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Synthesis of *N*-acetyl-*S*-(3-coumarinyl)-cysteine methyl ester and HPLC analysis of urinary coumarin metabolites

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Abstract

N-Acetyl-*S*-(3-coumarinyl)cysteine, a metabolite of coumarin in rodents, has been synthesized as methyl ester. A new synthetic route to prepare *N*-acetyl-*S*-(3-coumarinyl)-D,L-cysteine methyl ester (**1**) comprises reaction of 3-mercaptocoumarin (**3**) with *N*-acetyl-3-chloro-D,L-alanine methyl ester (**4**). *N*-acetyl-*S*-(4-coumarinyl)-L-cysteine (**10**) was obtained by reaction of 3-bromocoumarin (**12**) and *N*-acetyl-L-cysteine (**13**). A method for the determination of *N*-acetyl-*S*-(3-coumarinyl)cysteine as its methyl ester in urine by HPLC has been developed.

Keywords: Coumarin; *N*-Acetyl-*S*-(3-coumarinyl)cysteine; Metabolism; Synthesis; HPLC-determination

1. Introduction

Coumarin (2H-1-benzopyran-2-one (**11**)), is a widely occurring secondary plant metabolite that has been used as a fragrance in food and cosmetic products. After rapid absorption from the gastrointestinal tract coumarin is distributed throughout the body (Cohen, 1979; Fentem and Fry, 1993). Coumarin has also been found to be well absorbed through the skin (Beckley-Kartey et al., 1997; Yourick and Bronaugh, 1997). The daily intake of coumarin has been estimated to reach 0.02 mg kg^{-1} body weight via food and 0.04 mg kg^{-1} body weight from cosmetic products (Lake, 1999). Coumarin has been reported to be used clinically for treatment of lymphedemas, mainly in breast cancer patients (Burgos et al., 1999; Loprinzi et al., 1999); for treatment of renal cell carcinoma (Sagaster et al., 1995; Marshall et al., 1994); and for prophylaxis of thromboembolism in gynecologic oncology (Graf et al., 1998).

The biotransformation of coumarin is known to be species-dependent (Pearce et al., 1992; Bogan et al., 1996). It appears that 7-hydroxylation of coumarin is the major phase I pathway in the majority of human subjects (Rautio et al., 1992; Bogan et al., 1995), catalyzed predominantly by cytochrome P450 isoenzyme CYP2A6 (Li et al., 1997). In phase II biotransformation, 7-hydroxycoumarin is converted into glucuronide or sulfate

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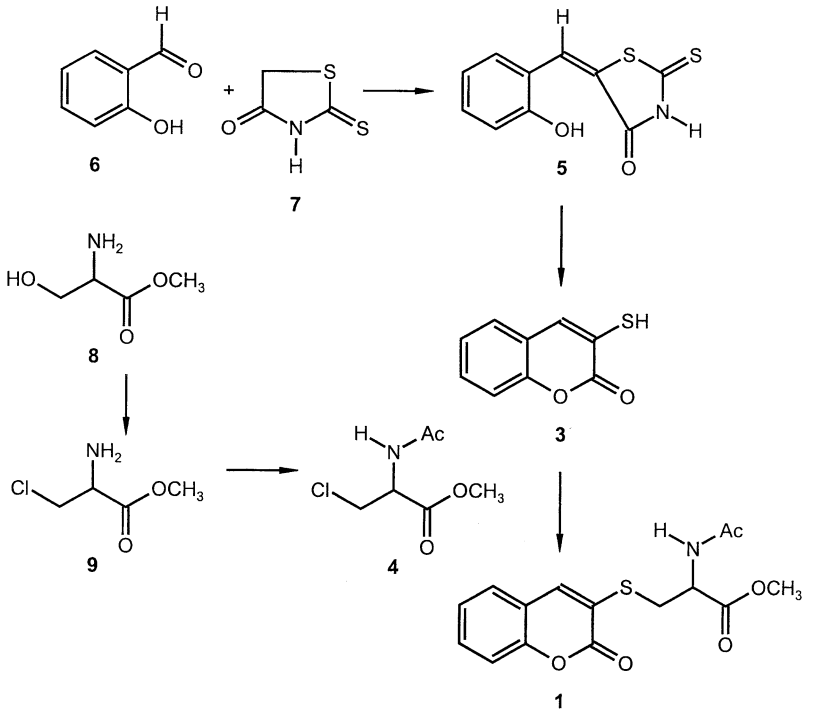
conjugates (Price et al., 1995; Duffy and O'Kennedy, 1998). 7-Hydroxycoumarin and its glucuronide or sulfate represent about 80% of human urinary coumarin metabolites following oral administration (Shilling et al., 1969). However, humans also convert coumarin to coumarin-3,4-epoxide as a minor metabolic pathway, resulting in the formation of 2-hydroxyphenylacetic acid. In 24 h urine, 2-hydroxyphenylacetic acid has been reported to account for about 4% of the coumarin dose administered orally (200 mg per person) (Shilling et al., 1969). CYP2A6 has been found to be expressed polymorphically. About 15% of Chinese subjects bear a defective CYP2A6 allele as compared with 1–3% of European populations (Oscarson et al., 1998, 1999). After oral administration of coumarin to subjects (2 mg) with a defective CYP2A6 allele, no 7-hydroxycoumarin was detected in the urine (0–8 h). However, about 50% of the dose was eliminated as 2-hydroxyphenylacetic acid (Hadidi et al., 1997). Single amino acid substitution (Leu160His) in CYP2A6 has been found to cause switching from 7- to 3-hydroxylation, resulting in predominant excretion of 2-hydroxyphenylacetic acid after oral coumarin (2 mg) (Hadidi et al., 1997). Among a population of two hundred, two individuals were deficient for 7-hydroxycoumarin formation after an oral dose of 5 mg coumarin (Oscarson et al., 1998). Thus, as a result of CYP2A6 polymorphism, biotransformation of coumarin via coumarin 3,4-epoxide might represent a major pathway in a very minor human population.

