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Analysis of bile acid glutathione thioesters by liquid chromatography/electrospray ionization–tandem mass spectrometry☆

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Abstract

The formation of thioester-linked glutathione (GSH) conjugates of bile acids (BAs) is presumed to occur via trans-acylation reactions between GSH and reactive acyl-linked metabolites of BAs. The present study examines the chemical reactivity of cholyl-adenylate and cholyl-CoA thioester, acyl-linked metabolites of cholic acid (CA), with GSH to form CA-GSH conjugate *in vitro*. The authentic specimen of CA-GSH was synthesized along with GSH conjugates of four common BAs found in the human body. Their structures were confirmed by proton-nuclear magnetic resonance spectroscopy and electrospray ionization (ESI)–tandem mass spectrometry in positive- and negative-ion modes. Incubation of cholyl-adenylate or cholyl-CoA thioester with GSH was carried out at pH 7.5 and 37 °C for 30 min, with analysis of the reaction mixture by liquid chromatography/ESI–tandem mass spectrometry, where CA-GSH was detected on the product ion mass chromatograms monitored with stable and abundant dehydrated positive-ion $[M+H-H_2O]^+$ at *m/z* 680.3 and fragmented negative-ion $[GSH-H]^-$ at *m/z* 306.0, and was definitely identified by CID spectra by comparison with those of the authentic sample. The results show that both cholyl-adenylate and cholyl-CoA thioester are able to acylate GSH *in vitro*.

Keywords: Bile acid; Glutathione; Glutathione conjugate; Liquid chromatography; Electrospray ionization; Mass spectrometry; LC–MS; Metabolite

1. Introduction

Bile acids (BAs) are one of a group of organic acids that are converted into *N*-acyl amino acid conjugates. They are synthesized *de novo* in the liver from cholesterol and have important detergent properties that assist the lipolysis and adsorption of fats in the hepatobiliary system and in the lumen of the upper small intestine [1]. The presence of the amino acid moiety lowers the pK_a of the BAs, thus preventing their precipitation at the acid pH of the upper gut [2]. Amidation of BAs with amino acids such as glycine and taurine is a two-step process involving the successive action of the enzymes bile acid: CoA ligase (BAL) and bile acid CoA:amino acid *N*-acyltransferase. In the initial reaction catalyzed by BAL, BAs are converted into the mixed anhydride with adenosine 5′-monophosphate (AMP) prior to the formation of the corresponding acyl-CoA thioester [3,4]. The acyl-adenylate has an electrophilic carbonyl-carbon and spontaneously reacts with the amino group of taurine [3], substance P [5], lysozyme [5], and histone [6] in a phosphate buffer solution, indicating a higher reactivity of the acyl-adenylate toward amino group. BAs are metabolized to their acyl-glucuronides [7–11] and react *in vitro* with proteins, resulting in BA-protein adducts [12]. The presence of bile acid-protein adducts in tissue [13] and histones [14] has been considered in relation to physiological disorders such as cholestasis and colon cancer. Many drugs containing carboxylic acid are metabolized to acyl-CoA [15] and acyl-glucuronide species [16] that can form irreversible adducts with proteins [12,17,18], and it has been suggested that such drug-protein adducts cause allergic reactions [19] and are hepatotoxic [20].

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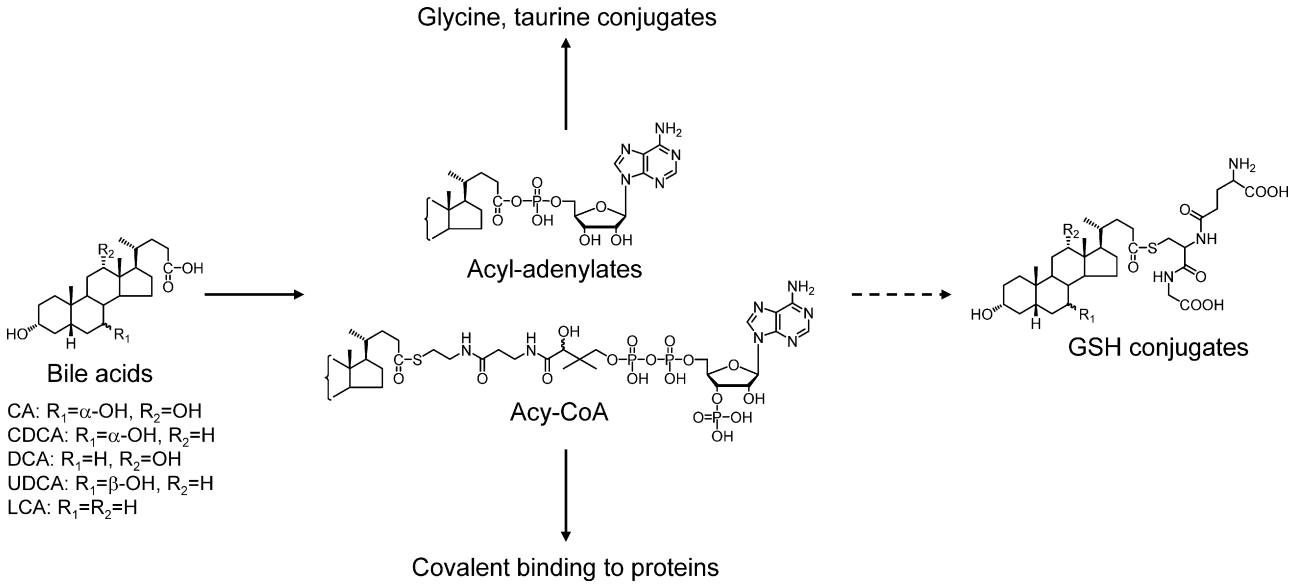

Fig. 1. Potential metabolic routes of bile acids and reactions of acyl-adenylates and acyl-CoA.

In addition to irreversible protein binding, drugs containing carboxylic acid are also converted *in vivo* and *in vitro* to a thioester-linked glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) conjugate [19–22]. The formation of GSH conjugate has been proposed to occur via *trans*-acylation reactions of reactive acyl-linked metabolites of drugs containing carboxylic acid with GSH [20]. Therefore it is likely that the acyl-linked metabolites of BAs are also converted into GSH conjugates (Fig. 1). Such GSH conjugates have never been isolated and characterized, however, because there have been neither suitable authentic specimens nor reliable methods for characterizing them.

Here we describe the chemical synthesis of authentic specimens of GSH conjugates of five major BAs in the human body and their characterization by liquid chromatography (LC)/electrospray ionization (ESI)–tandem mass spectrometry (MS). The nonenzymatic formation of a GSH conjugate by incubation of cholyl-adenylate (CA-AMP) and cholyl-CoA thioester (CA-CoA) with GSH in a buffer solution is also discussed.

2. Experimental

2.1. Materials and reagents

Cholic acid (CA), chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Lithocholic acid (LCA) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and ursodeoxycholic acid (UDCA) was kindly donated by Mitsubishi Pharma Co. (Osaka, Japan). Oxidized GSH (GSSG) was prepared from GSH (Peptide Institute Inc., Osaka, Japan) in our laboratories according to the reported method [23]. An Oasis HLB cartridge (60 mg, 3 ml) was provided by Waters Co. (Milford, MA, USA) and was successively conditioned with methanol (1 ml) and water (1 ml) prior to use. Acetonitrile and ammonium acetate of HPLC grade were purchased from Nacalai Tesque Inc. and distilled water of HPLC grade was purchased form Wako Pure Chemical Industries Ltd.

