, as methyl ester (**4**), γ-glutamyl-S-(2-carboxypropyl)cysteinylglycine (**5**) and γ-glutamyl-S-allylcysteine (**6**).

RESULTS

Profiles of the conjugate fraction obtained from the urine of healthy individuals usually contain a small peak (Fig. 2, peak 1, RI 1865). The mass spectrum (Fig. 3) of this GC peak shows fragments at mass 88, 144 and 176. The combined presence of these three ions was shown to be typical for N-acetylcysteine S-conjugates (mercapturic acids) [6,7].

Besides the molecular ion of mass 277, a very intense fragment of mass 218 is observed ($M^+ - 59$, mainly $M^+ - H_3C-CONH_2$, corresponding to high resolu-

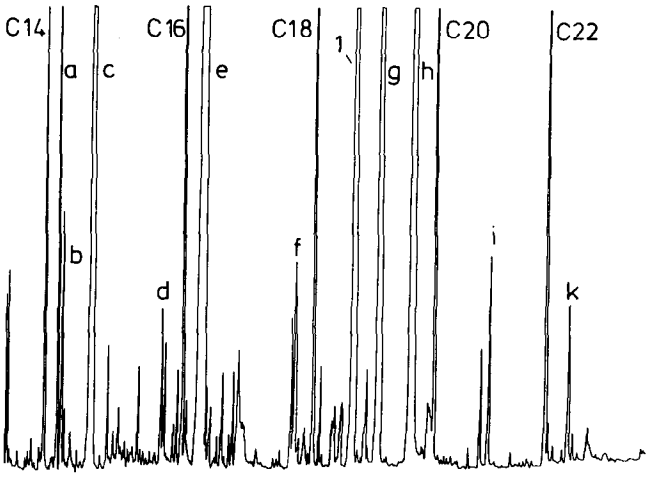

Fig. 5. Glass capillary gas chromatogram of urinary organic acids, after consumption of onions, separated as their methyl esters. Peaks: a = S-methyl-N-acetylcysteine; b = citric acid; c = furoylglycine; d = unknown; e = hippuric acid; f = unknown; g = *m*-methoxyhippuric acid; h = *p*-methoxyhippuric acid; i = dimethoxyhippuric acid; k = dimethoxycinnamoylglycine; 1 = N-acetyl-S-(2-carboxypropyl) cysteine.

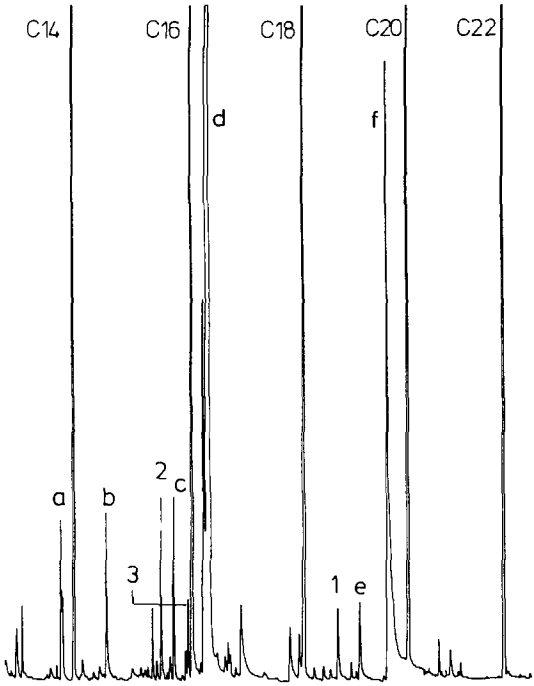

Fig. 6. Glass capillary gas chromatogram of urinary organic acids, after consumption of garlic, separated as their methyl esters. Peaks: a = 3-methylcrotonylglycine; b = furoylglycine; c = unknown; d = hippuric acid; e = *m*-methoxyhippuric acid; f = *p*-methoxyhippuric acid; 1 = N-acetyl-S-(2-carboxypropyl) cysteine; 2 = N-acetyl-S-allylcysteine; 3 = hexahydrohippuric acid.

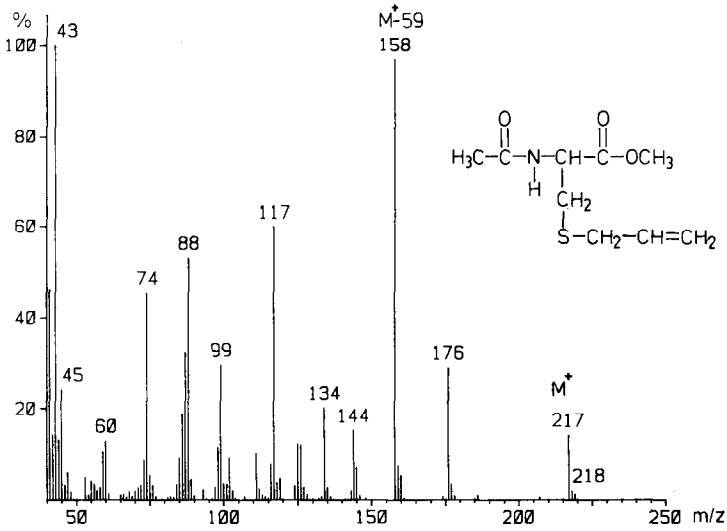

Fig. 7. Mass spectrum of N-acetyl-S-allylcysteine methyl ester.