In contrast to the normal human situation, 3,4-epoxidation represents the major metabolic route in rat and mouse, resulting in the formation of an unstable coumarin 3,4-epoxide which degrades spontaneously to form 2-hydroxyphenylacetaldehyde that may be subsequently converted to 2-hydroxyphenylethanol and 2-hydroxyphenylacetic acid (Lake, 1999; Lake et al., 1995; Born et al., 1997; Lovell et al., 1999). Recently, von Weymarn and Murphy have reported that the major coumarin metabolites formed by rat esophageal microsomes are 8-hydroxycoumarin, *o*-hydroxyphenylacetaldehyde, and *o*-hydroxyphenylacetic acid (von Weymarn and Murphy, 2001) *N*-Acetyl-*S*-(3-coumarinyl)cysteine has been reported to be excreted in the urine of rats given [benzene-^{14}C]coumarin, obviously as a result of coumarin-3,4-epoxide conjugation with glutathione (Huwer et al., 1991). Glutathione conjugation appears as an important detoxification reaction, since the formation of 2-hydroxyphenylacetaldehyde can be inhibited by glutathione (Zhuo et al., 1999).

It thus appeared of interest to quantify the metabolic pathway encompassing formation of coumarin 3,4-epoxide (Ford et al., 2001), with subsequent urinary excretion of *o*-hydroxyphenylacetic acid or, after glutathione conjugation, of *N*-acetyl-*S*-(3-coumarinyl)cysteine. *N*-Acetyl-*S*-(3-coumarinyl)cysteine until now has not been synthesized. Its identity as a urinary metabolite was established earlier by comparison of the ^{1}H-NMR and mass spectra of *N*-acetyl-*S*-(3-coumarinyl)cysteine isolated from rat urine with those of synthetic *N*-acetyl-*S*-(4-coumarinyl)cysteine (Huwer et al., 1991). In the present study, we report the synthesis of *N*-acetyl-*S*-(3-coumarinyl)-D,L-cysteine methyl ester (**1**) from 3-mercaptocoumarin (**3**) and *N*-acetyl-3-chloro-D,L-alanine methyl ester (**4**). *N*-Acetyl-*S*-(4-coumarinyl)-L-cysteine (**10**) was synthesized by reaction of 3-bromocoumarin (**12**) with *N*-acetyl-L-cysteine (**13**). A method for the determination of *N*-acetyl-*S*-(3-coumarinyl)cysteine in urine by HPLC as its methyl ester is described.