2.2. Apparatus

Proton-nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Mercury 300 (300 MHz, Varian Co. Ltd., Germany) or a JNM-ECA600 (600 MHz, JEOL Ltd., Tokyo, Japan) using tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet). Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (TOFMS) was carried out with a Kratos Kompact MALDI4 (Shimadzu Co., Kyoto, Japan). The samples were loaded onto the MALDI target plate by mixing 0.5 μl of each methanolic solution (1 mg/ml) with the same volume of a matrix solution prepared by dissolving 10 mg/ml of 2,5-dihydrobenzoic acid in 50% acetonitrile containing 0.1% of trifluoroacetic acid (TFA). Calibration was accomplished by using angiotensin II (Mw. 1047.20) as an internal standard. Preparative HPLC was carried out with a Shimadzu LC-10A *VP* (Shimadzu Co.) equipped with a UV detector (SPD-10A *VP* or SPD-6A, Shimadzu Co.).

2.3. LC/ESI–MS

The LC/MS analyses were carried out using a Finnigan LTQ ion-trap mass spectrometer (Thermo Electron, San Jose, CA, USA) equipped with an ESI source and coupled to a Paradigm MS4 pump (Michrom Bioresources Inc., Auburn, CA, USA) and an autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland). The ionization conditions for verifying the structures of BA-GSHs were as follows: ion source voltage, ±4 kV; capillary temperature, 270 °C; capillary voltage, ±20 V; sheath gas (nitrogen gas) flow rate, 50 arbitrary units (arb. units); auxiliary gas (nitrogen gas) flow rate, 5 arb. units; tube lens offset voltage, ±100 V. For tandem MS (MS^2) analysis, helium gas was used as the collision gas and the normalized collision

energy was set at 35%. The LC separations were conducted on a reversed-phase (RP) semi-micro column, TSKgel ODS-100 V (5 μm, 150 mm × 2.0 mm I.D.) from Tosoh Co. (Tokyo, Japan) by a linear gradient elution: 30% solvent B (acetonitrile) to 80% B against solvent A (5 mM ammonium acetate buffer, pH 6.0) over 30 min at a flow rate of 200 μl/min. Under these conditions, GSH conjugates of CA, CDCA, DCA, UDCA and LCA were, respectively, eluted at 5.6, 7.8, 8.1, 5.5, and 10.7 min.

2.4. Synthesis of CA-AMP and CA-CoA

CA-AMP and CA-CoA were chemically synthesized by the method previously reported [4]. The synthetic products were purified by preparative RP-HPLC with UV detection at 254 nm on a Cosmosil $5C_{18}$ column (250 mm × 10 mm I.D., Nacalai Tesque Inc.) by a linear gradient elution: 30% B (0.1% TFA in acetonitrile) to 80% B against solvent A (0.1% TFA) over 30 min at a flow rate of 4.7 ml/min. The fractions containing desirable product were collected and lyophilized to dryness. The structures of the purified CA-AMP and CA-CoA were confirmed by the negative-ion collision induced dissociation (CID) spectra of $[M-H]^-$ at *m/z* 736.4 and $[M-2H]^{2-}$ at *m/z* 577.8, respectively. The spectra show characteristic fragment ions at *m/z* 346.0 $[M-H–CA]^-$ for the former, and at *m/z* 1076.3 $[M-H–HPO_3]^-$, 1021.2 $[M-H–adenine]^-$ and 510.4 $[M-2H–adenine]^{2-}$ for the latter.

2.5. Syntheses of GSH conjugates of BAs (BA-GSHs)

2.5.1. Synthesis of [N,N′-bis(t-butoxycarbonyl)](oxidized glutathione) [(BocGS)$_2$]

To a solution of GSSG (0.4 g, 0.653 mmol) in water (10 ml) and acetone (10 ml) were added di-*t*-butyl dicarbonate (0.406 ml, 1.70 mmol) and $NaHCO_3$ (0.165 g, 1.96 mmol), and the mixture was stirred for 14 h at room temperature (r.t.). After the acetone was removed *in vacuo*, the aqueous solution was acidified with citric acid to pH 3 and saturated with NaCl. The resulting solution was extracted with AcOEt, and the combined extracts were washed with brine. The organic layer was dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give $(BocGS)_2$ as a colorless powder (0.463 g, 87.2%). The crude product was lyophilized from water to obtain as a fine powdery sample that without further purification was used for the next reaction. ^{1}H-NMR (DMSO-d_6) δ: 1.36 [18H, s, $-C(CH_3)_3 \times 2$), 1.72–1.92 (4H, m, Glu-β,β′-H × 2), 2.21 (4H, t, $J = 8.1$ Hz, Glu-γ,γ′-H × 2), 2.76–3.11 (4H, m, Cys-β,β′-H × 2), 3.73 (2H, d, $J = 6.3$ Hz, Gly-α-H × 2), 3.85 (2H, m, Glu-α-H × 2), 4.56 (2H, m, Cys-α-H × 2), 7.19 (2H, d, $J = 8.1$ Hz, Glu-NH × 2), 8.21 (2H, d, $J = 8.1$ Hz, Cys-NH × 2), 8.31 (2H, t, $J = 5.4$ Hz, Gly-NH × 2). MALDI-TOFMS: *m/z* 813.4 $[M+H]^+$, 836.0 $[M+Na]^+$, 858.5 $[M-H+2Na]^+$.

2.5.2. Synthesis of N-t-butoxycarbonylglutathione (Boc-GSH)

To a solution of $(BocGS)_2$ (1.14 g, 1.41 mmol) in water (10 ml) and acetone (10 ml) was added tributylphosphine (nBu$_3$P; 0.869 ml, 3.52 mmol), and the mixture was stirred for 1 h at r.t. After the acetone was removed *in vacuo*, the aqueous solution was washed with $CHCl_3$ and lyophilized to give Boc-GSH as a white powder (1.13 g, 98.2%), which without further purification was immediately used for the next reaction.

2.5.3. General method for preparation of bile acid succinimidyl esters (**1a–1e**)

To a stirred solution of each BA (0.2–3 g) in tetrahydrofuran (THF) (4–30 ml) and acetonitrile (1–3 ml) were added *N*-hydroxysuccinimide (1.2 eq) and a solution of *N*,*N*′-dicyclohexylcarbodiimide (1 eq) in THF (3–10 ml) at 0 °C, and the mixture was stirred for 24 h at r.t. The precipitated *N*,*N*′-dicyclohexylurea was filtered off, and the filtrate was concentrated *in vacuo*. The residue was dissolved in AcOEt, and the solution was washed with 5% $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give the desired esters **1a**–**1e**, which without further purification were used for the next reaction.

*2.5.3.1. CA 24-succinimidyl ester (**1a**).* The compound **1a** was obtained from CA (200 mg, 0.489 mmol) as colorless semi-solids; yield 209 mg (84.5%). ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-H), 0.88 (3H, s, 19-H), 1.01 (3H, d, $J = 5.9$ Hz, 21-H), 2.61 (2H, m, 23-H), 2.81 (4H, s, $-COCH_2CH_2CO-$), 3.46 (1H, m, 3β-H), 3.84 (1H, m, 7β-H), 3.96 (1H, m, 12β-H).

*2.5.3.2. CDCA 24-succinimidyl ester (**1b**).* The compound **1b** was obtained from CDCA (1.00 g, 2.55 mmol) as colorless semi-solids; yield 1.25 g (quant.). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-H), 0.91 (3H, s, 19-H), 0.97 (3H, d, $J = 6.0$ Hz, 21-H), 2.60 (2H, m, 23-H), 2.83 (4H, s, $-COCH_2CH_2CO-$), 3.46 (1H, m, 3β-H), 3.84 (1H, m, 7β-H).

*2.5.3.3. DCA 24-succinimidyl ester (**1c**).* The compound **1c** was obtained from DCA (3.00 g, 7.64 mmol) as colorless semi-solids; yield 3.25 g (86.8%). ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-H), 0.91 (3H, s, 19-H), 1.01 (3H, d, $J = 6.3$ Hz, 21-H), 2.60 (2H, m, 23-H), 2.84 (4H, s, $-COCH_2CH_2CO-$), 3.61 (1H, m, 3β-H), 3.98 (1H, m, 12β-H).