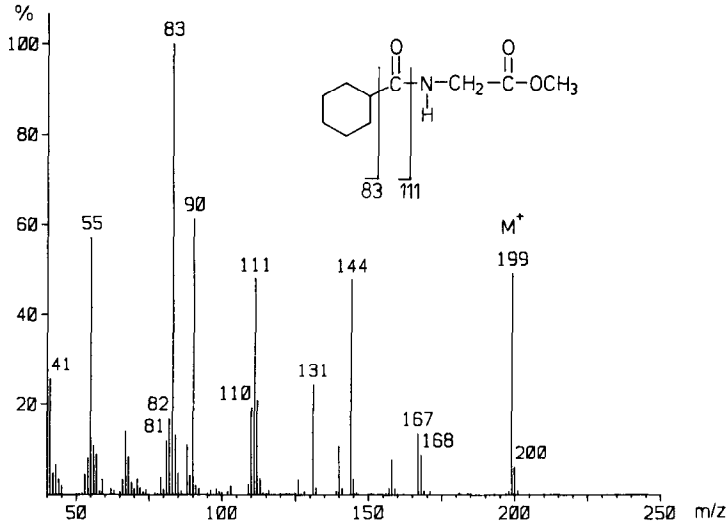

Fig. 8. Mass spectrum of hexahydrohippuric acid methyl ester.

tion measurement). If the mercapturic acid residue (m/z 176) is subtracted from the mass of the molecular ion, the remaining residue connected to the sulphur atom must contain 101 mass units. Furthermore, the residue must contain an additional carbomethoxy group, because from the ion m/z 218 two molecules of CH_3OH and one molecule of CO are eliminated ($218-CH_3OH=186$; $186-CO=158$; $158-CH_3OH=126$). Therefore the residue was assumed to be (C_3H_6)-$COOCH_3$.

The comparison of retention indices and mass spectra of the natural product with those of the synthetic N-acetyl-S-(2-carboxypropyl)cysteine (**1**) and its

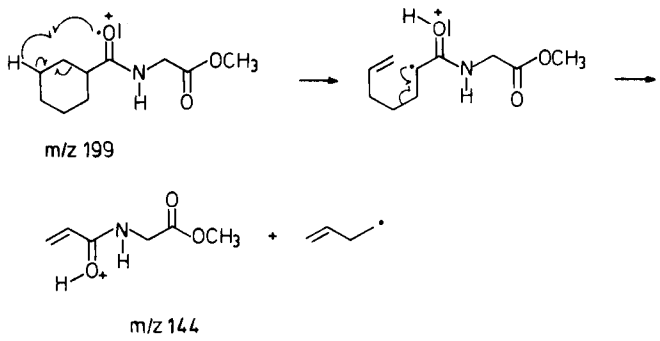

Fig. 9. Origin of the abundant ion of mass 144.

isomer N-acetyl-S-(1-methyl-2-carboxyethyl)cysteine (**4**) clearly confirmed structure **1** (Fig. 4.).

It is well known that mercapturic acids are metabolites of the corresponding glutathione conjugates [8]. Virtanen and Matikkala [9,10] found γ-glutamyl-S-(2-carboxypropyl)cysteinylglycine (**5**) in onions [9,10] and we suspected, therefore, that this compound might be the precursor of **1**. To verify this assumption, urine samples were collected after consumption of onions. Gas chromatograms of the methylated conjugate fraction showed a dramatic increase of peak 1 (Fig. 5).

Peptides similar to those occurring in onions are reported to be present in garlic. In addition, it was shown that garlic contains γ-glutamyl-S-allylcysteine (**6**) [10,11]. When garlic was consumed the collected urine samples showed the urine profile of the methylated conjugate fraction reproduced in Fig. 6. In addition to peak 1, we detected another mercapturic acid (peak 2, RI 1540), which turned out to be N-acetyl-S-allylcysteine (**2**), by comparison with the synthetic material.

Besides the typical ions of mass 88, 144, 176 and 158 ($M^+ - 59$), an intensive fragment of mass 117 is observed in the mass spectrum of **2** (Fig. 7). This peak can be derived from the ion *m/z* 158 ($M^+ - H_3C\text{–}CONH_2$) by loss of an allyl radical.

An additional compound, previously unknown in human urine corresponds to peak 3 (Fig. 6). Its mass spectrum (Fig. 8) shows a molecular ion of mass 199. The loss of a neutral fragment of 88 mass units, combined with the presence of a fragment of mass 90, is typical for a glycine conjugate; the latter ion is observed in dipeptides with C-terminal glycine [12]. The fragment of mass 83 is characteristic of a cyclohexyl residue. Thus it was suspected that the compound could be hexahydrohippuric acid (**3**). This assumption was confirmed by synthesis and comparison of the MS and RI values. The origin of the abundant ion of mass 144 can be explained in the following way as shown in Fig. 9.

Hexahydrohippuric acid was reported as a natural product for the first time in 1971. It was isolated from cattle urine [13], but could not be detected in human urine [14]. Labeling experiments proved later that this compound is a metabolite of shikimic acid [15].

DISCUSSION

S-Carboxyalkylcysteines are common compounds in human urine [16]. The corresponding N-acetylated derivatives (mercapturic acids) have not previously been detected. In humans, biosynthesis of mercapturic acid is an important detoxification mechanism of exogenic electrophilic compounds [8]. The metabolism of many xenobiotics occurs by this detoxification route. Mercapturic acids have been detected in the urine of rats dosed with halogenated alkanes [7], halogenated alkenes [17], acrylic esters [18] and styrene [19]. Also, the excretion of these thioethers is considered to be an indirect proof of human exposure to compounds of exogenous origin. N-Acetyl-S-(2-hydroxyethyl)cysteine was detected in the urine of workers exposed to vinyl chloride [20].

In this investigation, we demonstrated that at least compound **1** is nearly always present in human urine in minute amounts. The excretion of high levels of **1** and **2** was observed following consumption of onions or garlic. Hexahydrohippuric acid (**3**), which was previously detected only in the urine of herbivores [14], is also present in human urine if vegetables are consumed.

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