2. Chemistry

N-Acetyl-*S*-(3-coumarinyl)-D,L-cysteine methyl ester (**1**) was prepared by reaction of 3-mercaptocoumarin (**3**) with *N*-acetyl-3-chloro-D,L-alanine methyl ester (**4**) (Scheme 1). 3-Mercaptocoumarin (**3**) was obtained from 2-hydroxybenzylidenerhodanine (**5**) by refluxing in diluted ethanolic sodium hydroxide solution (Xing et al., 1987). Compound **5** was prepared by condensation of 2-hydroxybenzaldehyde (**6**) with rhodanine (**7**) (Mackie and Misra, 1954). D,L-Serine methyl ester hydrochloride (**8**) was used as starting material for preparation of **4**. Chlorination of D,L-serine methyl ester hydrochloride in acetyl chloride with PCl_5 yielded 3-chloro-D,L-alanine methyl ester hydrochloride



Scheme 1. Synthetic route of *N*-acetyl-*S*-(3-coumarinyl)-D,L-cysteine methyl ester (**1**).

(**9**), which was N-acetylated by refluxing with acetyl chloride in benzene.

N-Acetyl-*S*-(4-coumarinyl)-L-cysteine (**10**) was prepared by the reaction of 3-bromocoumarin (**12**) with *N*-acetyl-L-cysteine (**13**). 3-Bromocoumarin (**12**) can be easily obtained by bromination of coumarin (**11**) in chloroform in the presence of triethylamine (Brogden et al., 1974). Methylation of **10** to **2** was carried out using borone trifluoride in methanol (Hallas, 1965). The synthetic route of *N*-acetyl-*S*-(4-coumarinyl)-L-cysteine methyl ester (**2**) is demonstrated in Scheme 2.

Compounds **1** and **2** were characterized by elemental analysis; NMR-spectroscopy, IR-spectroscopy, UV-spectroscopy and mass spectrometry. The ^{1}H-NMR-spectra of **1** and **2** are given in Fig. 1. The ^{1}H-NMR-spectrum of **1** matched with the ^{1}H-NMR-spectrum of *N*-acetyl-*S*-(3-coumarinyl)cysteine isolated from urine of rats given coumarin orally (Huwer et al., 1991).

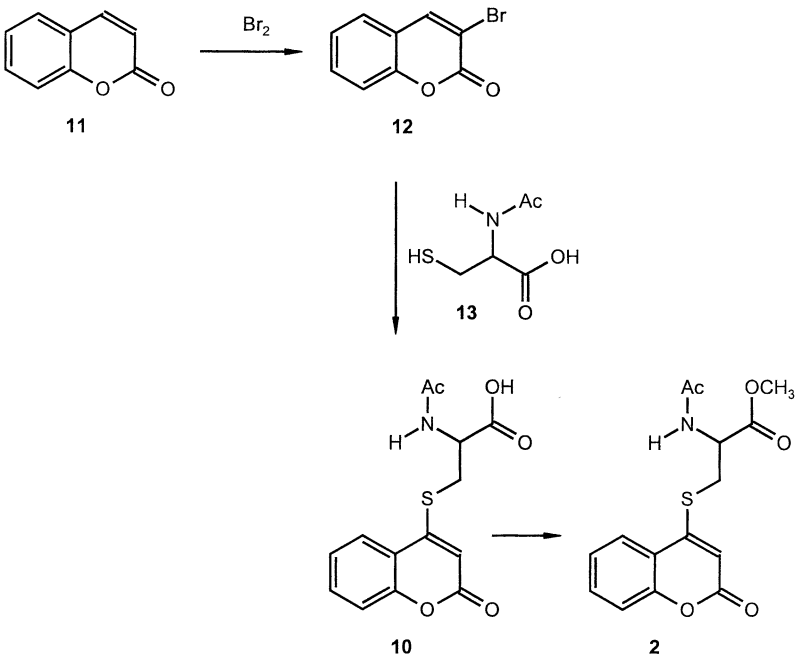
N-Acetyl-*S*-(4-coumarinyl)cysteine (**10**) in urine was concentrated using solid phase extraction on a Merck Lichrolut Extraction Cartridge, RP 18. After elution with methanol/buffer pH 2.5, it was converted to the methylester as described before, using borone trifluoride in methanol. Recoveries reached 65–90%.

N-Acetyl-*S*-(3-coumarinyl)-L-cysteine methyl ester (**1**) and its 4-coumarinyl counterpart **2** can be clearly separated under the HPLC conditions used (Fig. 2a). The separation profile of **11**, **14**, **15** and **1** is demonstrated in Fig. 2b.