*2.5.3.4. UDCA 24-succinimidyl ester (**1d**).* The compound **1d** was obtained from UDCA (1.00 g, 2.55 mmol) as colorless semi-solids; yield 1.25 g (quant.). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-H), 0.91 (3H, s, 19-H), 0.97 (3H, d, $J = 6.0$ Hz, 21-H), 2.60 (2H, m, 23-H), 2.84 (4H, s, $-COCH_2CH_2CO-$), 3.46 (1H, m, 3β-H), 3.84 (1H, m, 7α-H).

*2.5.3.5. LCA 24-succinimidyl ester (**1e**).* The compound **1e** was obtained from LCA (1.00 g, 2.66 mmol) as colorless semi-solids; yield 1.06 g (84.3%). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-H), 0.92 (3H, s, 19-H), 0.95 (3H, d, $J = 6.3$ Hz, 21-H), 2.59 (2H, m, 23-H), 2.83 (4H, s, $-COCH_2CH_2CO-$), 3.67 (1H, m, 3β-H).

2.5.4. General method for preparation of Boc-GSH derivatives of BAs (**2a–2e**)

To a solution of each *N*-succinimidyl ester of BA (**1a**–**1e**) and equimolar amount of Boc-GSH in *N*,*N*′-dimethylformamide

(10–50 ml) was added *N*,*N*-diisopropylethylamine (2 eq), and the mixture was stirred for 24 h at r.t. under Ar. After evaporation of the solvent *in vacuo*, the residue dissolved in AcOEt was washed with 10% citric acid. After the product in the organic layer was extracted with 5% $NaHCO_3$, the extract was then acidified to pH 3 with citric acid and the aqueous solution was extracted with AcOEt. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give the desired compounds.

*2.5.4.1. N-t-Butoxycarbonyl-S-(cholyl)glutathione (**2a**).* The compound **2a** was obtained from **1a** (1.10 g, 2.17 mmol) as colorless semi-solids; yield 1.16 g (67.0%). ^{1}H-NMR (DMSO-d_6) δ: 0.57 (3H, s, 18-H), 0.79 (3H, s, 19-H), 0.90 (3H, d, J = 6.3 Hz, 21-H), 2.56 (2H, m, 23-H), 2.93-3.23 (2H, m, Cys-β,β′-H), 3.20 (1H, m, 3β-H), 3.72 (2H, m, Gly-α,α′-H), 3.84 (1H, m, 7β-H), 3.96 (1H, m, 12β-H), 3.85 (1H, m, Glu-α-H), 4.42 (1H, m, Cys-α-H), 7.06 (1H, d, J = 8.1 Hz, Glu-NH), 8.13 (1H, d, J = 8.4 Hz, Cys-NH), 8.31 (1H, t, J = 5.7 Hz, Gly-NH). MALDI-TOFMS: m/z 820.7 $[M + Na]^+$, 843.1 $[M - H + 2Na]^+$.

*2.5.4.2. N-t-Butoxycarbonyl-S-(chenodeoxycholyl)glutathione (**2b**).* The compound **2b** was obtained from **1b** (269 mg, 0.550 mmol) as colorless semi-solids; yield 357 mg (83.0%). ^{1}H-NMR (DMSO-d_6) δ: 0.59 (3H, s, 18-H), 0.82 (3H, s, 19-H), 0.88 (3H, d, J = 6.3 Hz, 21-H), 2.18 (2H, t, J = 7.2 Hz, Glu-γ,γ′-H), 2.64 (2H, m, 23-H), 2.93–3.24 (2H, m, Cys-β,β′-H), 3.15 (1H, m, 3β-H), 3.61 (1H, m, 7β-H), 3.72 (2H, m, Gly-α,α′-H), 3.85 (1H, m, Glu-α-H), 4.42 (1H, m, Cys-α-H), 7.06 (1H, d, J = 7.8 Hz, Glu-NH), 8.13 (1H, d, J = 8.4 Hz, Cys-NH), 8.26 (1H, t, J = 6.0 Hz, Gly-NH). MALDI-TOFMS: m/z 804.4 $[M + Na]^+$, 826.6 $[M - H + 2Na]^+$.

*2.5.4.3. N-t-Butoxycarbonyl-S-(deoxycholyl)glutathione (**2c**).* The compound **2c** was obtained from **1c** (522 mg, 1.07 mmol) as colorless semi-solids; yield 434 mg (52.1%). ^{1}H-NMR (DMSO-d_6) δ: 0.58 (3H, s, 18-H), 0.83 (3H, s, 19-H), 0.89 (3H, d, J = 5.7 Hz, 21-H), 2.18 (2H, t, J = 7.5 Hz, Glu-γ,γ′-H), 2.59 (2H, m, 23-H), 2.94-3.24 (2H, m, Cys-β,β′-H), 3.15 (1H, m, 3β-H), 3.71 (2H, m, Gly-α,α′-H), 3.76 (1H, m, 12β-H), 3.85 (1H, m, Glu-α-H), 4.44 (1H, m, Cys-α-H), 7.06 (1H, d, J = 8.4 Hz, Glu-NH), 8.12 (1H, d, J = 8.4 Hz, Cys-NH), 8.26 (1H, t, J = 5.7 Hz, Gly-NH). MALDI-TOFMS: m/z 804.7 $[M + Na]^+$, 826.79 $[M - H + 2Na]^+$.

*2.5.4.4. N-t-Butoxycarbonyl-S-(ursodeoxycholyl)glutathione (**2d**).* The compound **2d** was obtained from **1d** (3.42 g, 6.98 mmol) as colorless semi-solids; yield 3.28 g (66.2%). ^{1}H-NMR (DMSO-d_6) δ: 0.60 (3H, s, 18-H), 0.86 (3H, s, 19-H), 0.88 (3H, d, J = 6.0 Hz, 21-H), 2.18 (2H, t, J = 7.5 Hz, Glu-γ,γ′-H), 2.64 (2H, m, 23-H), 2.93-3.24 (2H, m, Cys-β,β′-H), 3.15 (1H, m, 3β-H), 3.61 (1H, m, 7α-H), 3.72 (2H, m, Gly-α,α′-H), 3.86 (1H, m, Glu-α-H), 4.46 (1H, m, Cys-α-H), 7.06 (1H, d, J = 8.4 Hz, Glu-NH), 8.12 (1H, d, J = 8.7 Hz, Cys-NH), 8.26 (1H, t, J = 6.0 Hz, Gly-NH). MALDI-TOFMS: m/z 804.9 $[M + Na]^+$, 826.8 $[M - H + 2Na]^+$.

*2.5.4.5. N-t-Butoxycarbonyl-S-(lithocholyl)glutathione (**2e**).* The compound **2e** was obtained from **1e** (2.95 g, 6.23 mmol) as colorless semi-solids; yield 3.22 g (80.9%). ^{1}H-NMR (DMSO-d_6) δ: 0.59 (3H, s, 18-H), 0.84 (3H, s, 19-H), 0.86 (3H, d, J = 6.6 Hz, 21-H), 2.17 (2H, t, J = 7.8 Hz, Glu-γ,γ′-H), 2.64 (2H, m, 23-H), 2.93-3.28 (2H, m, Cys-β,β′-H), 3.15 (1H, m, 3β-H), 3.71 (2H, m, Gly-α,α′-H), 3.85 (1H, m, Glu-α-H), 4.44 (1H, m, Cys-α-H), 7.08 (1H, d, J = 8.1 Hz, Glu-NH), 8.13 (1H, d, J = 8.1 Hz, Cys-NH), 8.28 (1H, t, J = 5.4 Hz, Gly-NH). MALDI-TOFMS: m/z 788.4 $[M + Na]^+$, 810.6 $[M - H + 2Na]^+$.

*2.5.5. General method for preparation of BA-GSHs (**3a**–**3e**)*

Acid hydrolysis of Boc-GSH derivatives of BAs (**2a**–**2e**) was performed using 3 M HCl in AcOEt (5 ml) for 1 h at r.t. After evaporation of the solvent *in vacuo*, each of the crude products was purified by preparative RP-HPLC with UV detection at 220 nm on a Cosmosil 5C_{18}-AR column (250 mm × 20 mm I.D., Nacalai Tesque Inc.) by a linear gradient elution: 30% solvent B (0.1% TFA in acetonitrile) to 100% B against solvent A (0.1% TFA) over 30 min at a flow rate of 7 ml/min. The fractions containing desirable product were collected and lyophilized to dryness.

*2.5.5.1. S-(Cholyl)glutathione (CA-GSH; **3a**).* The compound **3a** was obtained from **2a** (10 mg, 1.31 μmol) as colorless semi-solids; yield 65.0%. ^{1}H-NMR (CD_3OD) δ: 0.70 (3H, s, 18-H), 0.91 (3H, s, 19-H), 1.00 (3H, d, J = 6.2 Hz, 21-H), 2.13-2.26 (2H, m, Glu-β,β′-H), 2.53 (2H, t, J = 7.0 Hz, Glu-γ,γ′-H), 2.51–2.66 (2H, m, 23-H), 3.13-3.41 (2H, m, Cys-β,β′-H), 3.37 (1H, m, 3β-H), 3.79 (1H, q, J = 2.8 Hz, 7β-H), 3.91 (2H, s, Gly-α,α′-H), 3.93 (1H, t, J = 2.8 Hz, 12β-H), 3.97 (1H, t, J = 6.0 Hz, Glu-α-H), 4.58 (1H, m, Cys-α-H). MALDI-TOFMS: m/z 698.9 $[M + H]^+$, 720.5 $[M + Na]^+$.

*2.5.5.2. S-(Chenodeoxycholyl)glutathione (CDCA-GSH; **3b**).* The compound **3b** was obtained from **2b** as colorless semi-solids; yield 73.3%. ^{1}H-NMR (CD_3OD) δ: 0.68 (3H, s, 18-H), 0.92 (3H, s, 19-H), 0.94 (3H, d, J = 6.5 Hz, 21-H), 2.13–2.24 (2H, m, Glu-β,β′-H), 2.52 (2H, t, J = 7.2 Hz, Glu-γ,γ′-H), 2.49–2.63 (2H, m, 23-H), 3.11–3.42 (2H, m, Cys-β,β′-H), 3.37 (1H, m, 3β-H),), 3.78 (1H, q, J = 2.4 Hz, 7β-H), 3.91 (2H, s, Gly-α,α′-H), 3.97 (1H, t, J = 6.4 Hz, Glu-α-H), 4.58 (1H, m, Cys-α-H). MALDI-TOFMS: m/z 682.8 $[M + H]^+$, 704.7 $[M + Na]^+$, 726.8 $[M - H + 2Na]^+$.

*2.5.5.3. S-(Deoxycholyl)glutathione (DCA-GSH; **3c**).* The compound **3c** was obtained from **2c** as colorless semi-solids; yield 74.1%. ^{1}H-NMR (CD_3OD) δ: 0.70 (3H, s, 18-H), 0.92 (3H, s, 19-H), 0.99 (3H, d, J = 6.4 Hz, 21-H), 2.13–2.20 (2H, m, Glu-β,β′-H), 2.53 (2H, t, J = 7.2 Hz, Glu-γ,γ′-H), 2.51–2.63 (2H, m, 23-H), 3.11–3.42 (2H, m, Cys-β,β′-H), 3.52 (1H, m, 3β-H), 3.91 (2H, s, Gly-α,α′-H), 3.94 (1H, t, J = 2.8 Hz, 12β-H), 4.00 (1H, t, J = 6.5 Hz, Glu-α-H), 4.58 (1H, m, Cys-α-H). MALDI-TOFMS: m/z 682.9 $[M + H]^+$, 704.7 $[M + Na]^+$, 720.7 $[M + K]^+$.

*2.5.5.4. S-(Ursodeoxycholyl)glutathione (UDCA-GSH; **3d**).* The compound **3d** was obtained from **2d** as colorless semi-solids; yield, 52.2%. ^{1}H-NMR (CD_3OD) δ: 0.70 (3H, s, 18-H), 0.95 (3H, d, J = 6.5 Hz, 21-H), 0.96 (3H, s, 19-H), 2.13–2.19 (2H, m, Glu-β,β′-H), 2.52 (2H, t, J = 6.7 Hz, Glu-γ,γ′-H), 2.50–2.62 (2H, m, 23-H), 3.11–3.43 (2H, m, Cys-β,β′-H), 3.37 (2H, m, 3β- and 7α-H), 3.91 (2H, s, Gly-α,α′-H), 3.92 (1H, t, J = 6.4 Hz, Glu-α-H), 4.58 (1H, m, Cys-α-H). MALDI-TOFMS: *m/z* 682.8 $[M+H]^+$, 704.7 $[M+Na]^+$, 726.8 $[M-H+2Na]^+$.

*2.5.5.5. S-(Lithocholyl)glutathione (LCA-GSH; **3e**).* The compound **3e** was obtained from **2e** as colorless semi-solids; yield 68.6%. ^{1}H-NMR (CD_3OD) δ: 0.67 (3H, s, 18-H), 0.94 (3H, s, 19-H), 0.94 (3H, d, J = 6.5 Hz, 21-H), 2.11–2.24 (2H, m, Glu-β,β′-H), 2.53 (2H, t, J = 7.1 Hz, Glu-γ,γ′-H), 2.51–2.68 (2H, m, 23-H), 3.11–3.42 (2H, m, Cys-β,β′-H), 3.53 (1H, m, 3β-H), 3.91 (2H, s, Gly-α,α′-H), 3.99 (1H, t, J = 6.4 Hz, Glu-α-H), 4.58 (1H, m, Cys-α-H). MALDI-TOFMS: *m/z*: 666.6 $[M+H]$, 688.5 $[M+Na]^+$.

2.6. Nonenzymatic transformation into CA-GSH

A solution of CA-CoA (500 nmol) and CA-AMP (136 nmol) in 10 mM phosphate buffer (pH 7.5) (0.5 ml for the former and 0.25 ml for the latter) was incubated for 1 h at 37 °C in the presence or absence of GSH (5 μmol for CA-CoA and 1.36 μmol for CA-AMP). After cooling on an ice bath, the incubation mixture was passed through an Oasis HLB cartridge. The cartridge was washed with water (2 ml) and then the desirable fraction was eluted with methanol (1 ml). After the methanol was evaporated under a stream of N_2 gas, the residue was redissolved in 30% (v/v) of acetonitrile, an aliquot of which was subjected to the LC/ESI–MS^n analysis on a TSKgel ODS-100 V column by isocratic elution using acetonitrile–5 mM ammonium acetate (pH 6) (3:7, v/v).

3. Results and discussion

3.1. Synthesis of BA-GSHs

For the ongoing characterization of biologically important trace compounds, it is preferable to use authentic specimens. Our initial efforts were therefore directed to the chemical synthesis of BA-GSHs. The synthesis of the target compounds required the selective condensation of the carboxyl group of BAs with the thiol group of GSH possessing three reactive functional moieties, i.e., the amino, carboxyl, and thiol groups. The method previously reported in the literature involves the direct coupling the thiol group of GSH with the mixed anhydride of a carboxyl group [24,25]. This method is not satisfactory for the synthesis of the target compounds because the coupling with the amino group of GSH occurs preferentially to that with the thiol group. This makes the yield of the desirable thioester compound lower than that of the amide compound, and their separation becomes difficult.