3. Discussion

N-Acetyl-*S*-(3-coumarinyl)-D,L-cysteine methyl ester (**1**) (coumarin 3-mercapturic acid methylester) is easily accessible by reaction of 3-mercaptocoumarin (**3**) with *N*-acetyl-3-chloro-D,L-alanine methyl ester (**4**). Formation of *N*-acetyl-*S*-(3-coumarinyl)cysteine has been suggested to result from conjugation of phase I metabolite coumarin-3,4-epoxide with glutathione.

The 4-mercapturic acid isomer **10** was prepared by the reaction of 3-bromocoumarin (**12**) with *N*-



Scheme 2. Synthetic route of *N*-acetyl-*S*-(4-coumarinyl)-L-cysteine methyl ester (**2**).

acetyl-L-cysteine (**13**). This reaction was found to proceed more smoothly and at higher yields than by using 4-chlorocoumarin, as described before.

As can be seen from the HPLC chromatograms, coumarin 3-mercapturic acid can be easily determined in urine as its methyl ester, in the presence of parent compound and further hydroxylated metabolites, with a limit of determination around 1–2 μg ml^{-1}. In clinical use, daily coumarin doses of 100–200 mg have been administered (Burgos et al., 1999; Shilling et al., 1969). On the assumption that normally about 4% of the administered coumarin will be metabolized via coumarin-3,4-epoxide, *N*-acetyl-*S*-(3-coumarinyl)cysteine present in 24 urine will be below the detection limit.

While the majority of humans metabolize coumarin to 7-hydroxycoumarin, it has also been reported that a deficiency in this pathway was observed in some individuals, apparently related to a genetic polymorphism in CYP2A6. Such individuals might metabolize coumarin preferentially by the 3,4-epoxidation pathway and possibly other alternative pathways (Oscarson et al., 1998, 1999; Hadidi et al., 1997; Ford et al., 2001; van Iersel et al., 1994; Hadidi et al., 1998; Meineke et al., 1998). In the case of a defective CYP2A6 allele, higher formation rates of coumarin 3,4-epoxide are to be expected. The urinary excretion of *N*-acetyl-*S*-(3-coumarinyl)cysteine in such subjects is expected to be quantifiable on the basis of the above HPLC method.

4. Experimental part

4.1. Chemistry

4.1.1. General

Coumarin, 2-hydroxybenzaldehyde, rhodanine, serine methyl ester, triethylamine, phosphorus pentachloride, acetyl chloride, and solvents were obtained from commercial sources. All reagents were of analytical grade.

Melting points were measured using Büchi 510 and are not corrected. Elemental analysis was carried out by Perkin Elmer 2400 CHN-Elemental-Analyzer; ^{1}H-NMR- and ^{13}C-NMR-spectra by Bruker AM 400; IR-spectra by Perkin Elmer FT-

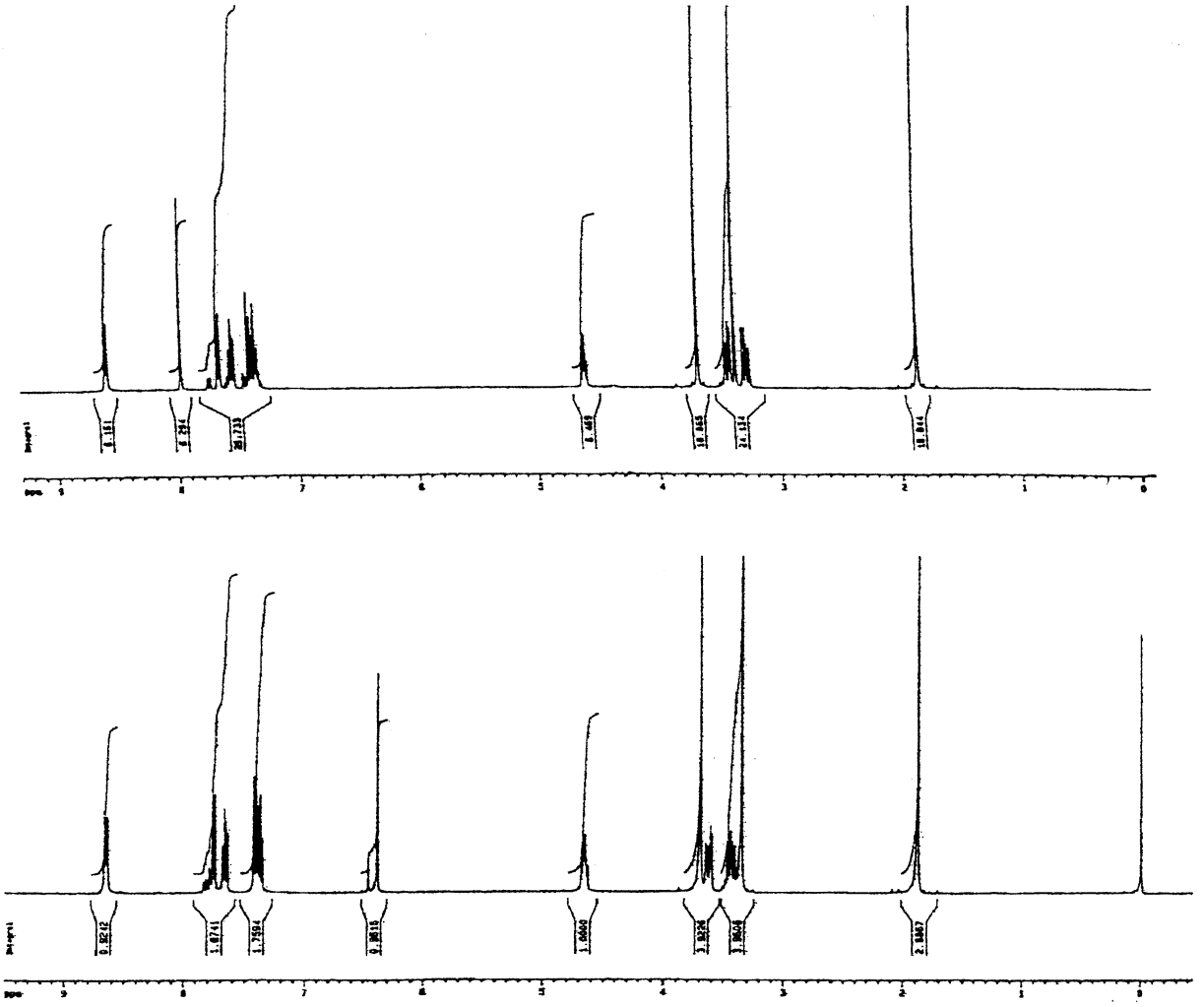


Fig. 1. ^{1}H-NMR-spectra of *N*-acetyl-*S*-(3-coumarinyl)cysteine methyl ester (**1**) (upper) and *N*-acetyl-*S*-(4-coumarinyl)cysteine methyl ester (**2**) (below).

IR 90; UV-spectra by Kontron-Uvikon 810; and mass spectra by Finnigan MAT 90 mass spectrometer.

4.2. *N*-Acetyl-*S*-(3-coumarinyl)-D,L-cysteine methyl ester (*1*)

Freshly distilled triethylamine (0.9 ml) was slowly added to a solution of 3-mercaptocoumarin (**3**) (500 mg, 3 mmol) and *N*-acetyl-3-chloro-D,L-alanine methyl ester (**4**) (500 mg, 3 mmol) in acetonitrile (60 ml) under argon atmosphere. The mixture was stirred for 4 h at 50 °C. Until termination of the reaction (DC control; DC-Alufoils, Kieselgel 60 F 254 Merck). The solvent was removed in vacuo; **1** was washed with *n*-hexane and water to yield a white, crystalline powder (400 mg, 47%), m.p. 175 °C.—$C_{15}H_{15}NO_5S$ (321.3) calc. C, 56.0; H, 4.7; N, 4.4. Found: C, 55.9; H, 4.8; N, 4.0%.—^{1}H-NMR (d_6-DMSO): δ (ppm) = 8.58 (d, 1H, N*H*), 7.96 (s, 1H, *H*-4), 7.56–7.35 (m, 4H, aromat), 4.60 (m, 1H, N–