We therefore wanted to use a GSH derivative on which the amino group is protected with a group that could be easily removed under mild and nonhydrolytic conditions. In the work reported here we used the *t*-butoxycarbonyl (Boc) group for the protection of GSH and prepared Boc-GSH by the conventional method as follows (Fig. 2). The amino groups of the GSSG were first protected with the Boc group and then the protected GSSG, i.e., [(Boc-GS)$_2$], was reduced again with nBu$_3$P to give Boc-GSH. Each BA was then converted to the corresponding succinimidyl ester, and the thus-obtained activated ester was coupled with the free thiol of Boc-GSH under basic conditions. The Boc group in each product was finally removed by treatment with 3 M HCl in AcOEt to give the desirable BA-GSH whose structure was confirmed by ^{1}H-NMR and ESI–MS^2.

3.2. Proton assignments of BA-GSHs in ^{1}H-NMR

The use of ^{1}H-^{1}H shift-correlated 2D NMR techniques is a powerful and reliable tool for structural and stereochemical elucidation of complex steroid conjugates [26]. The proton assignments were therefore carried out by using the correlation spectroscopy spectra, in which the following proton signals were assigned: angular H$_3$-18, H$_3$-19 (each s) and H$_3$-21 methyl protons in a higher field region of 0.695–1.001 ppm, H$_2$-23 methylene protons (each m) at 2.0–2.4 ppm, and H-3β, H-7 and/or H-12β methylene protons (occurring at 3.5–4.3 ppm as m) geminally attached to oxygen-containing functions in the aglycone moieties, and H$_2$-β,β′ in glutamic acid moieties at 2.146–2.264 ppm (m), H$_2$-γ,γ′ in glutamic acid moieties at 2.52–2.530 ppm (m), H$_2$-β,β′ in cysteine moieties at 3.130–3.419 ppm (m), H$_2$-α,α′ in glycine moieties at 3.908–3.911 ppm (each s), H-α in glutamic acid moieties at 3.929–4.005 ppm (m), and H-α in cysteine moieties at 4.577–4.580 ppm (m). These spectral data were consistent with the structures of BA-GSHs.

3.3. Collision-induced fragmentation of BA-GSHs

Mass spectrometry, in combination with tandem mass spectrometry, has been used to determine the structure of a variety of GSH conjugates in both *in vitro* and *in vivo* experiments. A general scheme for the collision-induced fragmentation of protonated GSH conjugates, based on FAB-MS/MS measurements, was reported by Pearson et al. more than 15 years ago [27,28], but the general behaviors of GSH conjugates of BAs in tandem mass spectrometry has not been extensively investigated and the fragmentation pathways of each conjugate have not been reported. We therefore examined the collision-induced fragmentation of BA-GSHs by using linear ion traps, which allow multiple tandem mass spectrometric experiments.

In the full-scan positive-ion mode, none of the BA-GSH conjugates formed doubly charged ions but did form singly charged ions $[M+H]^+$ indicating the covalently condensed product of BA and GSH. The product-ion spectra obtained by CID of the $[M+H]^+$ were very similar: the GSH conjugates of hydrophilic bile acids (CA, CDCA, and UDCA) showed $[M+H-H_2O]^+$ as the base peak with weak $[M+H-2H_2O]^+$ or no $[M+H-3H_2O]^+$, whereas the GSH conjugates of hydrophobic bile acids (DCA and LCA) showed a modest $[M+H-H_2O]^+$

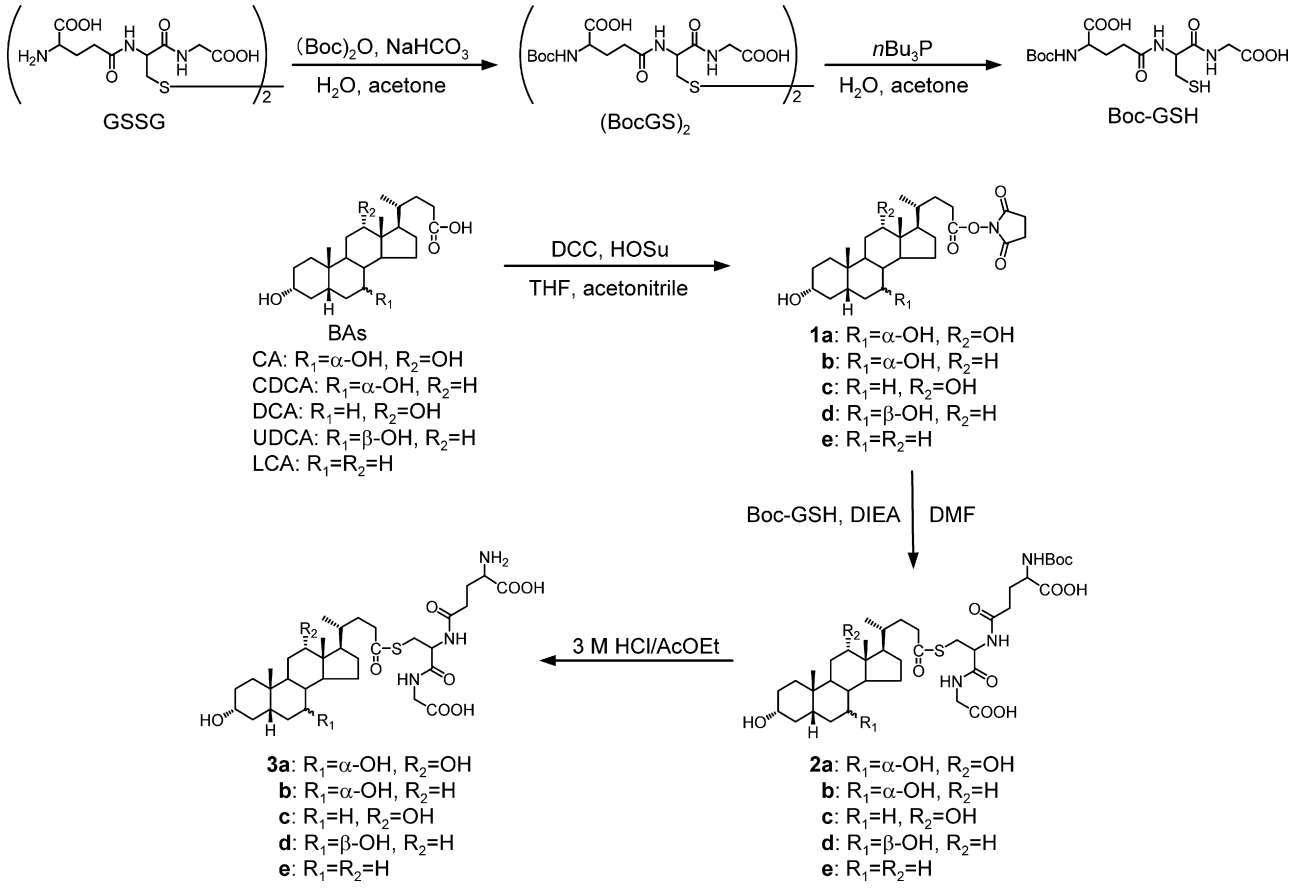

Fig. 2. Synthetic route of BA-GSHs.

ion with weak $[M+H-2H_2O]^+$ and $[M+H-3H_2O]^+$ ions. In addition to showing these dehydrated ions, all the product-ion spectra showed similar fragment ions formed by fragmentation of the GSH moiety, as is evident by the fragments observed from the cleavage of glycine (75 Da) and pyroglutamic acid (129 Da), and fragment ions formed by neutral loss of GSH (307 Da) from the aglycone moiety. These fragment ions are common in tandem mass spectra of GSH conjugates [29]. With lower abundance, consecutive loss of CO (28 Da) was also observed. The corresponding fragment ion of the protonated GSH at *m/z* 308 was found and further underwent classical fragmentation of GSH moieties to form less abundant ions (Table 1).

All the BA-GSHs behaved similarly in negative-ion ESI-MS, producing abundant deprotonated molecule $[M-H]^-$ with scarce $[M-2H+Na]^-$ and $[M-2H+K]^-$ ions. The fragmentation of the conjugates in negative-ion CID showed the same type of ions: ions formed by the loss of water and NH_2, ions formed by the loss of pyroglutamic acid and glycine, and ions derived from the GSH moiety. Negative-ion MS is thus complementary method to positive-ion MS, but the ion species and the relative abundances of the product ions differed significantly between the positive- and negative-ion modes. The CID spectra showed only a few $[M-H-H_2O]^-$, $[M-H-H_2O-NH_2]^-$, and $[M-H-2H_2O-NH_2]^-$, which are most likely charge-induced fragments from the GSH moiety. The ions $[M-H$ – pyroglutamic acid$]^-$, $[M-H-H_2O$–pyroglutamic acid–$NH_2]^-$, and $[M-H$–pyroglutamic acid–glycine–$CO]^-$, present in all the CID spectra, are formed by the loss of the GSH group. Similarly, the CID spectra of $[M-H]^-$ ion is characterized by a cleavage of the S–CH_2 bond, leading to a loss of 273 Da and the formation of the corresponding negative ions at *m/z* 423, 407, and 391. Unidentified fragment ions formed by losses of 186, 216, and 289 Da from $[M-H]^-$ ions are also characteristic of all the conjugates. The most abundant $[GSH-H]^-$ ion (*m/z* 306) is formed by the loss of the steroid moiety. Resulting from the localization of the negative charge at the GSH moiety, the frequently occurring fragments (*m/z* 288, 272, 254, 236, and 210) are formed by the loss of H_2O, H_2S, and CO_2 molecules in various combinations from the GSH fragment ion (Table 2). These ions are indicative of GSH conjugates but do not provide information about the steroid structure itself. Typical positive- and negative-ion CID spectra of CA-GSH are shown in Fig. 3.

3.4. Nonenzymatic transformation into glutathione conjugate

GSH exists at millimolar concentrations in the intracellular fluid of mammals. Because of its nucleophilic cysteinyl thiol group, GSH can react with electrophiles during biotransformation of both foreign compounds and endogenous compounds (either with the compound itself or its electrophilic metabolites)

Table 1
Diagnostically significant ions obtained from the CID spectra on the $[M+H]^+$ ions of GSH conjugates of bile acids

	m/z				
	CA-GSH 698.4[a]	CDCA-GSH 682.4[a]	DCA-GSH 682.4[a]	UDCA-GSH 682.4[a]	LCA-GSH 666.4[a]
Product ion					
$[M+H–H_2O]^+$	680.3 (100)	664.3 (100)	664.3 (76)	664.3 (100)	648.3 (31)
$[M+H–2H_2O]^+$	662.3 (36)	646.3 (6)	646.3 (13)	646.3 (9)	630.3 (1)
$[M+H–3H_2O]^+$	644.3 (6)	N.D.	628.4 (1)	N.D.	612.4 (1)
$[M+H–Gly]^+$	623.3 (18)	607.4 (3)	607.3 (16)	607.4 (2)	591.3 (27)
$[M+H–Gly–H_2O]^+$	605.3 (7)	589.3 (4)	589.3 (7)	589.3 (5)	573.3 (4)
$[M+H–Gly–2H_2O]^+$	587.3 (3)	571.3 (<1)	571.3 (2)	571.3 (1)	555.3 (1)
$[M+H–pyroGlu]^+$	569.3 (36)	553.3 (6)	553.3 (16)	553.2 (7)	537.3 (36)
$[M+H–pyroGlu–H_2O]^+$	551.2 (46)	535.3 (28)	535.3 (67)	535.3 (40)	519.3 (78)
$[M+H–pyroGlu–2H_2O]^+$	533.3 (49)	517.3 (21)	517.3 (100)	517.3 (38)	501.3 (100)
$[M+H–pyroGlu–3H_2O]^+$	515.3 (40)	499.3 (11)	499.3 (36)	499.3 (22)	N.D.
$[M+H–pyroGlu–4H_2O]^+$	497.3 (14)	N.D.	N.D.	N.D.	N.D.
$[M+H–Gly–pyroGlu]^+$	N.D.	N.D.	N.D.	478.3 (<1)	462.2 (12)
$[M+H–-Gly–pyroGlu–H_2O]^+$	476.3 (<1)	460.3 (2)	460.3 (5)	460.3 (5)	444.2 (54)
$[M+H–Gly–pyroGlu–2H_2O]^+$	458.3 (3)	442.2 (6)	442.3 (18)	442.2 (14)	N.D.
$[M+H–Gly–pyroGlu–3H_2O]^+$	440.37 (8)	N.D.	N.D.	N.D.	N.D.
$[M+H–pyroGlu–Gly–CO]^+$	466.3 (4)	450.3 (1)	450.3 (5)	450.3 (4)	434.3 (12)
$[M+H–pyroGlu–Gly–CO–H_2O]^+$	448.2 (4)	432.3 (2)	432.2 (4)	432.3 (4)	416.3 (12)
$[M+H–pyroGlu–Gly–CO–2H_2O]^+$	430.3 (2)	414.3 (1)	414.3 (3)	414.3 (2)	398.3 (<1)
$[M+H–pyroGlu–Gly–CO–3H_2O]^+$	412.3 (2)	N.D.	396.4 (<1)	396.3 (<1)	N.D.
$[M+H–GSH–H_2O]^+$	373.3 (<1)	N.D.	357.3 (2)	357.2 (<1)	341.3 (9)
$[M+H–GSH–2H_2O]^+$	355.2 (27)	339.3 (2)	339.3 (19)	339.3 (4)	323.3 (11)
$[M+H–GSH–3H_2O]^+$	337.3 (28)	321.3 (1)	321.2 (11)	321.3 (3)	N.D.
$[M+H–GSH–4H_2O]^+$	319.2 (7)	N.D.	N.D.	N.D.	N.D.
$[GSH+H]^+$	308.1 (6)	308.1 (1)	308.1 (5)	308.1 (2)	308.1 (5)

Values in parenthesis represent relative intensity.
N.D. = Not detected.
[a] Precursor ion

Table 2
Diagnostically significant ions obtained from the CID spectra on the $[M-H]^-$ ions of GSH conjugates of bile acids

	m/z				
	CA-GSH 696.3[a]	CDCA-GSH 680.3[a]	DCA-GSH 680.3[a]	UDCA-GSH 680.3[a]	LCA-GSH 664.3[a]
Product ion					
$[M-H–H_2O]^-$	678.3 (25)	662.3 (17)	662.3 (20)	662.3 (12)	646.3 (10)
$[M-H–H_2O–NH_2]^-$	662.3 (8)	646.3 (10)	646.4 (8)	646.3 (8)	630.4 (10)
$[M-H–2H_2O–NH_2]^-$	644.3 (14)	628.3 (15)	628.3 (13)	628.3 (14)	612.3 (16)
$[M-H–pyroGlu]^-$	567.3 (65)	551.3 (67)	551.3 (65)	551.3 (59)	535.3 (64)
$[M-H–pyroGlu–NH_2–H_2O]^-$	533.3 (10)	517.3 (14)	517.3 (11)	517.3 (13)	501.3 (15)
$[M-H–pyroGlu–Gly–CO]^-$	464.3 (23)	448.3 (24)	448.3 (24)	448.3 (23)	432.3 (24)
$[M-H–273]^-$	423.3 (2)	407.3 (1)	407.3 (1)	407.3 (1)	391.3 (1)
$[GSH–H]^-$	306.0 (100)	306.0 (100)	306.0 (100)	306.0 (100)	306.0 (100)
$[GSH–H–H_2O]^-$	288.0 (17)	288.0 (17)	288.0 (17)	288.0 (17)	288.0 (17)
$[GSH–H–SH_2]^-$	272.1 (30)	272.1 (30)	272.1 (30)	272.1 (30)	272.1 (30)
$[GSH–H–SH_2–H_2O]^-$	254.0 (21)	254.0 (21)	254.0 (21)	254.0 (21)	254.0 (21)
$[GSH–H–SH_2–2H_2O]^-$	236.0 (1)	236.0 (1)	236.0 (1)	236.0 (1)	236.0 (1)
$[GSH–H–SH_2–H_2O–CO_2]^-$	210.1 (3)	210.1 (3)	210.1 (3)	210.1 (3)	210.1 (3)
$[M-H–186]^-$	510.3 (3)	494.3 (4)	494.3 (4)	494.3 (4)	478.3 (4)
$[M-H–216]^-$	480.3 (2)	464.3 (2)	464.3 (2)	464.3 (2)	448.3 (2)
$[M-H–289]^-$	407.3 (3)	391.4 (1)	391.3 (3)	391.3 (1)	375.3 (1)