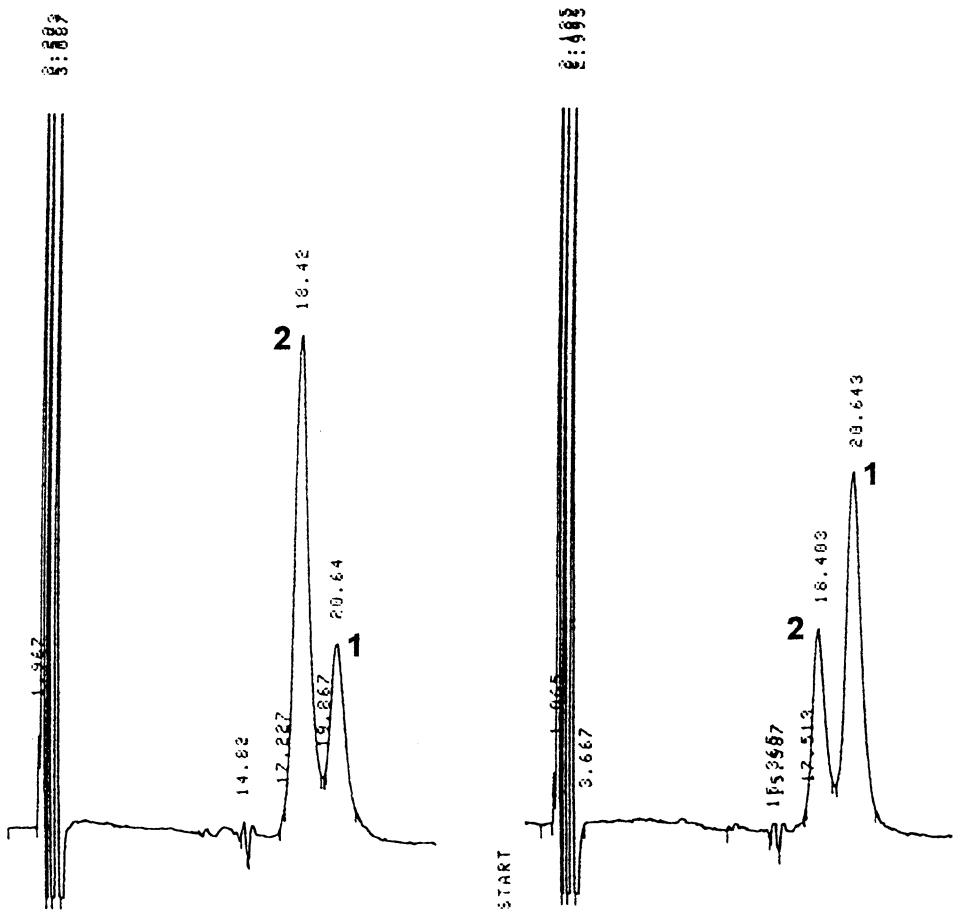
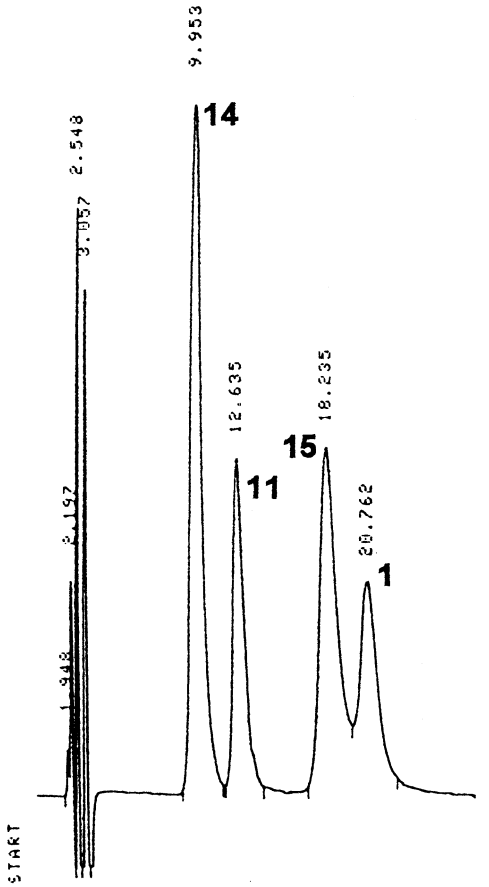


Fig. 2. HPLC chromatogram of a standard mixture composed of 3 μg *N*-acetyl-*S*-(4-coumarinyl)-L-cysteine methyl ester (**2**) and 2 μg *N*-acetyl-*S*-(3-coumarinyl)cysteine methyl ester (**1**) in 1 ml urine. UV-detector wavelength: 300 nm (right panel), 330 nm (left panel). (b) HPLC chromatogram of *N*-acetyl-*S*-(3-coumarinyl)-D,L-cysteine methyl ester (**1**), coumarin (**11**), 7-hydroxycoumarin (**14**), and 3-hydroxycoumarin (**15**). UV-Detector wavelength: 330 nm.