Values in parenthesis represent relative intensity.
N.D. = Not detected.
[a] Precursor ion

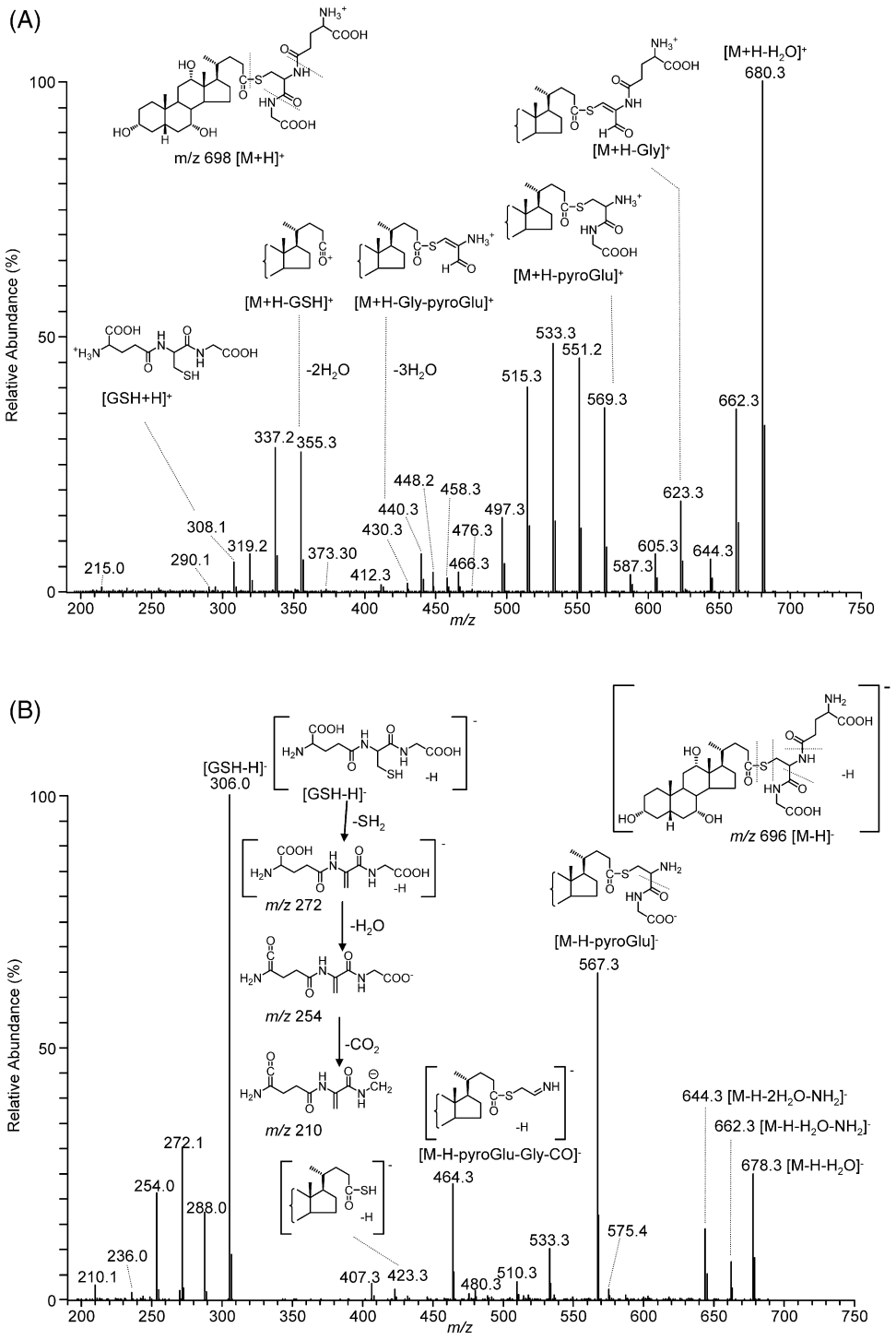

Fig. 3. Product-ion spectra obtained by CID of (A) $[M+H]^+$ and (B) $[M-H]^-$ ion of CA-GSH. Proposed structures of major fragments are depicted.

and thus affords detoxification. Some acyl-glucuronides and acyl-CoA thioesters, electrophilic metabolites formed during metabolism of drugs containing a carboxylic acid moiety, react with the thiol group of GSH to form thioester-linked GSH conjugates [19,20]. A recent finding suggest that CoA metabolites may be more reactive species than the acyl-glucuronides that have previously been associated with severe drug-related side effects [22]. The formation of thioester-linked GSH conjugates of BAs is therefore presumed to occur via trans-acylation reactions between GSH and reactive acyl-linked metabolites such as acyl-CoA and its reactive intermediate acyl-adenylate of CA. In the experiments, CA-AMP and CA-CoA were independently incubated with GSH in a phosphate buffer at 37 °C for 1 h. The incubation mixture was subjected to extraction on an Oasis HLB cartridge and a portion of the extract was subjected to LC/ESI-MS and -MS^2 analysis in both positive- and negative-ion mode. In positive-ion MS experiment, protonated molecules at *m/z* 1158.3, 738.3 and 698.3 for CA-CoA, CA-AMP and CA-GSH,

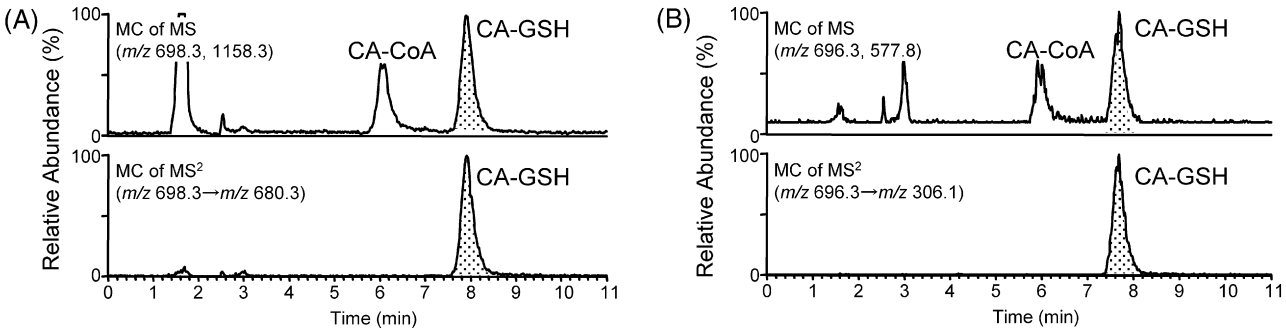

Fig. 4. Typical mass chromatograms in (A) positive- and (B) negative-ion mode of an incubation mixture of CA-CoA with GSH.

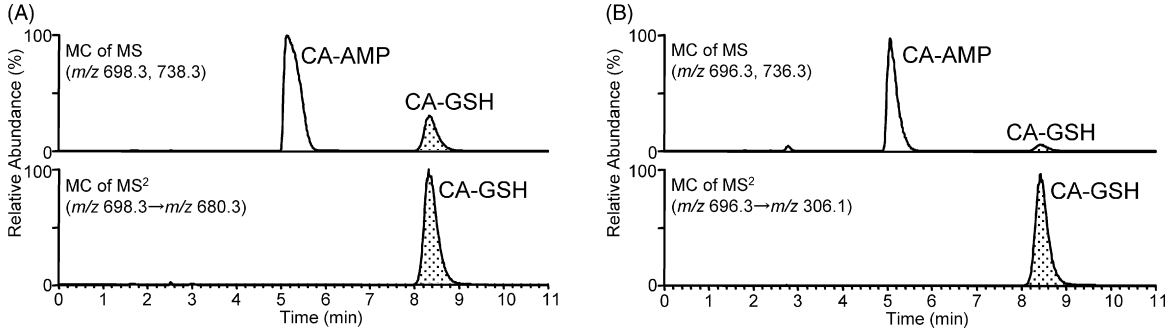

Fig. 5. Typical mass chromatograms in (A) positive- and (B) negative-ion mode of an incubation mixture of CA-AMP with GSH.

respectively, were monitored. Identification of the ion at *m/z* 698.3 $[CA\text{-}GSH + H]^+$ was confirmed by product ion scan MS^2 experiment leading to dehydrated ion $[M + H - H_2O]^+$ (*m/z* 680.3). The negative-ion ESI mode was also investigated by monitoring doubly charged ion *m/z* 577.8 for CA-CoA and deprotonated molecules *m/z* 736.3 for CA-AMP and *m/z* 696.3 for CA-GSH in MS experiment. Confirmation of ion at *m/z* 696.3 was done by product ion scan MS^2 experiment leading to product ion $[GSH - H]^-$ (*m/z* 306.1). The representative mass chromatograms and CID spectra were definitely identical to that of CA-GSH (Figs. 4 and 5), showing the formation of the acyl-adenylate and acyl-CoA through nucleophilic displacement. The above results are the first reported evidence for the formation of a GSH conjugates of BAs.

4. Conclusion

In the present study, we evaluated the chemical reactivity of CA-AMP and CA-CoA with GSH experimentally in order to investigate the acylating properties of these acyl-linked metabolites. We found that these metabolites react, in a trans-acylation type of reaction, with the nucleophilic cysteinyl thiol of GSH to form CA-GSH. The results of these *in vitro* studies show that acyl-adenylate and acyl-CoA thioester derivatives of BAs are reactive acylating species which can contribute to the hepatic biotransformation of BAs into GSH conjugates *in vivo*. Further studies on the enzymatic formation and detection in bile of GSH conjugates of BAs are now being conducted in our laboratories, and the results will be reported in the near future.

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References

[1] A.F. Hofmann, in: S.G. Schultz (Ed.), Handbook of Physiology-The Gastrointestinal System III, American Physiological Society, Bethesda, MD, 1989, p. 567.
[2] D.M. Small, in: P.P. Nair, D. Kritchevsky (Eds.), The Bile Acids, Chemistry, Physiology and Metabolism. Chemistry, vol. 1, Plenum Press, New York, 1971, p. 249.
[3] N. Mano, M. Uchida, H. Okuyama, I. Sasaki, S. Ikegawa, J. Goto, Anal. Sci. 17 (2001) 44.
[4] S. Ikegawa, H. Ishikawa, H. Oiwa, M. Nagata, J. Goto, T. Kozaki, M. Gotowda, N. Asakawa, Anal. Biochem. 266 (1999) 125.
[5] J. Goto, M. Nagata, N. Mano, N. Kobayashi, S. Ikegawa, R. Kiyonami, Rapid Commun. Mass Spectrom. 15 (2001) 104.
[6] N. Mano, K. Kasuga, N. Kobayashi, J. Goto, J. Biol. Chem. 279 (2004) 55034.
[7] I. Panfil, P.A. Lehman, P. Zimniak, B. Ernst, T. Franz, R. Lester, A. Radominska, Biochim. Biophys. Acta 1126 (1992) 221.
[8] J. Goto, N. Murao, C. Nakada, T. Motoyama, J. Oohashi, T. Yanagihara, T. Niwa, S. Ikegawa, Steroids 63 (1998) 186.
[9] S. Ikegawa, J. Oohashi, N. Murao, J. Goto, Biomed. Chromatogr. 14 (2000) 144.
[10] S. Ikegawa, H. Okuyama, J. Oohashi, N. Murao, J. Goto, Anal. Sci. 15 (1999) 625.
[11] N. Mano, K. Nishimura, T. Narui, S. Ikegawa, J. Goto, Steroids 67 (2002) 257.

[12] S. Ikegawa, N. Murao, M. Nagata, S. Ohba, J. Goto, Anal. Sci. 15 (1999) 213.
[13] N. Turjman, P.P. Nair, in: K.D.R. Setchell, D. Kritchevsky, P.P. Nair (Eds.), The Bile Acids: Chemistry, Physiology and Metabolism. Physiology and Metabolism, vol. 4, Plenum Press, New York, 1988, p. 373.
[14] P.P. Nair, G. Kessie, R. Patnaik, C. Guidry, Steroids 59 (1994) 212.
[15] S.D. Hall, Q. Xiaotao, Chem. Biol. Interact. 90 (1994) 235.
[16] J.A. Watt, A.R. King, R.G. Dickinson, Xenobiotica 21 (1991) 403.
[17] A. Ding, J.C. Ojingwa, A.F. McDonagh, A.L. Burlingame, L.Z. Benet, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 3797.
[18] A. Ding, P. Zia-Amirhosseini, A.F. McDonagh, A.L. Burlingame, L.Z. Benet, Drug Metab. Disps. 23 (1995) 369.
[19] M. Stogniew, C. Fenselau, Drug Metab. Dispos. 10 (1982) 609.
[20] L.J. Shore, C. Fenselau, A.R. King, R.G. Dickinson, Drug Metab. Dispos. 23 (1995) 119.
[21] M.P. Grillo, L.Z. Benet, Drug Metab. Dispos. 30 (2002) 55.
[22] J. Olsen, I. Bjørnsdottir, J. Tjørnelund, S.H. Hansen, J. Pharm. Biomed. Anal. 29 (2002) 7.
[23] V.-E. Rudolf, L. Christinane, S.-M. Jens, J. Peptide Res. 51 (1998) 365.
[24] M.P. Grillo, F. Hua, C.G. Knutson, J.A. Ware, C. Li, Chem. Res. Toxicol. 16 (2003) 1410.
[25] C.M. Dieckhaus, C.L. Fernández-Metzler, R. King, P.H. Krolikowski, T.A. Baillie, Chem. Res. Toxicol. 18 (2005) 630.
[26] T. Iida, Y. Kasuga, M. Arakawa, K. Mushiake, S. Ikegawa, J. Goto, T. Nambara, Magn. Reson. Chem. 39 (2001) 749.
[27] P.G. Pearson, M.D. Threadgill, W.N. Howald, T.A. Baillie, Biomed. Environ. Mass Spectrom. 16 (1988) 51.
[28] P.G. Pearson, W.N. Howald, S.D. Nelson, Anal. Chem. 62 (1990) 1827.
[29] K. Levsen, H.-M. Schiebel, B. Behnke, R. Dötzer, W. Dreher, M. Elend, H. Thiele, J. Chromatogr. A 1067 (2005) 55.