C*H*–CO_2), 3.66 (s, 3H, CO_2CH_3), 3.41 (dd, 1H, SCH_2), 3.26 (dd, 1H, SCH_2), 1.85 (s, 3H, $NCOCH_3$).—^{13}C-NMR (d_6-DMSO): δ (ppm) = 170.7 (s, CO_2), 169.6 (s, N*C*O), 158.4 (s, *C*2), 151.3 (s, *C*10), 136.0 (d, *C*4), 130.7 (d, *C*8), 127.1 (d, *C*7), 125.4 (s, *C*3), 124.9 (d, *C*6); 119.3 (s, *C*9), 116.0 (d, *C*5), 52.3 (q, CO_2CH_3), 50.8 (d, NH*C*H), 31.5 (t, SCH_2), 22.2 (q, $NCOCH_3$).—IR (KBr): $\upsilon = 3296$ cm^{-1} (amide), 1764 (ester), 1704 (lactone), 1530 (aromat), 754 (1,2-substituted aromat).—UV (CH_3OH): $\lambda = 330$ nm ($\varepsilon =$ 10 307), $\lambda = 235$ nm ($\varepsilon = 6154$).—MS: $m/z =$ 321.4 [M^+], 276.6 [$M^+ - COCH_2$], 246.6, 224.5.

4.3. 3-Bromocoumarin (*12*)

Coumarin (10 g, 68 mmol) was dissolved in 100 ml chloroform and bromine (10.9 g, 68 mmol) was added into the solution in an ice bath. The reaction mixture was stirred overnight until colorless, triethylamine (20 ml) was then added and the organic solution was washed two times with water. Compound **12** was obtained as a yellowish solid (11.5 g, 75%), m.p. 108 °C.—^{1}H-NMR ($CDCl_3$): δ (ppm) = 8.15 (s, 1H, *H*-4), 7.85–7.15 (m, 4H, aromat). The bromine substitution at position 3 is indicated by the signal at 8.15 ppm in the ^{1}H-



(b)

Fig. 2 (*Continued*)

NMR spectrum, showing the presence of a proton at position 4.

4.4. *N*-Acetyl-*S*-(4-coumarinyl)-L-cysteine (**10**)

3-Bromocoumarin (**12**) (10 g, 44 mmol) and *N*-acetyl-L-cysteine (**13**) (7.3 g, 44 mmol) were dissolved in dry methanol (100 ml). Triethylamine (15 ml) was added into the reaction mixture and stirred for 4 h at room temperature. After removing half of the solvent the reaction mixture was acidified with hydrochloric acid. The precipitate was collected on a filter, washed with *n*-hexane and water, and dried over P_2O_5 to yield **10** as a white, crystalline powder (6.1 g, 45%), m.p. 215–220 °C. $C_{14}H_{13}NO_5S$ (307.3) calc. C, 54.7; H, 4.3; N, 4.6. Found: C, 53.7; H, 4.2; N, 4.5%.—^{1}H-NMR (d_6-DMSO): δ (ppm) = 13.22 (s, 1H, CO_2H), 8.55 (d, 1H, N*H*), 7.79–7.39 (m, 4H, aromat), 6.42 (s, 1H, *H*-3), 4.61 (m, 1H, NC*H*CO_2), 3.62 (dd, 1H, SCH_2), 3.43 (dd, 1H, SCH_2); 1.89 (s, 3H, NCOCH_3). The protons of the methylene group present as two peaks at 3.62 and 3.43 ppm, indicating the occurrence of epimerization during the reaction. The ^{1}H-NMR data of **10** agree with those described by Huwer et al. (Huwer et al., 1991).—^{13}C-NMR (d_6-DMSO): δ (ppm) = 171.3 (s, *C*O_2H), 169.6 (s, N*C*O); 157.9 (s, *C*-2); 154.7 (s, *C*-4); 151.5 (s, *C*-10); 132.7 (d, *C*-8); 124.5 (d, *C*-7); 123.7 (d, *C*-6); 117.4 (s, *C*-9); 116.9 (d, *C*-5); 107.1 (d, *C*-3); 50.6 (d, NH*C*H); 31.5 (t, S*C*H_2); 22.3 (q, NCO*C*H_3).—UV (CH_3OH): λ =

300 nm ($\varepsilon = 11\,960$); $\lambda = 278$ nm ($\varepsilon = 10\,413$).—MS: $m/z = 307.4$ [M^+], 276.6 [$M^+ - COCH_2$].

4.5. N-Acetyl-S-(4-coumarinyl)-L-cysteine methyl ester (2)

50% boron trifluoride solution in methanol (1 ml) was added into a suspension of **10** (500 mg, 2 mmol) in dry methanol (10 ml). The mixture was heated up to 70 °C until it became a clear solution. Water (20 ml) was added after cooling. The precipitate was collected by filtration and dried in vacuo to give **2** as a white crystalline powder (420 mg, 82%), m.p. 155 °C.—$C_{15}H_{15}NO_5S$ (321.3) calc. C, 56.0; H, 4.7; N, 4.4. Found: C, 55.5; H, 4.5; N, 4.1%.—^{1}H-NMR (d_6-DMSO): δ (ppm) = 8.65 (d, 1H, N*H*), 7.76–7.36 (m, 4H, aromat), 6.38 (s, 1H, *H*-3); 4.66 (m, 1H, NC*H*CO_2), 3.69 (s, 3H, CO_2CH_3), 3.62 (dd, 1H, SCH_2), 3.42 (dd, 1H, SCH_2), 1.87 (s, 3H, $NCOCH_3$).—^{13}C-NMR (d_6-DMSO): δ (ppm) = 170.4 (s, CO_2), 169.7 (s, N*C*O), 157.9 (s, *C*-2), 154.5 (s, *C*-4), 151.5 (s, *C*-10), 132.7 (d, *C*-8), 124.5 (d, *C*-7), 123.7 (d, *C*-6), 117.4 (s, *C*-9), 116.9 (d, *C*-5), 107.2 (d, *C*-3), 52.4 (q, CO_2CH_3), 50.5 (d, NH*C*H), 31.2 (t, SCH_2), 22.2 (q, $NHCOCH_3$).—IR (KBr): $\upsilon = 3308$ cm^{-1} (amide), 1760 (ester), 1712 (lactone), 1654 (amide), 1530 (aromat), 760–740 (1,2-substituted aromat).

4.6. Determination of N-acetyl-S-(3-coumarinyl)cysteine methyl ester (1) in urine in a model experiment

4.6.1. Extraction and purification of N-acetyl-S-(4-coumarinyl)cysteine (10) from urine

Urine (1 ml) containing **10** (2 μg) was passed through a cartridge [Merck Lichrolut Extraction Cartridge (400 mg) RP 18] which was washed with buffer [3 g KH_2PO_4 l^{-1}, adjusted to pH 2.5 with H_3PO_4] and eluted with methanol/buffer 2:8 (v/v) collecting 1 ml fractions. Compound **10** was found in fractions 5–11, using HPLC under the following conditions: column: RP18, 250 × 4 mm, particle size 5 μm (Merck LiChorCart); UV-detector, 300 nm; solvent: methanol/buffer (pH 2.5) 4:6 (v/v); flow rate: 1 ml min^{-1}. Fractions 5–11 were combined and concentrated to 1 ml in vacuo.

4.6.2. HPLC determinations

Fractions containing **10** were combined and lyophilized. The dry residue was dissolved in 100 μl of a 25% BF_3/methanol solution and heated up to 70 °C for 10 min. Thereafter it was made up to 1 ml with water. Compound **2** was determined by HPLC under similar conditions as described for **10**, using methanol/buffer (pH 2.5) 1:1 (v/v) as solvent.

Compounds **1** (2 μg ml^{-1}) and **2** (3 μg ml^{-1}) in urine were isolated by solid extraction as described and determined by HPLC under the same conditions as described for **2** with an UV-detector (300 and 330 nm, respectively).

For determination of **11**, **14**, **15**, and **1** (2 μg per 1 ml) in urine, extraction and fractionation was as described for **10**, HPLC: column: RP18, 250 × 4 mm, particle size 5 μm (Merck LiChorCart); elution solvent: methanol/buffer (pH 2.5)/tetrahydrofurane/acetonitrile 153:20:17:10 (v/v); flow rate: 1 ml min^{-1}; UV-detector (300/330 nm).

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